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Postbiotic Production from *Bacillus* Consortium Enriched with *Avicennia marina* Leaves and Its Antibacterial Activity Against Aquaculture Pathogens

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ABSTRACT

Bacterial infections pose a critical challenge in aquaculture, causing mass mortality and financial losses. The present study aimed to formulate postbiotics from a *Bacillus* (*B.*) consortium (*B. safensis*, *B. mycoides*, and *B. pumilus*) enriched with *Avicennia marina* (*A. marina*) leaf powder (ALP) and evaluate their activity against *Streptococcus agalactiae* (*S. agalactiae*), *Aeromonas hydrophila* (*A. hydrophila*), *Vibrio* (*V.*) *harveyi*, *V. parahaemolyticus*, and *V. alginolyticus*. The postbiotics' antibacterial efficacy was assessed using broth microdilution to determine minimum inhibitory concentrations (MICs), followed by subculturing to determine minimum bactericidal concentrations (MBCs). The disk diffusion assay assessed the tolerance of *Bacillus* consortium to *A. marina* leaf extracts at 25, 50, 75, and 100 mg mL^{-1}, with ethanol as the negative and ciprofloxacin as the positive controls. Postbiotic production used the medium as the control and three graded concentrations of ALP at 1%, 2%, and 3%, followed by thermal inactivation, centrifugation, and filtration to ensure the absence of live cells. The postbiotic was examined using liquid chromatography-high-resolution mass spectrometry to determine its secondary metabolite profiles. The 3% ALP-enriched postbiotic was selected for final antibacterial efficacy analysis against aquaculture pathogens. The results demonstrated that the *Bacillus* consortium was resistant to leaf extracts at 25-100 mg mL^{-1}, with only minimal inhibition zones of 6.00 to 7.17 mm. In postbiotic production, 3% ALP enhanced bacterial viability to 9.08 log_{10} CFU mL^{-1}. The 3% ALP-enriched postbiotic increased the number of metabolites from 384 (baseline control) to 543, accompanied by upregulation of Cyclo(-L-Ala-L-Tyr), (R)-Lactaldehyde, an acylprolinamide derivative, and D-(-)-Ribose. *In vitro*, 3% ALP selectively inhibited *Vibrio* spp., demonstrating significant inhibition zones (11.33-15.30 mm), low MIC values (1.6-25% v/v), and low MBC values (6.3-50% v/v). Although disk diffusion produced no inhibition zones for either *A. hydrophila* or *S. agalactiae*, *A. hydrophila* exhibited susceptibility at 25% v/v in the MIC and at 50% v/v in the MBC. In contrast, *S. agalactiae* remained resistant at the highest concentration, with MIC and MBC values exceeding 50% v/v. Enriching the *Bacillus* consortium with ALP increased the active metabolites in *Bacillus* postbiotics, offering a promising control against *Vibrio* spp. in aquaculture.

Keywords: Aquaculture, *Avicennia marina*, *Bacillus* consortium, Metabolite, Postbiotic, Vibriosis

INTRODUCTION

Mangrove ecosystems contain several bioactive compounds, such as flavonoids, which act as antimicrobial, antioxidant, and immunomodulatory agents (Rahmania et al., 2025). One of the most promising mangrove species for the extraction of bioactive compounds is *Avicennia marina* (*A. marina*), due to its prevalence and natural abundance along tropical and subtropical coastlines, including the coastal regions of Indonesia (Rahmawati et al., 2019; Cerri et al., 2025). The interactions between *A. marina* and non-pathogenic bacteria have not been widely reported in the context of fermentation, largely because most of the bioactive compounds in *A. marina* have been documented primarily for their direct antimicrobial effects against pathogenic bacteria (Rahmania et al., 2025). Therefore, combination formulations that use plant-derived substrates and produce postbiotics hold great potential to enhance functional properties and health benefits (Sravya et al., 2025). Probiotics produced by *Bacillus* (*B.*) species, including *B. safensis*, *B. mycoides*, and *B. pumilus*, exhibit significant potential within the aquaculture and functional food industries owing to the enzymes and bioactive compounds they synthesize (Foysal et al., 2020; Wu et al., 2021; Raman et al., 2025). However, the integration and metabolic responses of *Bacillus* spp. with compounds derived from *A. marina* leaves have not been extensively studied (Saxena et al., 2020).

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Postbiotics are metabolic products that do not contain live microorganisms, making them safer and more stable than probiotics, which contain live cells. Nevertheless, postbiotics can support the immune response and protect aquatic animals against pathogens (Thakur et al., 2025). The use of *A. marina* leaf powder (ALP) as a substrate can support *Bacillus* spp. in secreting hydrolytic enzymes (cellulase) for degrading coarse fiber structures (Kurniawan et al., 2017). This biotransformation process broadens the range of metabolites through the production of functional organic acids and bacteriocins, thereby supporting the development of postbiotic formulations for health in the aquaculture sector (Rahman et al., 2020; Salminen et al., 2021; Rahman et al., 2023). The leaves of *A. marina* naturally contain several bioactive compounds, including phenolics, saponins, flavonoids, terpenoids, and alkaloids (Rozirwan et al., 2022). Plant-derived active compounds, such as phenolics at sub-inhibitory concentrations, act as mild stressors on *Bacillus* spp., thereby activating secondary metabolism and promoting the production of a broader diversity of bioactive compounds during fermentation (Wang et al., 2018; Hurtado-Navarro et al., 2025). Other plant-derived compounds from *A. marina* leaves, such as saponins, which are biosurfactants with amphiphilic and physicochemical properties, are functionally similar to surfactin or microbial-based biosurfactants (Karnwal et al., 2023). When these triterpenoid saponins are exposed to *Bacillus* spp. at sub-lethal concentrations, the bacteria undergo adaptive physiological changes rather than die, reprogramming the lipid composition of their membranes to survive (Uttlová et al., 2016). Furthermore, flavonoids at concentrations below the inhibitory threshold can cause subtle changes in cellular signaling and enzyme activity, driven by mild physiological stress rather than by lethal cellular damage (Patil et al., 2024). In addition, it has been reported that exposure to terpenoid and alkaloid groups can disrupt the structural integrity of *Bacillus* spp. (Huang et al., 2022a). However, at sub-inhibitory levels, these terpenoid and alkaloid compounds interfere only with the bacterial target site, without stopping its growth (Radeck et al., 2017).

Pathogenic bacterial infections are a major challenge in aquaculture, causing high mortality and significant economic losses due to reduced production (Sarjito et al., 2022). *Streptococcus agalactiae* (*S. agalactiae*) is a pathogen of concern in many cultured fish in aquaculture (Sapugahawatte et al., 2022). This concern emerges in the aquaculture sector due to high mortality rates, with *S. agalactiae* becoming more prevalent in many aquaculture-producing countries, including Indonesia (Lusiastuti et al., 2024). Furthermore, *Aeromonas hydrophila* (*A. hydrophila*) is a freshwater opportunistic bacterium responsible for motile aeromonas septicemia (MAS) and has been identified as one of the most pathogenic bacteria contributing to losses in different farmed fish species, such as *Oreochromis niloticus*, as well as crustaceans such as *Litopenaeus vannamei* (Tao et al., 2026). In brackish and marine aquaculture, *Vibrio* species such as *Vibrio harveyi* (*V. harveyi*), *Vibrio alginolyticus* (*V. alginolyticus*), and *Vibrio parahaemolyticus* (*V. parahaemolyticus*) are the primary agents of vibriosis, which frequently leads to disease outbreaks (Sarjito and Sabdono, 2021). *Vibrio* spp. are also known to resist multiple antibiotics, posing a challenge in aquaculture (Loo et al., 2020). The variety of bacterial threats in aquaculture systems necessitates safer and more stable alternatives for pathogen control. Therefore, the use of postbiotics is considered more efficient than the use of live cells or probiotics (Thakur et al., 2025). Significant knowledge gaps persist regarding the specific metabolic synergies within a *Bacillus* consortium in response to *A. marina* compounds, as well as the overall antimicrobial efficacy of the derived postbiotics against a broad range of aquaculture pathogens. The use of postbiotics based on the genus *Bacillus*, enriched with *A. marina* leaf substrate, has the potential to be a promising alternative for suppressing the growth of major pathogenic bacteria. The current study aimed to develop a postbiotic formulation by assessing the resistance of a *Bacillus* consortium, comprising *B. safensis*, *B. mycoides*, and *B. pumilus*, against *A. marina* leaf extract, monitoring postbiotic production, including bacterial viability and metabolite profiling through liquid chromatography-high-resolution mass spectrometry (LC-HRMS), as well as evaluating the efficacy of the postbiotic against prevalent aquaculture pathogens.

MATERIALS AND METHODS

Ethical approval

The procedures conducted in the present study adhered to the guidelines established by the Microbiology and Chemistry Laboratory of Ahli Usaha Perikanan (AUP) Polytechnic, Serang, Banten, Indonesia, the Laboratory Guidelines of the Fish and Environmental Health Testing Center, Serang, Banten, Indonesia, the Laboratory Guidelines for Fisheries Product Quality Testing and Implementation, Serang, Banten, Indonesia, and the Research Laboratory Guidelines of Corpora Science, Yogyakarta, Special Region of Yogyakarta, Indonesia.

Avicennia marina leaves preparation

Avicennia marina leaves were obtained from the Northern coastal area of Serang City, Banten Province, Indonesia. Mature leaves were randomly collected from 20 healthy trees, with a strict restriction of less than ten percent of total foliage per tree (300 g tree^{-1}). This restriction was implemented to prevent physiological stress and habitat disturbance,

as artificial defoliation up to 25% did not significantly impact mangrove growth (Tong et al., 2003). The implementation of the randomized, non-destructive harvesting protocol yielded a total wet mass of 6 kg of leaf samples for extraction. The ethanol extract of *A. marina* leaves was prepared according to the methodology of Bitwell et al. (2023), in which mature mangrove leaves were cleaned and dried at 23 °C for 72-96 hours until the moisture content reached ≤ 10%, then ground into powder. A total of 100 g of the powder was macerated in 96% food-grade ethanol (1:10; Malindo Raya Industrial Ltd., Indonesia) for 72 hours, with periodic stirring using a magnetic stirrer (without heating) at 150 rpm for five minutes every eight hours in two replicates. All filtrates were combined in an evaporator at 40-50 °C and 100 mBar, followed by final drying in a universal laboratory oven (Memmert, Germany) at 45 °C for two hours, yielding a paste-like crude extract of *A. marina*.

Bacillus consortium bacterial culture

The *Bacillus* consortium, comprising three strains previously isolated from mangrove sediments and identified via a molecular approach, included *B. safensis* LC849759.1, *B. mycoides* MT509769.1, and *B. pumilus* LC852608.1, was sourced from the Microbiology Laboratory at the Faculty of Fisheries and Marine Sciences, Diponegoro University, Indonesia (Chrisnawati et al., 2023; Fadhila et al., 2023; Widya et al., 2024). The prepared bacterial consortium was a powder formulation coated with calcium carbonate ($CaCO_3$) at a 1:1:1 ratio and a density of 10^8 CFU g^{-1}. For the initial plating suspension, 0.1 g of the *Bacillus* consortium powder was empirically weighed, suspended in sterile 0.85% sodium chloride (NaCl) solution to a final volume of 10 mL, and homogenized with a vortex mixer. A total of 100 µL of the homogenized bacterial suspension was spread onto sterile nutrient agar (Merck KGaA, Germany) and incubated for approximately 24 hours at 30°C. After the incubation period, colonies were harvested using a sterile loop needle based on their specific morphological characteristics to ensure that the three bacterial species were collected. *Bacillus mycoides* was identified macroscopically and confirmed with a biological microscope at 40x magnification (Nikon Corporation, Japan) by rhizoid-shaped colonies resembling circular root filaments, grayish-white in color, and dull in surface texture (Di Franco et al., 2002). *Bacillus pumilus* was identified by its circular, undulate (wavy-edged) colonies, whitish to cream color, and matte, raised appearance (Irkitova et al., 2021). *Bacillus safensis* was characterized by small, round colonies with flat to slightly wavy edges, milky white or shiny cream in color, and smooth in texture (Satomi et al., 2006). The entire colony biomass was then suspended in a sterile NaCl solution, and cell density was adjusted by adding colonies or diluting until the turbidity reached the 0.5 McFarland standard (Feliatra et al., 2021).

Disk diffusion assay of _Avicennia marina_ extract against _Bacillus_ consortium

The disk diffusion test was conducted according to CLSI (2020) guidelines, using a sterile cotton swab dipped into the bacterial consortium suspension at a density of 0.5 McFarland. After swirling the swab against the tube wall to remove excess liquid, the inoculum was spread in three directions across the Mueller-Hinton agar (MHA, pH 7; Merck KGaA, Germany) to ensure a uniform layer. The paste-like crude extract of *A. marina* was dissolved in 96% food-grade ethanol and subjected to vigorous vortex mixing (Thermolyne, United States) at approximately 2500 rpm for three minutes to ensure complete, particle-free solubilization, yielding four treatment groups (Azis et al., 2022). The treatment groups included 25 mg mL^{-1} of *A. marina* was dissolved in l mL 96% food-grade ethanol or 2.5% w/v (Group B), 50 mg mL^{-1} or 5% w/v (Group C), 75 mg mL^{-1} or 7.5% w/v (Group D), and 100 mg mL^{-1} or 10% w/v (Group E), respectively (Ibrahim et al., 2022; Priambodo et al., 2024). Prior to impregnation, the laminar airflow cabinet (Medfuture Biotech Co., Ltd., China) was sterilized using UV radiation. Immediately prior to impregnation, each treatment solution was shortly vortexed, after which sterile paper discs (6 mm diameter, MN 827 ATD, Macherey-Nagel GmbH & Co. KG, Germany) were impregnated with 10 µL of *A. marina* leaf extract and then dried under aseptic conditions for approximately 30 minutes with the airflow blower on and the UV lamp off, ensuring complete evaporation of the ethanol vehicle (Mohideen et al., 2026). The prepared discs containing different concentrations (groups B, C, D, and E) were placed on the consortium bacterial inoculum medium, with approximately 24 mm distances between discs. Next, the treatment medium was incubated upside down at approximately 30°C for 24 hours. For comparison, ciprofloxacin (Oxoid Ltd., England) at a concentration of 5 µg was used as a positive control (Group F) because of its activity against *Bacillus* spp. (Adewumi et al., 2009; Zhai et al., 2023). Meanwhile, a 96% food-grade ethanol solution, dried under conditions identical to those used for the treatment groups, served as a negative control (Group A). After incubation, the inhibition zone was measured using a digital caliper with an accuracy of 0.01 mm.

Postbiotics production with _Bacillus_ consortium enriched with _Avicennia marina_ leaf powder

The postbiotic production procedure begins with preparing a basal medium. All medium ingredients, including peptone (1.0% w/v), sodium hydrogen phosphate (Na2HPO4, 0.4% w/v; Merck KGaA, Germany), sodium dihydrogen phosphate (NaH2PO4, 0.4% w/v; Merck KGaA, Germany), magnesium sulfate heptahydrate (MgSO4.7H2O, 0.02%

w/v; Merck KGaA, Germany), and Tween 80 (0.10% w/v; Merck KGaA, Germany), were dissolved in 100 mL of sterile water, homogenized on a magnetic stirrer and hotplate at 45°C for 10 minutes, and sterilized in an autoclave at 121°C for 15 minutes, following standard basal media for Bacillus cultivation (Atlas, 2010). After sterilization, the liquid medium was cooled to 30°C, and 1.5% (w/v) glucose was added to all treatments. After the glucose dissolved with magnetic stirring, sterile water was added to bring the final volume to 198 mL before inoculation. All treatments were carried out in three replicates. For postbiotic production, dried ALP, prepared as previously described, was used directly as a substrate without prior solvent extraction. The basal medium without any ALP addition was designated as the baseline control group (BC). In the treatment groups, ALP was added at concentrations of 1.0%, 2.0%, and 3.0% (w/v). All groups, including the BC and graded ALP treatments, were provided with a standard 1.5% (w/v) glucose concentration as the main carbon source. The pH was adjusted to 7.0, and sterile water was added to bring the final volume to 198 mL before inoculation. The same 0.5 McFarland standard suspension (approximately 1.5×10^8 CFU mL was used as the inoculum. All treatments were inoculated with 2.0 mL of the *Bacillus* consortium to achieve a final concentration of 1% (v/v) in a total volume of 200 mL, then incubated at 30°C for 24 to 48 hours (Tong et al., 2023; Chen et al., 2026).

Bacterial viability analysis

Bacterial viability assessment was conducted in two stages, with three replications. The initial analysis was performed on liquid cultures obtained prior to the inactivation steps of postbiotic production, by counting bacteria (30-300 colonies) in serial dilutions (10^{-1} to 10^{-5}), expressed as CFU mL^{-1}. Postbiotic production was carried out through inactivation (heating) steps using a universal laboratory oven at 65°C (Memmert, Germany) for 10 minutes, two centrifugations at 10,000 and 12,000 rpm for 10 minutes each, and two filtrations using 25 mm hydrophilic polyethersulfone syringe filters (0.22 µm membrane; OneMed, Indonesia), with the final product being a postbiotic suspension (Paul Beulah and Rajasekar, 2024). The second test, using the same counting method as the initial test, was conducted on the postbiotic suspension to enumerate bacterial colonies.

Liquid chromatography-high-resolution mass spectrometry

The LC-HRMS was employed for untargeted metabolomic analysis following the protocols outlined by Susanto et al. (2026). Metabolomic analysis began with sample preparation. A 200 µL aliquot of the postbiotic suspension sample was immediately mixed with a precooled extracting solution of methanol, acetonitrile, and an aqueous solution (2:2:1, v/v) to induce rapid cooling and quenching, then homogenized by vortexing. The separation of chemical components was carried out using the Vanquish Horizon UHPLC System (Thermo Scientific™, United States). This separation process used an Accucore™ C18 column with dimensions of 100 mm × 2.1 mm (internal diameter) and a particle size of 2.6 µm. For molecular mass identification, an Orbitrap Exploris 240 HRMS instrument (Thermo Scientific™, United States) was operated in full-scan mass spectrometry (Full MS) and data-dependent tandem mass spectrometry (dd-MS2) modes. The resolution levels applied to these measurements were 60,000 full width at half maximum (FWHM) for Full MS and 22,500 FWHM for MS2, respectively. Data processing was performed using Compound Discoverer version 3.3 software (Thermo Scientific™, United States). Potential metabolite annotation was performed using an automated workflow that leveraged multiple structural repositories. High-resolution MS/MS spectra were matched against the mzCloud (Jansen et al., 2022; HighChem LLC, 2025), LIPID MAPS (Sud et al., 2007), the Arita Lab Flavonoid Database (Arita and Suwa, 2008), the ChEBI database (Hastings et al., 2012), KEGG (Kanehisa and Goto, 2000), the Natural Product Atlas (van Santen et al., 2022), and PubMed (Sayers et al., 2022) references for metabolite structure verification.

Antibacterial activity test of postbiotics against aquaculture pathogenic bacteria

Antibacterial activity was assessed against five types of pathogenic bacteria in aquaculture using postbiotic samples derived from an ALP-enriched *Bacillus* consortium. The antibacterial activity analysis employed disc diffusion, broth microdilution, and subculture techniques. *Streptococcus agalactiae* ATCC 12386, *A. hydrophila* ATCC 7966, *V. harveyi* ATCC 14126, *V. parahaemolyticus* ATCC 17802, and *V. alginolyticus* ATCC 17749 were obtained from the collections of the laboratories of the Center for Brackish Water Aquaculture (Jepara, Indonesia), the Center for Fish Health and Environmental Testing (Serang, Indonesia), and the Technical Implementation Unit for Testing and Application of Fishery Product Quality (Serang, Indonesia). The bacterial suspension was adjusted to a 0.5 McFarland turbidity standard, which corresponded to approximately 1.5×10^8 CFU mL^{-1}. The actual CFU count was then confirmed by serial dilution and plate counting.

Disk diffusion assay of postbiotic against aquaculture pathogenic bacteria

The disc diffusion assay using postbiotic samples followed the CLSI (2020) guidelines and was performed on MHA for *A. hydrophila*, on MHA supplemented with 5% (0.05 mL mL^{-1}) sterile lysed horse blood for *S. agalactiae*, and on

MHA supplemented with 1% (10 mg mL^{-1}) analytical-grade sodium chloride (NaCl) for the three *Vibrio* species. The inoculation was performed by dipping a sterile cotton swab into the standardized bacterial suspension and rotating it firmly against the inside wall of the tube to remove excess liquid. The swab was then streaked evenly in three directions across the entire agar surface to ensure a uniform bacterial load and avoid liquid pooling. Following inoculation, sterile 6-mm paper discs pre-impregnated with 10 µL of undiluted postbiotic (100% v/v) were placed onto the inoculated agar medium at approximately 24 mm apart. The treatment medium was then inverted and incubated at approximately 28°C for approximately 24 hours.

Minimum inhibitory concentration of postbiotic against aquaculture pathogenic bacteria

The broth microdilution method was performed to determine the minimum inhibitory concentration (MIC) in accordance with CLSI (2020) guidelines, using twofold serial dilutions with three replicates. Standard Cation-Adjusted Mueller-Hinton Broth (CAMHB; Merck KGaA, Germany) was used for *A. hydrophila*, whereas for *S. agalactiae*, the CAMHB medium was supplemented with 5% sterile lysed horse blood. Additionally, CAMHB supplemented with 1% NaCl was utilized for the three *Vibrio* species. In the initial stage, 50 µL of sterile medium was added to all wells of a 96-well microplate (well rows 2 to 12). Then, 100 µL of undiluted postbiotic stock (100% v/v) was added to well column 1. A total of 50 µL of stock extract was transferred to well column 2, which already contained 50 µL of media, and homogenized using a micropipette with the up-and-down pipetting technique. This dilution process (1:2) was repeated sequentially up to the tenth well column, until a uniform volume (50 µL) was obtained in all well columns. The initial concentration ranged from well columns 1 to 10, from 100% v/v to 0.19% v/v. In the final step, 50 µL of bacterial suspension was prepared in media at a density of 1×10^6 CFU mL^{-1} and inoculated into each well (except well 11), resulting in a concentration in wells 1 to 10 of 50% to 0.09% v/v while achieving a final standard inoculum concentration of 5×10^5 CFU mL^{-1}. Well 11 served as the growth control (bacteria without postbiotic). The 96-well microtiter plates containing the treatments were then incubated at 28°C for 24 hours. MIC values were considered valid only when the growth control exhibited turbidity.

Minimum bactericidal concentration of postbiotic against aquaculture pathogenic bacteria

A subculture assay was performed to determine the minimum bactericidal concentration (MBC), with three replicates. To determine the MBC value, 50 µL was taken from the well with a concentration equal to double the MIC (2× MIC) and from the growth control well. This suspension was inoculated onto standard MHA for *A. hydrophila*, MHA with 5% sterile lysed horse blood for *S. agalactiae*, and MHA with 1% NaCl for the three *Vibrio* species. The agar plate containing the inoculum was incubated at 28°C for 24 hours. After the incubation period, the number of live colonies (30-300) was counted, expressed as CFU mL^{-1}.

Data analysis

Experimental data, including quantitative bioassays, semi-quantitative evaluations, and untargeted chemical profiling, were analyzed using IBM SPSS Statistics version 26.0 (IBM Corp., USA). Quantitative zones of inhibition for extract-treated *Bacillus* consortium (six treatments, three replicates) and postbiotic-treated aquaculture pathogens (five treatments, three replicates) were assessed using the Kruskal-Wallis H-test to determine the overall effects of the treatments and presented as mean ± SD. Subsequently, Dunn's pairwise comparisons ($p < 0.05$) were performed, with Bonferroni-adjusted p-values applied solely to the extract-treated groups. Categorical postbiotic data from broth microdilution and subculture outcomes for pathogens were analyzed using Fisher's exact test followed by pairwise Z-tests. Bacterial viability counts (4 treatments, 3 replicates) were subjected to Log_{10} transformation and subsequently analyzed using a one-way ANOVA with Tukey's post-hoc test, following verification of normality (Shapiro-Wilk) and homogeneity of variances (Levene's) assumptions. Finally, the chemical profiles, which included one BC group and three postbiotic dosage treatment groups containing ALP at 1%, 2%, and 3% (w/v), were filtered by peak area using a stringent fold-change (FC) threshold. Confident identified metabolites exhibiting a two-fold or greater enrichment ($FC \geq 2.0$) compared to the BC group were highlighted as dose-responsive indicators to capture prominent biological changes (Smith et al., 2006). However, due to the single-replicate design of the metabolomics analysis, a more conservative FC threshold of ≥ 5.0 was subsequently applied to filter confidently identified metabolites and minimize false positives. Subsequently, the antibacterial potency of the identified metabolites was predicted *in silico* via PASS Online by submitting the canonical Simplified Molecular-Input Line-Entry System (SMILES) string for each metabolite, retrieved from the PubChem database. Compounds with a probability of being active (*Pa*) greater than the probability of being inactive (*Pi*) were considered to have potential antibacterial activity (Ks et al., 2026).

RESULTS

Bacillus consortium resistance against *Avicennia marina* leaf extract

The present results revealed a significant overall difference in inhibition zone diameters among the treatment groups ($p < 0.05$; Figure 1). Further comparative analysis confirmed that the negative control (Group A) and the 25 mg mL^{-1} *A. marina* leaf extract treatment (Group B) had the same mean diameter (6.00 ± 0.00 mm), indicating no inhibitory activity. A gradual increase in inhibition zone diameters was observed in groups C (6.42 ± 0.01 mm), D (6.83 ± 0.02 mm), and E (7.17 ± 0.04 mm), resulting in minor inhibition zones. The present results indicated that groups C and D did not differ significantly from Group A ($p > 0.05$). Group E differed significantly from groups A and B ($p < 0.05$), while no significant differences were observed among groups C, D, and E ($p > 0.05$). The current results indicated no significant difference among groups C, D, and E ($p > 0.05$). The positive control produced significantly larger inhibition zones (23.78 ± 0.16 mm) compared to all extract-treated groups ($p < 0.05$). All extract treatment groups (B to E) produced inhibition zones below the 10 mm resistance threshold, categorizing the bacterial consortium as resistant to the *A. marina* leaf extract (Burhanuddin et al., 2019; Irkitova et al., 2021).

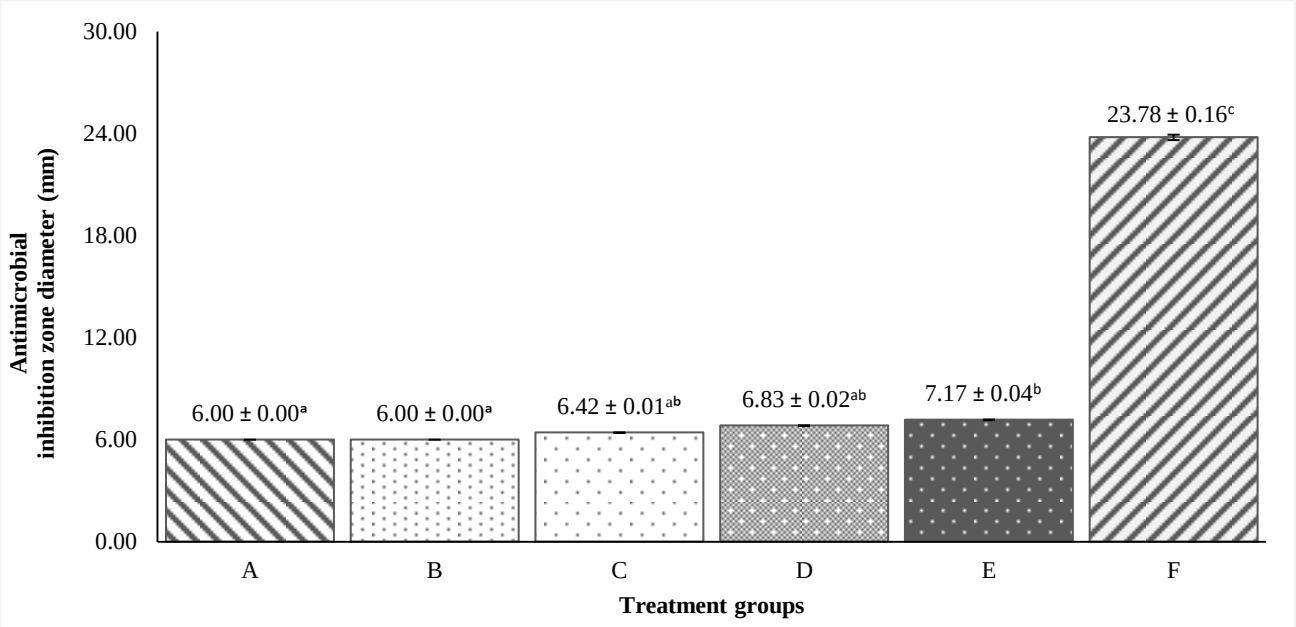

Figure 1. Antimicrobial inhibition zone diameters in the disk diffusion assay of *Bacillus* consortium enriched with *Avicennia marina* leaf extract. [a,b,c] Mean different superscript letters indicate significant differences across the groups ($p < 0.05$). **A:** Negative control group (ethanol), **B:** 25 mg mL^{-1} of *A. marina* leaf extract, **C:** 50 mg mL^{-1} of *A. marina* leaf extract, **D:** 75 mg mL^{-1} of *A. marina* leaf extract, **E:** 100 mg mL^{-1} concentration of *A. marina* leaf extract, **F:** Positive control group (Ciprofloxacin). Data are presented as mean ± SD.

Postbiotic bacterial viability

Bacterial viability was assessed to determine the effects of different ALP concentrations (1%, 2%, and 3%) and the BC group before and after thermal inactivation, centrifugation, and filtration (Figure 2). The viability of the *Bacillus* consortium in the BC group was 7.86 ± 0.012 $\log_{10}$ CFU mL^{-1}. Prior to inactivation, centrifugation, and filtration, the addition of ALP resulted in a significant, concentration-dependent increase in bacterial viability compared to the BC group ($p < 0.05$). Specifically, the bacterial viability increased to 8.80 ± 0.007 $\log_{10}$ CFU mL^{-1} in the 1% ALP group, 8.98 ± 0.009 $\log_{10}$ CFU mL^{-1} in the 2% ALP group, and 9.08 ± 0.010 $\log_{10}$ CFU mL^{-1} in the 3% ALP group, with all treatment groups being significantly different from both the control and each other ($p < 0.05$). In contrast, the viability of the *Bacillus* consortium at all postbiotic concentrations (including the control) after inactivation, centrifugation, and filtration demonstrated no significant differences ($p > 0.05$). The bacterial viability values across all treatment groups, including the BC group, were zero, indicating that the postbiotic suspension contained no live bacteria. Comparison of these findings indicated that adding a substrate (prebiotic) before inactivation increased bacterial viability in culture, with higher ALP concentration promoting greater bacterial growth. Meanwhile, the bacterial viability values after inactivation, centrifugation, and filtration indicated compliance with the scope of postbiotics, which defines postbiotics as preparations without live microorganisms (Thakur et al., 2025).

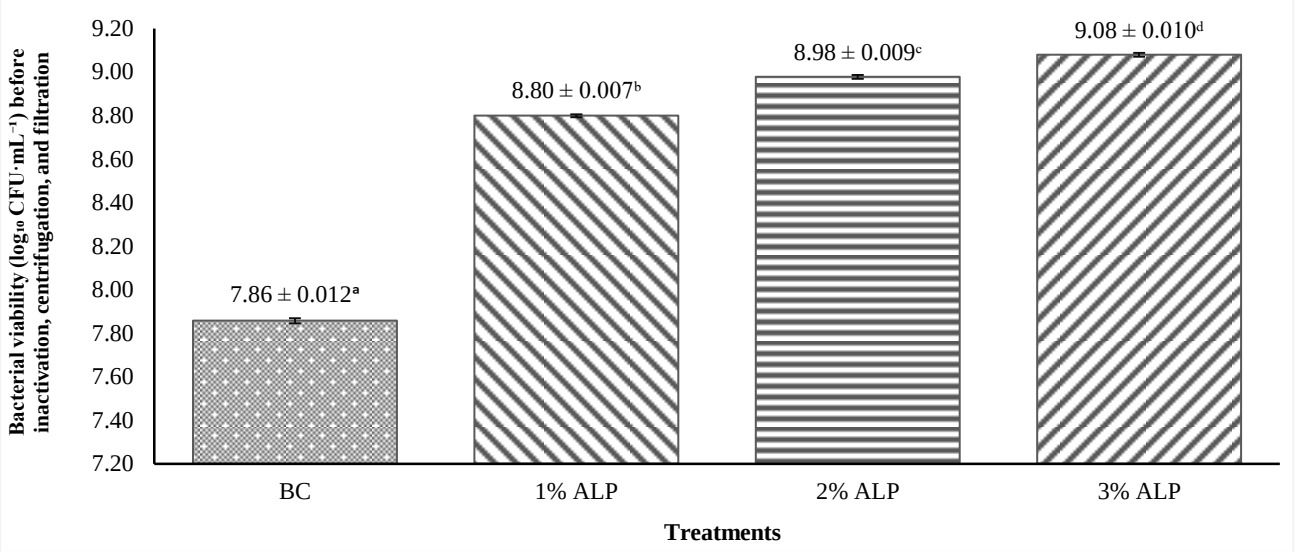

Figure 2. Bacterial viability values before inactivation, centrifugation, and filtration of *Avicennia marina* leaf powder. [a,b,c,d] Mean different superscript letters indicate significant differences across the treatment groups ($p < 0.05$). BC: Baseline control group, 1% ALP: *A. marina* leaf powder at 1%, 2% ALP: *A. marina* leaf powder at 2%, 3% ALP: *A. marina* leaf powder at 3%. Data are presented as mean ± SD.

Metabolomics profiling and *in silico* screening of antibacterial potency of postbiotic

Initial untargeted metabolomics screening via LC-HRMS revealed a concentration-dependent increase in the total number of identified metabolites. In the BC group, 384 metabolites were detected, increasing to 493 in the 1% ALP group, 515 in the 2% ALP group, and peaking at 543 in the 3% ALP group. The peak area of each identified metabolite was normalized to its relative abundance (constant-sum normalization). Initial screening using a fold-change threshold of FC > 2.0 compared to the BC group yielded 21 candidate metabolites with four accumulation profile trends (Table 1). According to Table 1, Profile 1, the kinetic pattern demonstrated a linear increase and was characterized by three main metabolites, with the largest increase in peak area observed in the 3% ALP group for Cyclo(-L-Ala-L-Tyr), corresponding to an FC of 8.00. The Versicomide D metabolite exhibited the largest increase in the 2% ALP group, with an FC of 12.70, followed by (R)-Lactaldehyde (FC = 10.34; Table 1, Profile 2). In contrast to the increasing trend observed in Profile 2, Profile 3 demonstrated a consistent decrease in metabolite accumulation as ALP concentration increased. The sharpest decrease in the 3% ALP group was for Tenacibactin B metabolite, with an FC of 4.73. Finally, Table 1, Profile 4 exhibited a biphasic trend dominated by D-(-)-Ribose, which had a sharp increase in the 1% ALP group (FC = 40.99), followed by a sharp decline in the 2% ALP group (FC = 20.56), and a subsequent increase in the 3% ALP group (FC = 20.66).

In addition to the *in vitro* kinetic profile evaluation, an *in silico* analysis using the online PASS tool was conducted to predict the likelihood of metabolite antibacterial activity. The PASS software predicted antibacterial activity when the *Pa* value is higher than *Pi*. A *Pa* value greater than 0.3 indicated moderate activity with high structural novelty, and a value greater than 0.7 suggested a high probability of structural similarity to existing medicines (Ks et al., 2026). In general, the resulting exposure parameters indicated that most identified secondary metabolite candidates had *Pa* values greater than *Pi* (*Pa* > *Pi*), suggesting strong experimental potential for antibacterial activity. Among the main metabolites identified in Profile 1, the metabolite Cyclo-(L-Ala-L-Tyr) demonstrated the highest probability of antibacterial activity, with a *Pa* value of 0.325 and a very low *Pi* value of 0.051. Several key metabolites identified in the present study exhibited notable antibacterial potential, including (R)-Lactaldehyde, Cereusitin A, and FL6DDAGI0001. Conversely, 3-(Hexanoylamino)-2-(pentyloxy)propyl 2-(trimethylammonio)ethyl phosphate was detected as having no antibacterial activity, with *Pa* and *Pi* values of zero. In the present study, based on the FC value (FC > 2) and interpretation criteria (*Pa* > 0.3), the addition of ALP to the basic growth medium of *Bacillus* consortium bacteria indicated successful enrichment of 10 antibacterial metabolites. However, because this study used a single-replicate design for metabolomics analysis, biological variance could not be distinguished from technical artifacts. Therefore, a highly conservative FC threshold (FC > 5) was subsequently applied to filter confidently identified metabolites and minimize false positives. Applying a FC limit, MS/dd-MS2 fragmentation analysis detected the accumulation of five key compounds, including Cyclo-(L-Ala-L-Tyr), (R)-Lactaldehyde, (+)-Flavipucine, 1-[(2R)-3-Cyclopentyl-2-{[formyl(hydroxy)amino]methyl}propanoyl]-N-(ethylcarbamoyl)-L-prolinamide, and D-(-)-Ribose. Prioritizing metabolites with FC > 5 provided a strong indication that the substantial accumulation of these compounds resulted from ALP supplementation.

Table 1. Metabolomic profiling, graded fold-change kinetics, and *in silico* antibacterial potency scores (*Pa*/*Pi*) of the 21 selected candidate secondary metabolites from the postbiotic made by the *Bacillus* consortium enriched with *Avicennia marina* leaf powder

Identified metabolite name	Molecular formula	ADM (ppm)	Calc.MW	m/z	RT (minutes)	Reference ion	Peak area (BC)	Fold-change (FC) > 2.0			*Pa*	*Pi*
								1% ALP	2% ALP	3% ALP		
								Kinetic profile 1				
Cyclo-(L-Ala-L-Tyr)	$C_{12}H_{14}N_2O_3$	1.55	234.1008	235.10807	2.406	[M+H]+1	1478946.4	6.53	7.88	8.00	0.325	0.051
leu-gly-leu	$C_{14}H_{27}N_3O_4$	0.84	301.20041	302.20768	3.905	[M+H]+1	4520442.6	3.87	5.59	7.47	0.188	0.130
Tenacibactin A	$C_{15}H_{28}N_2O_5$	-0.33	316.19972	315.19267	5.518	[M-H]-1	1330985.2	4.33	5.60	6.32	0.251	0.082
								Kinetic profile 2				
Thymidine	$C_{10}H_{14}N_2O_5$	0.47	242.09038	243.09769	0.889	[M+H]+1	14654097.8	2.08	2.41	2.26	0.432	0.024
3-(Hexanoylamino)-2-(pentyloxy)propyl 2-(trimethylammonio)ethyl phosphate	$C_{19}H_{41}N_2O_6P$	-3.79	424.26862	425.27591	3.991	[M+H]+1	269221675.7	2.48	2.75	2.19	0.000	0.000
met-pro	$C_{10}H_{18}N_2O_3S$	1.27	246.10413	247.1114	1.878	[M+H]+1	17140378.3	2.28	2.94	2.60	0.178	0.027
N-Acetyl-D-alloisoleucine	$C_8H_{15}NO_3$	0.55	173.10529	172.098	3.54	[M-H]-1	9238325.8	2.84	3.97	2.70	0.277	0.069
Succinic acid	$C_4H_6O_4$	0.25	118.02664	117.01936	0.902	[M-H]-1	417138243.6	5.07	9.02	8.11	0.288	0.065
(R)-Lactaldehyde	$C_3H_6O_2$	-0.03	74.03678	73.0295	0.902	[M-H]-1	50343018.9	5.34	10.34	9.02	0.390	0.033
trans-Cinnamic acid	$C_9H_8O_2$	0.45	148.0525	147.04522	3.912	[M-H]-1	26890389.2	2.41	4.38	2.71	0.312	0.056
TRILAURYLAMINE	$C_{36}H_{75}N$	0.08	521.58999	522.59727	18.761	[M+H]+1	15551314.7	2.83	3.03	2.83	0.224	0.099
Versicomide D	$C_{19}H_{25}N_3O_4$	1.61	359.18508	360.19236	3.438	[M+H]+1	11940373.3	10.31	12.70	11.22	0.193	0.124
								Kinetic profile 3				
Cereusitin A	$C_{25}H_{34}N_4O_6$	0.42	486.24804	485.24051	5.899	[M-H]-1	2251035.9	2.59	2.40	2.05	0.345	0.044
Indole-3-lactic acid	$C_{11}H_{11}NO_3$	0.13	205.07392	238.10739	4.353	[M+H+MeOH]+1	6855156.2	6.20	5.30	4.15	0.127	0.108
Tenacibactin B	$C_{14}H_{26}N_2O_5$	0.41	302.1843	301.17706	4.547	[M-H]-1	1408088.6	9.03	5.48	4.73	0.276	0.069
								Kinetic profile 4				
(+)-Flavipucine	$C_{12}H_{15}NO_4$	0.59	237.10025	236.09297	4.346	[M-H]-1	8444567.6	5.81	4.94	5.35	0.439	0.023
1-[(2R)-3-Cyclopentyl-2-{[formyl(hydroxy)amino]methyl}propanoyl]-N-(ethylcarbamoyl)-L-prolinamide	$C_{18}H_{30}N_4O_5$	1.12	382.22205	381.21385	3.097	[M-H]-1	4423280.0	7.87	7.02	7.13	0.452	0.021
Acetyl-L-carnitine	$C_9H_{17}NO_4$	0.32	203.11582	202.10855	3.823	[M-H]-1	36399307.2	3.27	2.33	2.94	0.186	0.131
D-(-)-Ribose	$C_5H_{10}O_5$	0.23	150.05286	195.05106	0.761	[M+FA-H]-1	11045182.7	40.99	20.56	20.66	0.378	0.036
FL6DDAGI0001	$C_{22}H_{26}O_6$	0.03	386.17295	387.18023	8.846	[M+H]+1	5282553.8	3.06	2.23	4.16	0.464	0.020
Streptodiketopiperazine A	$C_{13}H_{14}N_2O_2$	1.03	230.10577	231.11304	2.999	[M+H]+1	5412984.0	4.79	4.14	5.86	0.358	0.041

ADM: Annotated deltamass (ppm), Calc. MW: Calculated molecular weight, m/z: Mass-to-charge ratio, RT: Retention time (minutes), ALP: *A. marina* leaf powder, *Pa*/*Pi*: *In silico* antibacterial potency scores, *Pa*: Probability of activity, *Pi*: Probability of inactivity, BC: Baseline control group, 1% ALP: *A. marina* leaf powder at 1%, 2% ALP: *A. marina* leaf powder at 2%, 3% ALP: *A. marina* leaf powder at 3%.

Antibacterial activity of postbiotics against pathogenic bacteria

To assess the broad-spectrum antimicrobial effectiveness of postbiotics, 3% ALP was selected for its highest pre-inactivation cell biomass and greatest chemical diversity. The antimicrobial efficacy of postbiotics was evaluated by measuring the inhibition zone diameter against five target aquaculture bacterial pathogens (Figure 3). There were significant differences in the distribution of antimicrobial inhibition zones among the evaluated pathogenic bacteria ($p < 0.05$). The current results demonstrated that *V. parahaemolyticus* had the largest inhibition zone diameter, at 15.30 ± 0.53 mm, which was not significantly different from that of *V. alginolyticus*, at 14.85 ± 0.22 mm ($p > 0.05$). A moderate inhibitory effect was observed against *V. harveyi*, which indicated an average clear zone diameter of 11.33 ± 0.58 mm. No inhibition zone was observed against *S. agalactiae* or *A. hydrophila* (disc diameter remained at 6.00 mm). The lack of variation for *S. agalactiae* and *A. hydrophila* reflected the absence of inhibition beyond the disc diameter. This negative result indicated a limitation of the current study, suggesting that the antimicrobial efficacy of this postbiotic might have been selective or strain-specific.

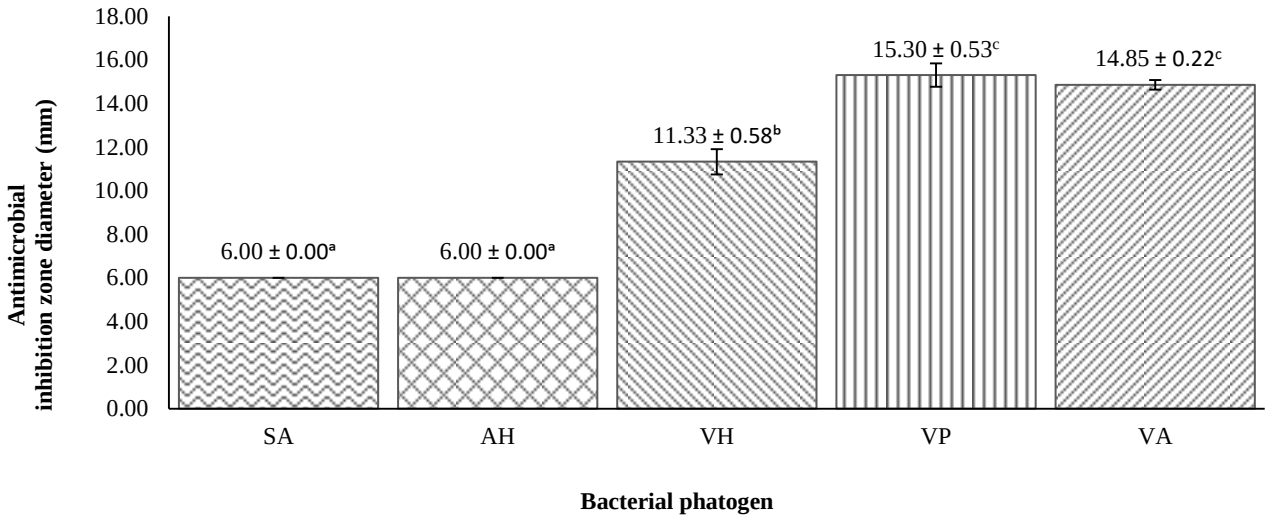

Figure 3. Antimicrobial inhibition zones against aquaculture pathogenic bacteria in the disk diffusion assay, using a 100% (v/v) concentration of the postbiotic derived from a *Bacillus* consortium enriched with 3% *Avicennia marina* leaf powder. [a,b] Mean different superscript letters indicated significant differences among the five evaluated aquaculture pathogens ($p < 0.05$). SA: *Streptococcus agalactiae*, AH: *Aeromonas hydrophila*, VH: *Vibrio harveyi*, VP: *Vibrio parahaemolyticus*, and VA: *Vibrio alginolyticus*. Data are presented as mean ± SD.

The antibacterial potency of postbiotics was further determined by evaluating the MIC and classifying the aquaculture pathogens into susceptibility categories (Table 2). There was a significant relationship between bacterial species and their respective susceptibility profiles ($p < 0.05$). The analysis results divided the five pathogens into two statistically distinct groups based on their sensitivity patterns. *Streptococcus agalactiae* was classified into a separate subgroup, exhibiting complete resistance to the postbiotic, with MIC values surpassing the highest concentration tested (>50% v/v; $p < 0.05$). Compared to the absolute resistance of *S. agalactiae*, all three biological replicates of the other four species (*A. hydrophila, V. harveyi, V. parahaemolyticus*, and *V. alginolyticus*) demonstrated significantly complete susceptibility to the postbiotic at concentrations ≤ 50% v/v ($p < 0.05$). Among the susceptible strains, different degrees of postbiotic sensitivity were observed based on actual MIC values. *Vibrio harveyi* indicated the highest sensitivity, requiring only a very low concentration of the postbiotic (1.6% v/v) to inhibit its growth. Moderate susceptibility was observed in *V. parahaemolyticus* at an MIC value of 12.5% v/v, followed by *A. hydrophila* and *V. alginolyticus*, both of which required a higher concentration of the postbiotic (25 % v/v) to inhibit growth. Negative disc diffusion results but positive MIC results for *A. hydrophila* highlighted the limitations of the present study. This discrepancy between negative disk diffusion results and positive MIC results for *A. hydrophila* might be due to poor diffusion of compounds in the agar and indicated that the antimicrobial efficacy of this specific postbiotic may be selective, highly strain-dependent, or influenced by the test medium.

To determine the bactericidal capacity of postbiotics, the MBC was evaluated, and pathogens were classified into the appropriate susceptibility categories (Table 3). There was a significant relationship between bacterial species and their bactericidal susceptibility profiles ($p < 0.05$). Further analysis divided the five aquaculture pathogens into two statistically distinct groups, consistent with the trend observed in the MIC analysis. *Streptococcus agalactiae* was isolated into a separate (resistant) sub-group, whereas *A. hydrophila*, *V. harveyi*, *V. parahaemolyticus*, and *V. alginolyticus* formed the susceptible group. Compared with the susceptible group, the actual MBC values for *S. agalactiae* at concentrations exceeding 50% v/v indicated that the postbiotic had no bactericidal activity against this species within the tested concentration range ($p < 0.05$). Conversely, all three biological replicates for these four species

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demonstrated a significant susceptibility to the postbiotic treatment at concentrations ≤ 50% v/v ($p < 0.05$). Examination of the actual volumetric concentrations within the susceptible groups revealed different degrees of bactericidal tolerance. *Vibrio harveyi* was the most susceptible pathogen, with the lowest MBC of 6.3% v/v. A moderate bactericidal susceptibility was observed for *V. parahaemolyticus* at 25% v/v. Meanwhile, *A. hydrophila* and *V. alginolyticus* indicated higher tolerance, requiring a postbiotic concentration of 50% v/v to achieve complete bacterial eradication. Based on the inhibition zone, MIC, and MBC values, the postbiotic exhibited selective antibacterial activity against aquaculture pathogens, with greatest efficacy against *Vibrio* species.

Table 2. Minimum inhibitory values and susceptibility categorization of the postbiotic made by the *Bacillus* consortium enriched with *Avicennia marina* leaf powder against aquaculture bacterial pathogens

Bacterial pathogen	Actual MIC value (% v/v)	Susceptible (≤ 50% v/v)	Resistant (> 50% v/v)	Z-Test grouping
	Statistical model	n (%)	n (%)	
Streptococcus agalactiae	> 50	0 (0.0%)	3 (100.0%)	a
Aeromonas hydrophila	25	3 (100.0%)	0 (0.0%)	b
Vibrio harveyi	1.6	3 (100.0%)	0 (0.0%)	b
Vibrio parahaemolyticus	12.5	3 (100.0%)	0 (0.0%)	b
Vibrio alginolyticus	25	3 (100.0%)	0 (0.0%)	b

Statistical model: The most frequently observed inhibitory concentration value among the biological replicates. Z-Test grouping: Different letters indicate statistically significant differences among the bacterial pathogens ($p < 0.05$). a: Resistant pathogen, b: Susceptible pathogens. n: Total number of replicates

Table 3. Minimum bactericidal values and susceptibility categorization of the postbiotic made by the *Bacillus* consortium enriched with *Avicennia marina* leaf powder against aquaculture bacterial pathogens

Bacterial pathogen	Actual MBC Value (% v/v)	Susceptible (≤ 50% v/v)	Resistant (> 50% v/v)	Z-Test grouping
	Statistical model	n (%)	n (%)	
Streptococcus agalactiae	> 50	0 (0.0%)	3 (100.0%)	a
Aeromonas hydrophila	50	3 (100.0%)	0 (0.0%)	b
Vibrio harveyi	6.3	3 (100.0%)	0 (0.0%)	b
Vibrio parahaemolyticus	25	3 (100.0%)	0 (0.0%)	b
Vibrio alginolyticus	50	3 (100.0%)	0 (0.0%)	b

Statistical model: The most frequently observed inhibitory concentration value among the biological replicates. Z-Test grouping: Different letters in the same column indicated statistically significant differences among the bacterial pathogens ($p < 0.05$). a: Resistant pathogen, b: Susceptible pathogens. n: Total number of replicates

DISCUSSION

The preliminary experiment demonstrated that the *Bacillus* consortium, comprising *B. safensis*, *B. mycoides*, and *B. pumilus*, exhibited a notably high level of tolerance to *A. marina* leaf extract. The inhibition zone observed in the disc diffusion assay was deemed biologically insignificant because its size was below the resistance threshold (<10 mm; Burhanuddin et al., 2019; Irkitova et al., 2021). The disc diffusion assay demonstrated that the *A. marina* extract exhibited negligible inhibitory activity against the *Bacillus* consortium in the present study. Previous studies have reported that *A. marina* leaf extract had direct antimicrobial activity against several Gram-positive bacteria (Okla et al., 2021; Sravya et al., 2025). However, the absence of an inhibitory zone in the present study indicated the biological compatibility between the halophyte plant substrate and the targeted bacterial isolate (Seukep et al., 2020). The present study was consistent with the findings of Ibrahim et al. (2022), who reported that ethanol, acetone, and ethyl acetate extracts of *A. marina* (100 mg mL^{-1} in dimethyl sulfoxide) exhibited small inhibition zones (< 7.6 mm) against *B. subtilis*. Species of the genus *Bacillus* are known to have robust adaptive mechanisms to environmental stressors and phytochemical compounds, primarily through alterations in the lipid composition of cell membranes and the activation of efflux systems (Diomandé et al., 2015; Seukep et al., 2020). Moreover, metabolic interactions within a multi-strain consortium often have greater collective protection than single cultures, due to extracellular matrix secretion or metabolic coordination (Azeem et al., 2025). Therefore, the negligible inhibition zones observed at concentrations of 50 to 100 mg mL^{-1}, along with the absence of an inhibition zone at 25 mg mL^{-1}, justified the utilization of ALP as a safe functional substrate in postbiotic production processes.

The incorporation of ALP was shown to notably enhance the viability of the bacterial consortium prior to the inactivation phase in a dose-dependent manner. The increase in bacterial viability from 7.86 ± 0.012 $\log_{10}$ CFU mL^{-1} (baseline control) to 9.08 ± 0.10 $\log_{10}$ CFU mL^{-1} in the 3% ALP group indicated the functional role of the mangrove leaf extract as a potential prebiotic carbon source. This finding is consistent with Goh et al. (2022), who reported that structural polysaccharides and plant-derived natural fibers can serve as non-digestible prebiotics. These compounds are selectively fermented by *Bacillus* spp. and provide a primary energy source for cell replication. The improved growth kinetics can be attributed to the effective use of complex sugars present in mangrove leaves, which are broken down into simple glucose by the innate extracellular hydrolytic enzymes of the *Bacillus* consortium (Sam-on et al., 2023). In addition, the nutrient profile of coastal halophyte plants has been reported to be rich in osmoprotectant compounds and specific micronutrients that synergistically trigger microbial anabolic metabolism, thereby enabling a massive increase in bacterial population density before entering the stationary phase (Rudnyckyj and Thomsen, 2026). In contrast to this massive pre-inactivation growth, after inactivation by heating, centrifugation, and double filtration, the residual viability of all postbiotic suspensions dropped to zero in the present experiment. The complete absence of viable cells confirmed that the final preparations were completely free of living microbial cells and met the International definition of a safe postbiotic preparation (Thakur et al., 2025).

Metabolomics analysis via LC-HRMS revealed potential changes in secondary metabolite profiles across different ALP concentrations. The 3% ALP treatment increased metabolites, and five key compounds were detected, including Cyclo(-L-Ala-L-Tyr), (R)-Lactaldehyde, (+)-Flavipucine, D-(-)-Ribose, and 1-[(2R)-3-Cyclopentyl-2-{[formyl(hydroxy)amino]methyl}propanoyl]-N-(ethylcarbamoyl)-L-prolinamide, which were classified as an Acylprolinamide derivative according to the NCBI (2026a) and Axten et al. (2012). Based on the results of *in silico* screening, the majority of the detected compounds indicated *Pa* values greater than 0.3, suggesting potential antibacterial activity.

The presence of Cyclo(L-Ala-L-Tyr) in the BC group, according to the mechanism supported by Ryan et al. (2009), indicated that Cyclo(L-Ala-L-Tyr) originated from the utilization of free amino acids in the peptone component as the sole source of organic nitrogen in the base medium. Diketopiperazines are secondary metabolites that have been confirmed to be synthesized by *Bacillus* spp. and function as potential antimicrobial agents against target microbes, as well as serve as intercellular communication signaling molecules (Lu et al., 2009; Bofinger et al., 2017). The high level of Cyclo(L-Ala-L-Tyr) following the addition of ALP was likely due to an influx of plant-derived amino acids, which increased the nutrient reservoir in the fermentation medium, as evidenced by the findings of Hinokidani et al. (2020). Additionally, NATURAL phenolic compounds in the mangrove leaf powder act as organic elicitors, serving as low-level environmental stressors. According to the findings of Bofinger et al. (2017) and Patil et al. (2024), the environmental stress induced by the phenolic compounds triggers a bacterial defense response that upregulates gene transcription, resulting in much higher excretion levels of defense peptides such as Cyclo(L-Ala-L-Tyr).

Furthermore, other compounds, such as (R)-Lactaldehyde in the BC group, were derived from the catabolism of glucose, the main carbon source in the baseline medium. This observation was consistent with the findings of Wang et al. (2024), who described that lactaldehyde served as a key biochemical intermediate in the methylglyoxal pathway during glucose catabolism. The notable rise in (R)-Lactaldehyde levels following ALP addition may have resulted from increased glycolytic flux, driven by additional glucose released from mangrove fiber degradation, consistent with the findings of Mehta et al. (2024). The accumulation of (R)-Lactaldehyde contributed to the antibacterial potential of the postbiotic prepared in the present study, which was aligned with the findings of Aljaafari et al. (2022), who reported that (R)-Lactaldehyde could function as a reactive metabolite compound possessing natural inhibitory properties against the growth of pathogenic bacteria.

The initial LC-HRMS annotation identified (+)-Flavipucine, yet manual investigations revealed that this metabolite was taxonomically and biologically irrelevant to the candidate compounds in the *Bacillus* spp. expression system and their antibacterial potential. (+)-Flavipucine is unlikely to produce a dominant signal in negative ionization mass spectrometry (Loesgen et al., 2011; Kruve et al., 2014; NCBI, 2026b). Therefore, the $[M-H]^-$ signal indicates a higher likelihood of N-Lactoylphenylalanine (Lac-Phe), a pseudo-dipeptide that possesses a similar molecular formula and molecular weight (NCBI, 2026c). In the present study, conducting manual investigations of these structural and taxonomic metabolites was crucial for eliminating automated misannotations and directly guiding subsequent antibacterial screening towards a biologically plausible metabolite produced by the *Bacillus* spp. system. In the present study, the detected $[M-H]^-$ signal yielded an experimental m/z value of 236.09297, with a high mass accuracy of 0.59 ppm, perfectly matching the exact theoretical mass of Lac-Phe (Hoene et al., 2022). From a chemotaxonomic perspective, N-Lactoylphenylalanine (Lac-Phe) is a metabolite more likely to be synthesized by *Bacillus* spp. in the medium (Udrea et al., 2026). However, the *in silico* prediction screening results for Lac-Phe indicated *Pa* (0.180) and *Pi* (0.137) values that did not meet the criteria for antibacterial activity. Manual selection confirmed that the metabolite was

N-Lactoylphenylalanine (Lac-Phe) rather than the database-annotated (+)-Flavipucine. However, Lac-Phe was excluded as an antibacterial candidate due to a low predicted activity score (*Pa* < 0.3).

The presence of acylprolinamide derivatives in the control group indicated that the *Bacillus* consortium likely synthesized acyl-amide-based secondary metabolites naturally, using the growth medium to support their basal metabolic activity (Stecker et al., 2022; Shiri et al., 2023). The increase in compound production following the addition of mangrove leaf powder might be linked to environmental stress-elicitation mechanisms (Razzaq et al., 2025). A 1% ALP treatment was believed to induce a hormetic effect strong enough to activate a bacterial defense response. Meanwhile, fluctuations in the results of 2% and 3% ALP treatments suggested that exposure to mangrove secondary metabolites at higher doses induced environmental stress, potentially shifting cellular energy from secondary biosynthesis to essential survival processes. The accumulation of acylprolinamide derivatives was believed to contribute to antibacterial activity, as reported by Axten et al. (2012), that the acylprolinamide group has the potential for broad-spectrum antibacterial activity. The detection of D-(-)-Ribose in the BC group might be attributed to pentose phosphate pathway metabolism, which occurred during glucose oxidation in the basal media (Liu et al., 2023). However, the D-(-)-Ribose peak area increased following the addition of ALP, likely because the inherent hemicellulosic components of *A. marina* leaves (Almardeai et al., 2017) provided the *Bacillus* consortium with additional precursors for pentose sugar accumulation. The D-(-)-Ribose peak demonstrated a notable increase in the overall group of pentose sugars. The accumulation of D-(-)-Ribose in postbiotics in the present study was an important finding because it has been shown to be effective as an adjuvant that restores the sensitivity of resistant bacteria to standard antibiotics such as gentamicin (Zhou et al., 2022).

The present study focused exclusively on four key compounds found in both the control and treatment groups, namely Cyclo(-L-Ala-L-Tyr), (R)-Lactaldehyde, an acylprolinamide derivative, and D-(-)-Ribose. The focus on these four metabolites was driven by the specific objective of assessing the impact of ALP supplementation, as a quantitative stimulant, on the abundance of metabolites identified in the *Bacillus* expression system.

The antibacterial efficacy of the postbiotic, based on a *Bacillus* consortium enriched with 3% ALP, showed a distinctive selectivity pattern, with the highest inhibitory activity against marine pathogenic *Vibrio* bacteria. Based on the diameter of the inhibition zone, the most extensive response was recorded against *V. parahaemolyticus* and *V. harveyi*. The Current findings strongly indicate the underlying functional capacity of key compounds that exhibited increased abundance following ALP supplementation. According to Leyton et al. (2012), the genus *Bacillus* produces diketopiperazine compounds that act as quorum-sensing antagonists against *Vibrio* spp., strongly reducing virulence and biofilm formation. Furthermore, the increased abundance of D-(-)-Ribose in the postbiotic formulation enriched with 3% ALP was believed to provide a unique synergistic effect. Consistent with the present study, Niazy (2021) reported that at specific concentrations, D-ribose can interfere with the quorum-sensing pathway (AI-2) and downregulate luxS expression, thereby remarkably inhibiting biofilm maturation in *Vibrio* species.

In the microdilution evaluation assays, *V. harveyi* was identified as the most sensitive strain with an MIC of 1.6% v/v and an MBC of 6.3% v/v. The observed high sensitivity indicated that the outer membrane of *V. harveyi* was especially susceptible to the enzyme-targeting small molecules present within the postbiotic. The physiological susceptibility of *V. harveyi* aligned with the ability of acylprolinamide derivatives to penetrate the Gram-negative membrane and competitively inhibit peptide deformylase (PDF), thereby preventing the post-translational maturation of essential proteins (Mamelli et al., 2009; Axten et al., 2012). The observed intracellular enzymatic inhibition was indicated to act in conjunction with (R)-Lactaldehyde (Jabri et al., 2023). In the present study, these reactions likely facilitated the detoxification of methylglyoxal and maintained the overall efficacy of the postbiotic matrix against pathogenic bacteria.

In contrast to the susceptibility observed in the Gram-negative strain (*V. harveyi*), the 3% ALP postbiotic exhibited no meaningful antibacterial efficacy against the Gram-positive pathogen, *S. agalactiae*, indicating absolute resistance, with MIC and MBC values exceeding the maximum tested concentration (> 50% v/v). This result contradicted some previous studies that found *A. marina* leaf extracts generally exhibit broad-spectrum activity against Gram-positive bacteria due to the absence of a selective outer membrane (Okla et al., 2021; Sravya et al., 2025). Nevertheless, the observed resistance of *S. agalactiae* was consistent with previous findings in which Gram-positive bacteria exhibited lower susceptibility to *A. marina* extracts, which are characterized by predominantly polar antimicrobial agents (Ibrahim et al., 2022). This disparity indicated that the antibacterial activity of the 3% ALP postbiotic formulation was not caused by a general membrane disruption, as observed in typical organic solvent extracts, but instead resulted from specific molecular interactions. The resistance of S. agalactiae could be due to a structural barrier, such as a thick peptidoglycan layer, or the lack of specific molecular targets that Cyclo(L-Ala-L-Tyr) compounds and acylprolinamide derivatives can recognize (Fieulaine et al., 2016). Meanwhile, the moderate response of *A. hydrophila* to the 3% ALP postbiotic indicates differential susceptibility, likely due to variations in its lipopolysaccharide structure or efflux pump system.

These differences may necessitate a higher concentration of the active compound to effectively disrupt its cellular defense mechanisms (Gonzalez-Avila et al., 2021; Huang et al., 2022b).

CONCLUSION

The postbiotic formulation produced by the *Bacillus* consortium demonstrated favorable compatibility with the *A. marina* leaf substrate. The absence of an inhibition zone at 25 mg mL^{-1} of *A. marina* leaf extract, along with minimal zones (6.42 ± 0.02 mm to 7.17 ± 0.02 mm) exhibited at 50 to 100 mg mL^{-1}, indicated that *A. marina* leaf extract effectively acted as a growth-supporting factor. The 3% ALP treatment increased bacterial viability before the inactivation phase and enriched the identified metabolite profile, from 384 metabolites (baseline control) to 543 metabolites. Consequently, this process resulted in the accumulation of four key antibacterial candidate compounds, namely Cyclo(-L-Ala-L-Tyr), (R)-Lactaldehyde, an acylprolinamide derivative, and D-(-)-Ribose. *In vitro*, the postbiotic derived from the *Bacillus* consortium enriched with 3% ALP demonstrated selective antibacterial efficacy against the pathogenic *Vibrio* group, as evidenced by prominent inhibition zones ranging from 11.33 to 15.30 mm, low MIC (1.6-25% v/v), and low MBC (6.3-50% v/v) values. While both *S. agalactiae* and *A. hydrophila* indicated no inhibition zones in the disk diffusion assay, *A. hydrophila* demonstrated susceptibility in the broth microdilution method, in contrast to the complete resistance of *S. agalactiae* up to the highest tested concentration (MIC > 50% v/v). Although enhanced bacterial viability and enriched metabolite profiles indicated the postbiotic's antibacterial potential, the metabolomic screening was performed on a single biological replicate per treatment group; therefore, future studies should include biological replicates to strengthen the metabolomic data analysis.

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Authors' contributions

Ashari Fahrurrozi conducted visualization, methodology, data collection, formal analysis, data management, conceptualization, and manuscript drafting. Slamet Budi Prayitno and Sarjito Sarjito contributed to the study design, conceptualization, methodology, supervision, and manuscript drafting. Aninditia Sabdaningsih managed supervision, administration, data processing, and drafted the manuscript. All authors have read and approved the final edition of the manuscript.

Availability of data

All relevant data generated during the study are included in this published article and are available upon reasonable request from the corresponding author.

Competing interests

The authors declared that they have no conflicts of interest or personal relationships that could affect this article.

Ethical considerations

The authors declared that the manuscript is original, has not been published elsewhere, and that the latest edition was confirmed before publication. The authors declared the data's originality prior to submission and reaffirmed it during the peer-review process, assuming full responsibility for its originality. The authors declared that no AI tools were used for writing and preparing the article.

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