Bionatura Journal
Ibero-American Journal of Biotechnology and Life Sciences

Article

Initial analysis of profiles from *Pseudovibrio denitrificans* and *Vibrio parahaemolyticus* antagonism by LC-MS-based metabolomics

Ricardo Palma[1], Bolivar Chalén[1], Noroska G.S. Mogollón[2], Jenny Rodríguez[1], Cristian Quiroz-Moreno[2,3*]*

[1] *ESPOL Polytechnic University, Escuela Superior Politécnica del Litoral, ESPOL, Centro Nacional de Investigaciones Marinas (CENAIM), Campus Gustavo Galindo Km. 30.5 Vía Perimetral, P.O. Box 09-01-5863, Guayaquil, Ecuador. B bochalen@espol.edu.ec , jenrodri@espol.edu.ec*
[2] *Biomolecules Discovery Group, Universidad Regional Amazónica Ikiam, Km 7 Via Muyuna, Tena, Napo, Ecuador.; noroska.salazar@ikiam.edu.ec. ORCID:*
[3] *Horticulture and Crop Science Deparment, Ohio State University, USA, quirozmoreno.1@osu.edu,*
** Correspondence: rspalma@espol.edu.ec, quirozmoreno.1@osu.edu*
Available from. http://dx.doi.org/10.21931/BJ/2024.01.02.4

ABSTRACT

Research on bioprospecting bioactive secondary metabolites from marine bacteria has rapidly expanded in the past few decades. The complexity of the environment in which bacterial communities live involves complex interactions with other communities competing for resources for survival. Previously, the ability of Pseudovibrio denitrificans has been demonstrated to inhibit the growth and virulence of different pathogens such as Vibrio parahaemolyticus. The antagonism of these bacteria has been studied, considering the latter's importance in producing farmed shrimp. The molecules produced by P. denitrificans may be fundamental for its antibacterial effect. In the present work, we examine LC/MS profiles of the interaction under two different circumstances: direct and delayed antagonism. The results show marked differences in the chemical composition between both interactions and the tentative identification of antibacterial and antibiofilm compounds. The observed profile could be part of P. denitrificans chemical arsenal to halt V. parahaemolyticus growing. The presented data is relevant to designing future studies on bacterial antagonism with relevant implications for the aquaculture industry.

Keywords: vibriosis, probiotics, chemometrics, white shrimp.

INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative bacterium commonly present in aquatic environments responsible for infections in humans and several marine organisms. Virulent strains of *V. parahaemolyticus* are the leading causative agent of acute hepatopancreatic disease (AHPND) that affects the production of relevant shrimp species such as Litopennaeus vannamei. The shrimp industry is estimated to lose one billion dollars annually due to AHPND outbreaks. The emergence of resistant strains to current antimicrobial treatments and the ability to form bacterial biofilms promote the persistence of pathogenic V. parahaemolyticus in aquaculture systems. In this scenario, exploring new alternatives to neutralize the impact of the disease is crucial.

https://bionaturajournal.com/

Marine biodiversity is an exciting source of microorganisms that can be applied in shrimp farms. Bacteria associated with marine eukaryotic organisms represent an impressive source of desirable molecules that have been discovered through the application of metabolomics techniques[1]. Bacteria isolated from marine invertebrates such as the Pseudovibrio genus have raised interest due to the antimicrobial compounds they produce[2]. Previously, Harrington et al. [3] identified the presence of molecules capable of inhibiting the growth and attenuating the virulence of V. parahaemolyticus, some of which are responsible for the loss of farmed shrimp production.

In nature, the interaction between different organisms involves competition for resource availability. This is typical of bacterial communities, where antagonism represents a strategy to compete for primary resources mediated by the production of compounds to deter competitors [4,5]. For instance, these molecules can act as antibiotics or inhibit communication (quorum sensing) of the antagonist by interfering with processes such as aggregation, bacterial motility and the release of toxins [6]. Because of this, molecules that emerge due to this ecological interaction have raised the interest of different studies, considering their potential against pathogenic organisms[7,8].

Metabolome comprises low molecular weight organic compounds, including bioactive molecules resulting from bacterial interactions[8]. Recent advances in analytical techniques have allowed the study of organisms' response to external stimuli, which result in changes in the metabolome within complex biological samples[9]. Metabolomic studies of changes in *Pseudovibrio spp*. profiles driven by its coculture with *V. parahaemolyticus* are relevant, considering its potential as a source of compounds for aquaculture activities[10,11]. In the present work, we study the metabolomic profiles of *Pseudovibrio denitrificans* comparing three different conditions: axenic culture, direct and delayed antagonism with Vibrio parahaemolyticus in order to detect the most significant changes at metabolomic level between the evaluated conditions. Our results provide new insights to identify the direction of future studies for elucidation of compounds of interests.

MATERIALS AND METHODS

Pseudovibrio denitrificans was isolated from sessile marine invertebrate organisms from the El Pelado Marine Reserve in Santa Elena, Ecuador. *Vibrio parahaemolyticus* (BA94C2A) was used to induce antagonism and was obtained from the National Center for Aquaculture and Marine Research (CENAIM-ESPOL) strain collection.

Three treatments were prepared to evaluate different conditions of the antagonism. First, *V. parahaemolyticus* was cultured in TSB for 12 h and adjusted to an absorbance of 0.3 ($1x10^8$ cells/ml). The bacteria were suspended in a 2% NaCl solution (JT Baker, USA). Then, 200 µl of the suspension was taken and cultured by extension in marine agar (Difco, United States). *P. denitrificans strain* was cultured in conditions similar to the one described above. After incubation, *P. denitrificans* colonies were collected and placed in a plate as cumulus with a diameter of ~ 2-3 mm on marine agar where *V. parahaemolyticus* suspension was previously spread. Then, the plates identified as delayed antagonism were maintained at 28 °C for 72 h (Heratherm® IGS 180, Thermo Scientific, Germany). As direct antagonism treatment, *P. denitrificans* and *V. parahaemolyticus* were inoculated simultaneously in a petri dish for 72 h at 28 °C. Samples from the culture medium inoculated only with P. denitrificans were included as the axenic culture treatment, and plates without bacteria were used as the negative control. Each treatment was performed in triplicate.

Sample preparation for LC-MS

The culture agar was suspended in a 1:1 acetone/methanol solution (v/v) for extraction. The controls of bacteria cultured separately and the culture medium without inoculation received the same treatment. After 12 h at 20 °C, samples were filtered to obtain the solid residues. Acetone/methanol solution was recovered and evaporated on a rotary evaporator (Buchi, RII, Switzerland) and subjected to Solid Phase Extraction (SPE) with SPE C18 cartridges (Thermo Scientific, HYPERSEC, United States), using five different eluents, 10 mL each: H2O (F1), H2O: MeOH 1: 1 (F2), MeOH (F3) (JT Baker, Trinidad and Tobago), MeOH: CH2CL2 1: 1 (F4), and CH2CL2 (F5) (SupraSolv®, Merck, Germany). Later, MeOH fraction (F3) was dried and later reconstituted in the same solvent filtered through 0.22 µm filters and injected into the ultra-high efficiency liquid chromatograph accoupled to a mass spectrometer (UPLC-MS) (Waters, ACQUITY, USA). Chromatographic runs were performed with a reversed-phase column (BEH C18) of 2.1x150 mm and 1.7 µm in particle size (ACQUITY, Waters, Ireland). The mobile phase consisted of acetonitrile (Sigma-Aldrich, South Korea) and grade I water, acidified with 0.1% trifluoroacetic acid (Sigma-Aldrich, France). A gradient of water (A) and acetonitrile (B) of 0-2 minutes was performed at 10% B, of 2-10 minutes B increased to 100%, of 10-12 minutes B was maintained and finally of 12-15 minutes B dropped to 10%. After separation, samples were analyzed using electrospray ionization (4.5 kV, 0.2 bar, positive ion mode) by TOF-MS in a mass range of 100 to 800 Da. We used pooled quality control samples to assess instrument variability and correct for any source of unwanted variation introduced by the performance of the mass spectrometer. Pooled quality control samples (QC) were made by collecting a 10 µL aliquot of each sample and pooled together. After ten samples, quality control samples were injected to capture and correct for equipment variation.

Data analysis

Raw mass spectrometry data (.raw) was converted to .mzXML files using MSconvert[12], and MS-DIAL was used for peak picking and chromatogram deconvolution[13]. Parameters suggested in MS-DIAL documentation were used for peak deconvolution. Briefly, the MS tolerance was set to 0.01 Da, samples were aligned to QCs samples, and the weights for the alignment were set to 25% in the retention time dimension and 75% in the m/z dimension. Once we obtained the feature table, we followed a rigorous data quality control using R jointly with the notame package (https://github.com/antonvsdata/notame)[14]. First, we diagnosed the drift effect after visual inspection, steadily decreasing the signal throughout the injection. This unwanted source of variation caused quality control samples not to cluster together. Therefore, we correct this effect with a robust linear regression method described in the BatchCorrMetabolomics package (https://github.com/rwehrens/BatchCorrMetabolomics)[15]. We also accounted for feature redundancy by removing features highly correlated with each other based on network analysis. Briefly, this pipeline removes in-source fragments and adducts deconvoluted as individual features rather than being grouped in. a single cluster.

Once the feature table passed all quality control assessments, a partial least square discriminant analysis (PLS-DA) was conducted to find the features that drive the most differences between antagonism interactions. The high-quality feature table was mean-centered, and the PLS-DA model was limited to the three first latent variables. A cross-validation was performed using a k-fold approach, where k = 5. Finally, we filter essential features using the variable importance prediction (VIP) metric. Features with VIP values > 1 created heatmaps and Manhattan plots.

RESULTS AND DISCUSSION

Currently, various platforms are available for metabolomic analysis. One of them is liquid chromatography coupled to mass spectrometry, which is highly sensitive for detecting variations in the dynamics of metabolism[16]. When LC-MS is combined with non-targeted analytical approaches, it enables the identification of metabolites with scarce or no prior knowledge of the composition of the samples, as well as showing known and unknown metabolic changes[16]. However, analysis of the obtained data can be complicated considering the complexity of biological samples[17]. Chemometric methods offer a robust and dependable means to obtain results, aiding in interpreting and rectifying inherent errors associated with this technique in such scenarios[18]. The tools mentioned may help identify metabolome variations from interaction with other organisms[9,17-19].

In this study, we employed partial least squares discriminant analysis (PLS-DA) to observe differences between treatments. The results show a significant difference in the metabolomic profile of the delayed interaction treatment compared to the other conditions (Figure 1). This difference would be related to the advantage of the accumulation of nutrients that V. parahaemolyticus had when it was first inoculated. This allowed the bacteria to adapt, modify the environment, and eventually proliferate in new niches[20]. On the other hand, the dispersion observed between the classes suggests that the compounds produced in the axenic culture and in the direct interaction share similarities. This would indicate that the production of some antagonistic metabolites in P. denitrificans is constitutive[3].

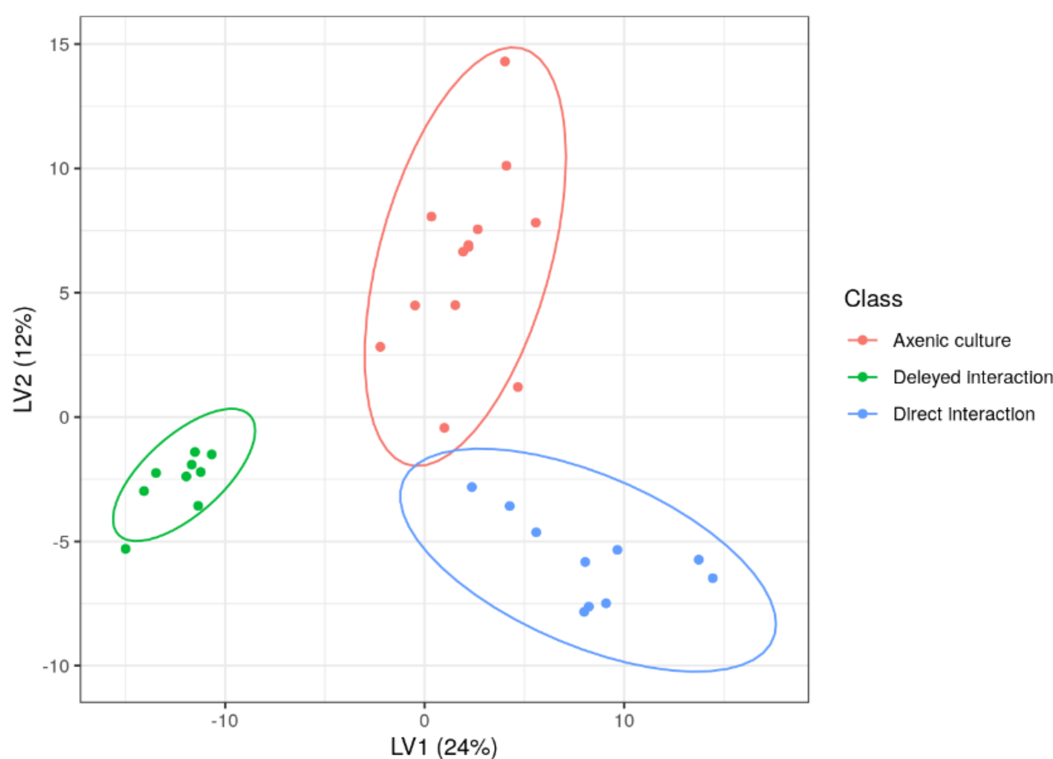

Figure 1. Partial Least-Squares Discriminant Analysis (PLS-DA) of mass spectra of antagonism interaction between P. denitrificans and V. parahaemolyticus (axenic culture of P. denitrificans, delayed interaction and direct interaction).

Previously, *P. denitrificans* has been shown to exert inhibitory activity against five species of Vibrio, including Vibrio parahaemolyticus[10]. A significant increase in the survival rates of white shrimp (larvae and juveniles) was observed in experimental ponds. The ability of Pseudovibrio to compete and displace Vibrio can be related to various mechanisms. For instance, LaSarre and Federle Michael [21] identified that quorum sensing (QS) plays a pivotal role in virulence expression in V. parahaemolyticus. Meanwhile, Pseudovibrio

https://bionaturajournal.com/

could produce QS inhibitors, negatively affecting communication in the Vibrio culture, preventing it from remaining in the medium and attenuating its virulence[3].

The heatmap presents similar information to what was observed in the PLS-DA analysis (Figure 2). Delayed interaction products are grouped in a different and deeper clade than the other samples, evidencing the pronounced separation noted previously. Meanwhile, the axenic culture of *P. denitrificans* and direct interaction appear to originate from the same cluster, indicating similarities. Although the distinction between axenic culture and direct interaction is not as pronounced, it is evident that there are differences in their profiles. These changes may be related to the compounds from V. parahaemolyticus metabolism in the last sample.

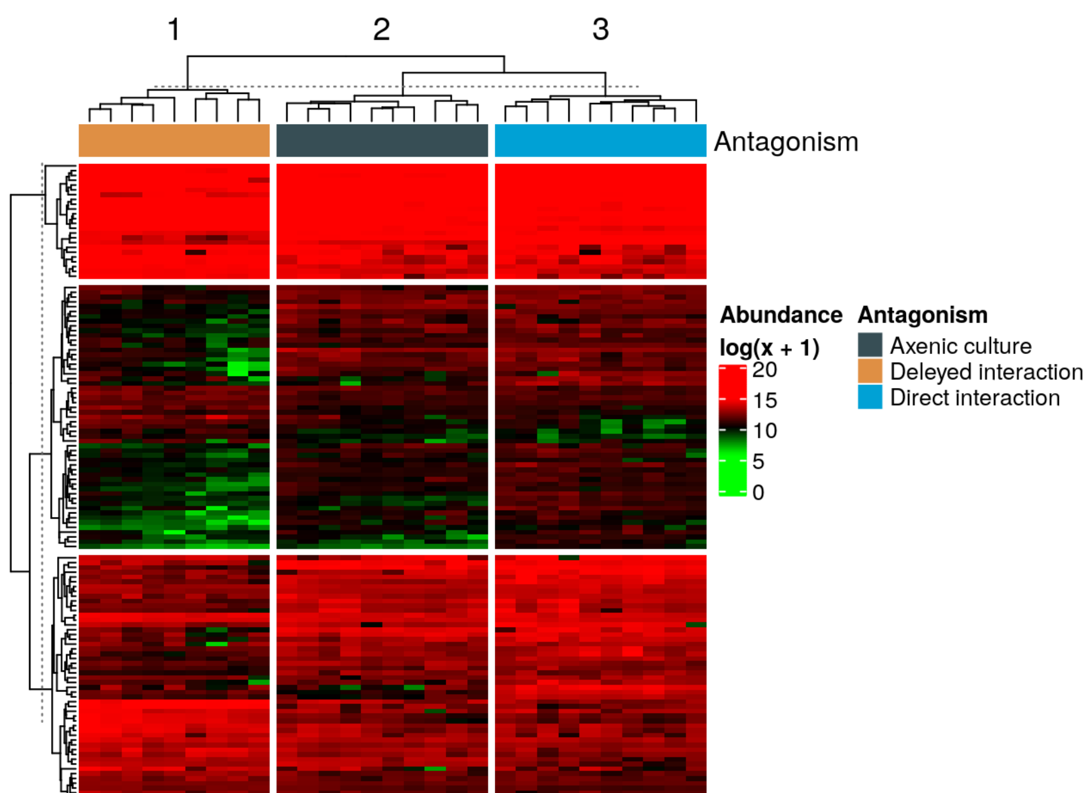

Figure 2. Heat map of mass spectra of antagonism interaction between P. denitrificans and V. parahaemolyticus (axenic culture of P. denitrificans, delayed interaction and direct interaction).

The heatmap also illustrates three defined cluster features for each sample, representing the abundance of a specific signal derived from the chromatograms. A study of the metabolic products of *Pseudoalteromonas piscicid*a and *Vibrio harveyi* revealed a greater diversity of compounds in coculture conditions than in monoculture[22]. The antagonistic effect produced an enrichment and differential expression of a group of metabolites. Similarly, in this work, the direct interaction (coculture) profile exhibited the highest accumulation and variety of compounds according to the heatmap (Figure 2). The relative abundance in delayed interaction is lower than the other two conditions. In the delayed antagonism, V. parahaemolyticus was previously cultured, having access to the nutrients in the medium. After 48 h, the resources available in the agar are limited for P. denitrificans, resulting in altered culture conditions. The profile of secondary metabolites varies according to environmental conditions, as has been observed in recent works[11,23].

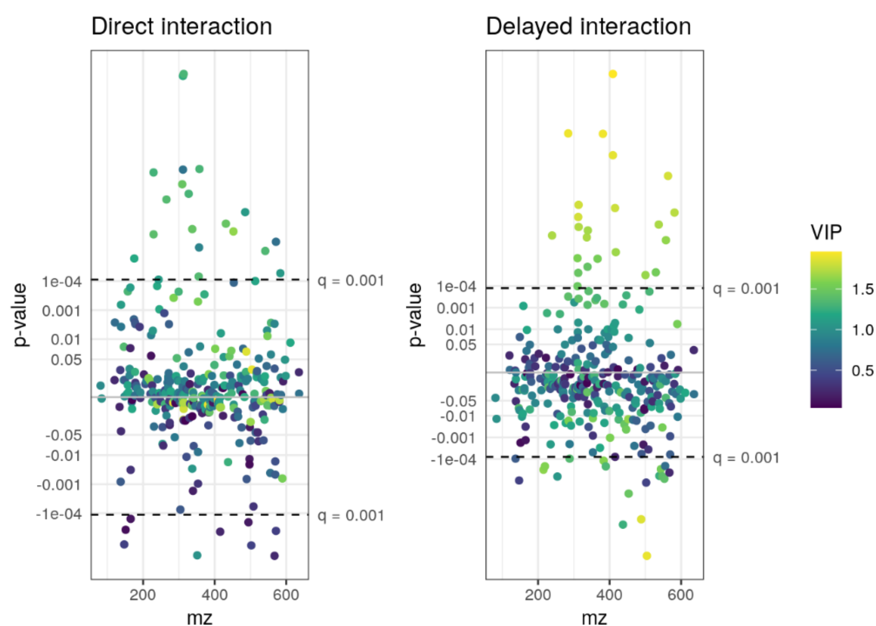

Figure 3. Manhattan plot of the antagonism between P. denitrificans and V. parahaemolyticus. The x-axis of the graph indicates the mass-to-charge ratio of the compounds in the samples. The y-axis illustrates the statistical significance of the p-value of the fold change for the represented features.

The Manhattan plot of direct and delayed interaction reveals the presence of a notable group of features within the delayed interaction profile that does not follow a uniform distribution. VIP values highlight the metabolites that are present in a highly significant amount, which generates a separation from the other data analyzed (Figure 3). Meanwhile, for the direct interaction, the analysis shows uniformity in the p values, which suggests that the abundance of the compounds in this sample is similar [24]. This data is related to the observed results in the PLS-DA and heatmap analysis, which showed that the chemical composition of each interaction is particular. The compounds' abundance and diversity change between the conditions studied in the present work.

Compound	KEGG ID
Pyridoxamine	C00534
o-Nitrobenzoate	C16234
Hexadecasphinganine	C13915
Cytisine	C10763
11-Deoxytetrodotoxin	C20026
1,2-Dihydrostilbene	C14685

Table 1. Identified compounds using the KEGG database.

Lastly, data from delayed interaction was analyzed using the KEGG database to identify compounds in the sample (Table 1). Among them is cytisine, previously characterized as an alkaloid with activity against Escherichia coli[25]. Another reported compound was deoxy tetrodotoxin, described as a neurotoxin produced by Vibrio alginolyticus[26]. Furthermore, the analysis suggests the presence of dihydrostilbene in the delayed interaction. This molecule has been tested in a previous study, showing an effective natural marine antifoulants[27]. It can reduce the activity of bacteria by allowing them to form biofilms, including strains resistant to multiple antibiotics. V. parahaemolyticus virulence and persistence in aquaculture systems have been related to its capacity to produce biofilms[28]. Due to this, several recent investigations have focused on proposing mechanisms to eliminate or reduce biofilms to prevent or treat infections[29-31].

The findings of the present work suggest a significant abundance of compounds in the chemical arsenal of Pseudobrio. The tentative identification showed interesting molecules that could intervene during the antagonism interaction, allowing the displacement of the pathogen. In subsequent works, it will be crucial to confirm the presence of these compounds in the analyzed fractions since they may be essential in the Pseudovibrio mechanism to counteract the growth of Vibrio.

CONCLUSIONS

In this work, we have successfully dived into the antagonism interaction between *P. denitrificans* and *V. parahaemolyticus* using untargeted metabolomics through liquid chromatography coupled with mass spectrometry. Supervised discriminant analysis (PLS-DA) assisted in finding metabolic features that drive the difference between antagonism interactions. By using publicly available databases, we were able to suggest potential metabolites that could be considered biomarkers, such as cytisine, deoxy tetrodotoxin, and dihydrostilbene. However, it is worth noting that the tentative identification of these biomolecules was carried out using mass spectrometry with the monoisotopic mass and the predicted formula from this mass. Therefore, to enhance the confidence in the identity of these molecules, targeted mass spectrometry experiments or comparisons against authentic standards are necessary for an accurate identification.

Author Contributions: Conceptualization, C.Q-M. and BC; methodology, C.Q.-M. and BC; formal analysis, C.Q-M..; investigation, RP and C.Q-M..; resources, NGSM and JR; writing—original draft preparation, RP and C.Q-M..; writing—review and editing, RP, BC, C.Q-M. NGSM and JR; supervision, NGSM and JR; project administration, RP and C.Q-M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Data are available through the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

1 O' Halloran, J. A. et al. Diversity and antimicrobial activity of Pseudovibrio spp. from Irish marine sponges. Journal of Applied Microbiology **110**, 1495-1508, doi:https://doi.org/10.1111/j.1365-2672.2011.05008.x (2011).

2 Penesyan, A. et al. Identification of the Antibacterial Compound Produced by the Marine Epiphytic Bacterium Pseudovibrio sp. D323 and Related Sponge-Associated Bacteria. Marine Drugs **9**, 1391-1402 (2011).

3 Harrington, C. et al. Characterisation of Non-Autoinducing Tropodithietic Acid (TDA) Production from Marine Sponge Pseudovibrio Species. Marine Drugs **12**, 5960-5978 (2014).

4 Russel, J., Røder, H. L., Madsen, J. S., Burmølle, M. & Sørensen, S. J. Antagonism correlates with metabolic similarity in diverse bacteria. Proceedings of the National Academy of Sciences **114**, 10684-10688 (2017).

5 Singh, A. A., Singh, A. K. & Nerurkar, A. Bacteria associated with marine macroorganisms as potential source of quorum-sensing antagonists. Journal of Basic Microbiology **60**, 799-808 (2020).

6 Meschwitz, S. M. et al. Antagonism of Quorum Sensing Phenotypes by Analogs of the Marine Bacterial Secondary Metabolite 3-Methyl-N-(2'-Phenylethyl)-Butyramide. Mar Drugs **17**, doi:10.3390/md17070389 (2019).

7 Esteves, A. I., Cullen, A. & Thomas, T. Competitive interactions between sponge-associated bacteria. FEMS Microbiol Ecol **93**, doi:10.1093/femsec/fix008 (2017).

8 Chalén Alvarado, B., Quiroz Moreno, C., Mogollón, N., Domínguez, C. & Rodríguez, J. Estudio del metaboloma en el antagonismo microbiano a través de cromatografía líquida con algoritmos quimiométricos. (2019).

9 Farrés, M., Piña, B. & Tauler, R. LC-MS based metabolomics and chemometrics study of the toxic effects of copper on Saccharomyces cerevisiae. Metallomics **8**, 790-798, doi:10.1039/c6mt00021e (2016).

10 Domínguez-Borbor, C. et al. The marine symbiont Pseudovibrio denitrificans, is effective to control pathogenic Vibrio spp. in shrimp aquaculture. Aquaculture **508**, 127-136, doi:https://doi.org/10.1016/j.aquaculture.2019.04.077 (2019).

11 Crowley, S. P., Gara, F., Sullivan, O., Cotter, P. D. & Dobson, A. D. W. Marine Pseudovibrio sp. as a Novel Source of Antimicrobials. Marine Drugs **12**, 5916-5929 (2014).

12 Adusumilli, R. & Mallick, P. in Proteomics: Methods and Protocols (eds Lucio Comai, Jonathan E. Katz, & Parag Mallick) 339-368 (Springer New York, 2017).

13 Tsugawa, H. et al. MS-DIAL: data-independent MS/MS deconvolution for comprehensive metabolome analysis. Nature Methods **12**, 523-526, doi:10.1038/nmeth.3393 (2015).

14 Klåvus, A. et al. "Notame": Workflow for Non-Targeted LC–MS Metabolic Profiling. Metabolites **10**, 135 (2020).

15 Wehrens, R. et al. Improved batch correction in untargeted MS-based metabolomics. Metabolomics **12**, 88, doi:10.1007/s11306-016-1015-8 (2016).

16 Choudhary, A., Naughton, L. M., Dobson, A. D. W. & Rai, D. K. High-performance liquid chromatography/electrospray ionisation mass spectrometric characterisation of metabolites produced by Pseudovibrio sp. W64, a marine sponge derived bacterium isolated from Irish waters. Rapid Communications in Mass Spectrometry **32**, 1737-1745, doi:https://doi.org/10.1002/rcm.8226 (2018).

17 Cook, D. W. & Rutan, S. C. Chemometrics for the analysis of chromatographic data in metabolomics investigations. Journal of Chemometrics **28**, 681-687, doi:https://doi.org/10.1002/cem.2624 (2014).

18 Boccard, J. & Rudaz, S. Harnessing the complexity of metabolomic data with chemometrics. Journal of Chemometrics **28**, 1-9, doi:https://doi.org/10.1002/cem.2567 (2014).

19 Yi, L. et al. Chemometric methods in data processing of mass spectrometry-based metabolomics: A review. Analytica Chimica Acta **914**, 17-34, doi:https://doi.org/10.1016/j.aca.2016.02.001 (2016).

20 Li, L. et al. RNA-seq analysis unveils temperature and nutrient adaptation mechanisms relevant for pathogenicity in Vibrio parahaemolyticus. Aquaculture **558**, 738397, doi:https://doi.org/10.1016/j.aquaculture.2022.738397 (2022).

21 LaSarre, B. & Federle Michael, J. Exploiting Quorum Sensing To Confuse Bacterial Pathogens. Microbiology and Molecular Biology Reviews **77**, 73-111, doi:10.1128/mmbr.00046-12 (2013).

22 Ren, W. et al. Multi-Costimulatory Pathways Drive the Antagonistic Pseudoalteromonas piscicida against the Dominant Pathogenic Vibrio harveyi in Mariculture: Insights from Proteomics and Metabolomics. Microbiology Spectrum **10**, e02444-02422, doi:doi:10.1128/spectrum.02444-22 (2022).

23 Conde-Martínez, N., Bauermeister, A., Pilon, A. C., Lopes, N. P. & Tello, E. Integrating Molecular Network and Culture Media Variation to Explore the Production of Bioactive Metabolites by Vibrio diabolicus A1SM3. Mar Drugs **17**, doi:10.3390/md17040196 (2019).
24 Hur, M. et al. A global approach to analysis and interpretation of metabolic data for plant natural product discovery. Nat Prod Rep **30**, 565-583, doi:10.1039/c3np20111b (2013).
25 Yotsu-Yamashita, M. et al. First Identification of 5,11-Dideoxytetrodotoxin in Marine Animals, and Characterization of Major Fragment Ions of Tetrodotoxin and Its Analogs by High Resolution ESI-MS/MS. Marine Drugs **11**, 2799-2813 (2013).
26 Strand, M. et al. The Bacterial (Vibrio alginolyticus) Production of Tetrodotoxin in the Ribbon Worm Lineus longissimus-Just a False Positive? Mar Drugs **14**, doi:10.3390/md14040063 (2016).
27 Moodie, L. W. K. et al. Design and Biological Evaluation of Antifouling Dihydrostilbene Oxime Hybrids. Mar Biotechnol (NY) **20**, 257-267, doi:10.1007/s10126-018-9802-z (2018).
28 Roy, P. K. et al. Elimination of Vibrio parahaemolyticus biofilms on crab and shrimp surfaces using ultraviolet C irradiation coupled with sodium hypochlorite and slightly acidic electrolyzed water. Food Control **128**, 108179, doi:https://doi.org/10.1016/j.foodcont.2021.108179 (2021).
29 Lu, C., Liu, H., Shangguan, W., Chen, S. & Zhong, Q. Antibiofilm activities of the cinnamon extract against Vibrio parahaemolyticus and Escherichia coli. Archives of Microbiology **203**, 125-135, doi:10.1007/s00203-020-02008-5 (2021).
30 Sathiyamoorthi, E., Lee, J.-H., Tan, Y. & Lee, J. Antimicrobial and antibiofilm activities of formylchromones against Vibrio parahaemolyticus and Vibrio harveyi. Frontiers in Cellular and Infection Microbiology **13**, doi:10.3389/fcimb.2023.1234668 (2023).
31 Zhu, W. et al. Anti-Biofilm Activity of Laurel Essential Oil against Vibrio parahaemolyticus. Foods **12**, 3658 (2023).

Received: April 5, 2024 / Accepted: May 22, 2024 / Published: June 15, 2024.

Citation: Palma R, Chalén B, Mogollón NGS, Rodríguez J, Quiroz-Moreno C. Initial analysis of profiles from *Pseudovibrio denitrificans* and *Vibrio parahaemolyticus* antagonism by LC-MS-based metabolomics. Bionatura Journal 2024; 1 (2) 4. http://dx.doi.org/10.21931/BJ/2024.01.02.4

Additional information
ISSN 3020-7886
Correspondence should be addressed to *rspalma@espol.edu.ec, quirozmoreno.1@osu.edu*

Peer review information. Bionatura Journal thanks the anonymous reviewers for their contribution to the peer review of this paper using https://reviewerlocator.webofscience.com/.
All articles published by Bionatura Journal are freely and permanently accessible online immediately upon publication, with no subscription fees or registration barriers.
Editor's note: Bionatura Journal remains neutral regarding jurisdictional claims in published maps and institutional affiliations.
Copyright: © 2024 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).