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Article

High-Resolution Comparative and Quantitative Proteomics of Biogenic-Amine-Producing Bacteria and Virulence Factors Present in Seafood

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ABSTRACT: The presence of biogenic amines (histamine, tyramine, putrescine, and cadaverine) in seafood is a significant concern for food safety. This review describes for the first time a shotgun quantitative proteomics strategy to evaluate and compare foodborne strains of bacteria that produce biogenic amines in seafoods. This approach recognized 35,621 peptide spectrum matches, belonging to 20,792 peptides, and 4621 proteins. It allowed the determination of functional pathways and the classification of the strains into hierarchical clusters. The study identified a protein−protein interaction network involving 1160 nodes/10,318 edges. Proteins were related to energy pathways, spermidine biosynthesis, and putrescine metabolism. Label-free quantitative proteomics allowed the identification of differentially regulated proteins in specific strains such as putrescine aminotransferase, arginine decarboxylase, and L-histidine-binding protein. Additionally, 123 peptides were characterized as virulence factors and 299 peptide biomarkers were selected to identify bacterial species in fish products. This study presents the most extensive proteomic repository and progress in the science of food biogenic bacteria and could be applied in the food industry for the detection of bacterial contamination that produces histamine and other biogenic amines during food processing/storage.

KEYWORDS: *bacteria, bacterial detection, biogenic amine, fish, LC−MS/MS, shotgun quantitative proteomics*

INTRODUCTION

Biogenic amines (BAs) are low-molecular-weight degradation products generated by a variety of bacteria as part of their normal metabolism; they are organic compounds, derived from amino acids, that are naturally present in many foods.[1] However, certain BAs, when consumed in large amounts, can cause adverse reactions in some individuals. Well-known BAs include histamine, tyramine, cadaverine, and putrescine; with histamine the best-known and, probably, the principal common BA responsible for food poisoning. Histamine is a well-characterized primary mediator, implicated in a diversity of physiological activities such as inflammation, neurotransmission, allergic responses, gastric acid production, and cell proliferation, as well as having therapeutic applications as an immunostimulant drug.[2]

As mentioned above, BAs can cause adverse reactions when ingested; hence, their presence in food is a serious cause for concern. In people, these reactions can range from mild symptoms, like headaches, nausea, and vomiting, to more severe conditions, such as hypertension and allergic and anaphylactic reactions. The food items mainly associated with the presence of BAs are either fermented or aged products, like cheese, sausages, certain types of fish (i.e., scombroids), wine, and beer. While the specific bacteria responsible for BA production can vary depending on the food product, it is essential to handle and store foods properly to prevent excessive BA formation and accumulation. BAs are naturally occurring compounds found in various foods and can be formed through the enzymatic decarboxylation of amino acids by certain microorganisms. The formation of BAs in foods can be influenced by several factors, such as microbial activity, amino acid content, alkaline conditions, storage conditions, processing methods, and hygiene and food safety practices. Necessary precautionary measures include maintaining appropriate temperatures during storage, avoiding cross-contamination between different foodstuffs, and consuming perishable foods within their recommended shelf life. In addition, individuals who are particularly sensitive to BAs need to limit their intake of these foods known to contain these compounds; people with a sensitivity or intolerance to BAs should consult a healthcare professional, who can provide personalized advice and guidance.

BA-producing bacteria usually constitute part of the "common" microbiota that live in animals and plants; these microbes comprise species of well-known bacterial families, such as the Enterobacteriaceae (i.e., *Enterobacter*, *Hafnia alvei*, *Proteus* spp., *Klebsiella* spp., *Serratia* spp., and *Salmonella* spp.) and Vibrionaceae (i.e., *Vibrio alginolyticus*), as well as *Pseudomonas*-like species. Taking into account that these

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Table 1. Biogenic-Amine-Producing Bacterial Strains Included in This Study. Spanish Type Culture Collection (CECT)

sample	bacterial strain	code	original code	source	T^a	Niven's medium
S1	*Enterobacter aerogenes*	EbA01	ATCC 13048	CECT 684 T	30 °C	positive
S2	*Enterobacter cloacae*	Ebc11	ATCC 13047	CECT194	30 °C	positive
S3	*Hafnia alvei*	HaA02	ATCC 9760	CECT 157	30 °C	positive
S4	*Klebsiella oxytoca*	KlOx11	ATCC 13182	CECT 860 T	37 °C	positive
S5	*Klebsiella pneumoniae*	KlPn22	ATCC 29665	CECT 997	37 °C	positive
S6	*Proteus mirabilis*	PrM01	ATCC 14153	CECT 4101	37 °C	positive
S7	*Proteus vulgaris*	PrV22	Sard1	Sardine	37 °C	positive
S8	*Providencia rettgeri*	PvR61	ATCC 29944	CECT 865	37 °C	positive
S9	*Providencia stuartii*	PvS51	ATCC 29914	CECT 866	37 °C	positive

bacteria are generally a normal microbiota of the foodstuff, either plant or animal, and the ability of microorganisms to rapidly multiply, the best approach is to speed up the food preservation procedure, as well as rapidly, and unequivocally, identify the important microbial population existing in foods. In order to identify biogenic amine bacteria, Takahashi et al. published in 2003 a polymerase chain reaction (PCR) approach for the amplification of histidine decarboxylase genes for the rapid evaluation of histamine-producing Gram-negative bacteria present in food.[3] On their part, Coton and Coton further developed the procedure in 2005, using multiplex PCR for the identification of the histidine decarboxylase genes existent in Gram-positive bacteria (*Streptococcus*, *Enterococcus*, and *Lactococcus*), as these represent the microorganism described as the principal originators of BAs in fermented food.[4] The use of real-time PCR was also reported for the quantification of the histamine present in fish, wine, and cheese derivatives.[5] Moreover, high-performance liquid chromatography (HPLC) was also applied for the evaluation of BAs in fermented milk products.[6] More recently, the development of novel and advanced procedures, such as liquid chromatography coupled to tandem mass spectrometry (LC−MS/MS)-based proteomics, has achieved the fast identification of the relevant bacterial species, as well as the detection of bacteriophages present in pathogenic bacteria.[7,8] However, its qualitative proteomic application for biogenic-amine-producing bacteria is limited to date.[9]

Fishes are tremendously perishable foods due to their high moisture content, neutral pH, and nutrient-rich composition, which provide an ideal environment for the growth of microorganisms. The primary microbiota involved in the spoilage of fish are psychrotrophic bacteria (*Pseudomonas* spp. and *Shewanella* spp), lactic acid bacteria (*Lactobacillus* spp. and *Leuconostoc* spp.), Enterobacteriaceae (*Escherichia coli* and *Salmonella*), *Clostridium* species, and *Protobacterium* spp. The fish can also be a major reservoir for BAs when contaminated by such bacterial species, particularly those with the ability to produce BAs.[10] Here, we present a novel shotgun quantitative proteomic technique that can be utilized for the fast, and straightforward, comparative analysis of 9 different biogenic-amine-producing bacteria that commonly contaminate seafood. The procedure involves label-free quantification (LFQ), a relative-quantitative proteomics technique that allows the identification of up/downregulated proteins. The data obtained was compared to the protein repository for functional bioinformatics analyses, identifying proteins involved in gene ontology (GO), functional pathways, and hierarchical clustering, as well as protein networks. The research also identified virulence factors and provided specific species-specific peptide biomarkers that could be used to identify foodborne BA-producing bacteria by the food control authorities and food industry in order to guarantee the quality and safety of the consumers.

This work represents the most extensive quantitative proteomic analysis of food biogenic bacteria and could be applied in the food industry for the detection of bacterial contamination that produces histamine and other biogenic amines during food processing/storage. Principally, the present research is important to the fish industry, as several peptide biomarkers for significant biogenic-amine-producing bacteria in seafood were recognized.

MATERIALS AND METHODS

Bacterial Strains. In this research, 9 different seafood biogenic-amine-producing bacteria were investigated (Table 1). The strains were obtained from the Spanish Type Culture Collection (CECT) except the strain *Proteus vulgaris* that was obtained directly from sardine. All of the strains were tested in either Niven's medium or Niven's modified medium.[11] A strain was considered as a potential histamine producer if it displayed a positive reaction in either of these two media, i.e., an increase in the pH of the medium, which was visualized by a change in color.[11] We used Gram-negative bacteria because they are involved in scombroid poisoning and generate much greater concern in seafood. The bacterial strains were previously characterized by 16S rRNA sequencing and matrix-assisted laser desorption ionization−time-of-flight mass spectrometry (MALDI-TOF-MS).[12] Bacterial cultures involved activation in brain-heart infusion (BHI) and incubation, in liquid suspension, at 31 °C for 24 h. The bacteria were then grown on histamine-specific Niven's medium, at either 30 or 37 °C depending on the strain, for 24 h.[12] The confirmation of histamine and other BAs production was achieved by HPLC following the report of Ben-Gigirey et al.[11] All samples were prepared in triplicates.

Protein Extraction. Extract of proteins were prepared as reported by Carrera et al.[7] Briefly, a fresh inoculation loop of bacterial culture was disrupted in 100 μL of a lysis buffer containing 5 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma Chemical Co. St. Louis, MO), 1% dithiothreitol (DTT) (Sigma Chemical), 1% lauryl maltoside (Sigma Chemical), and 60 mM Tris-HCl (Sigma Chemical) pH 7.5. Cell rupture was achieved with the addition of glass beads and several beating cycles, for 10 min at 4 °C. After centrifugation for 10 min at 40,000*g* (J221-M centrifuge, Beckman, CA), the solubilized polypeptides were moved to a new tube, and the protein content was evaluated by the bicinchoninic acid approach (Sigma Chemical).

Peptide Sample Preparation. Proteins were trypsinized as designated previously.[13] For that, 100 μg of protein was dried using a speedvac vacuum concentrator and resuspended in 8 M urea in 25 mM ammonium bicarbonate, pH 8.0 (25 μL). Afterward 5 min of sonication and the addition of DTT at a final concentration of 10 mM, the samples were incubated at 37 °C for 1 h. Iodoacetamide was added at a final concentration of 50 mM, followed by a further incubation at room temperature (20−22 °C), in obscurity, for 1 h. The samples were then diluted four times, with 25 mM ammonium

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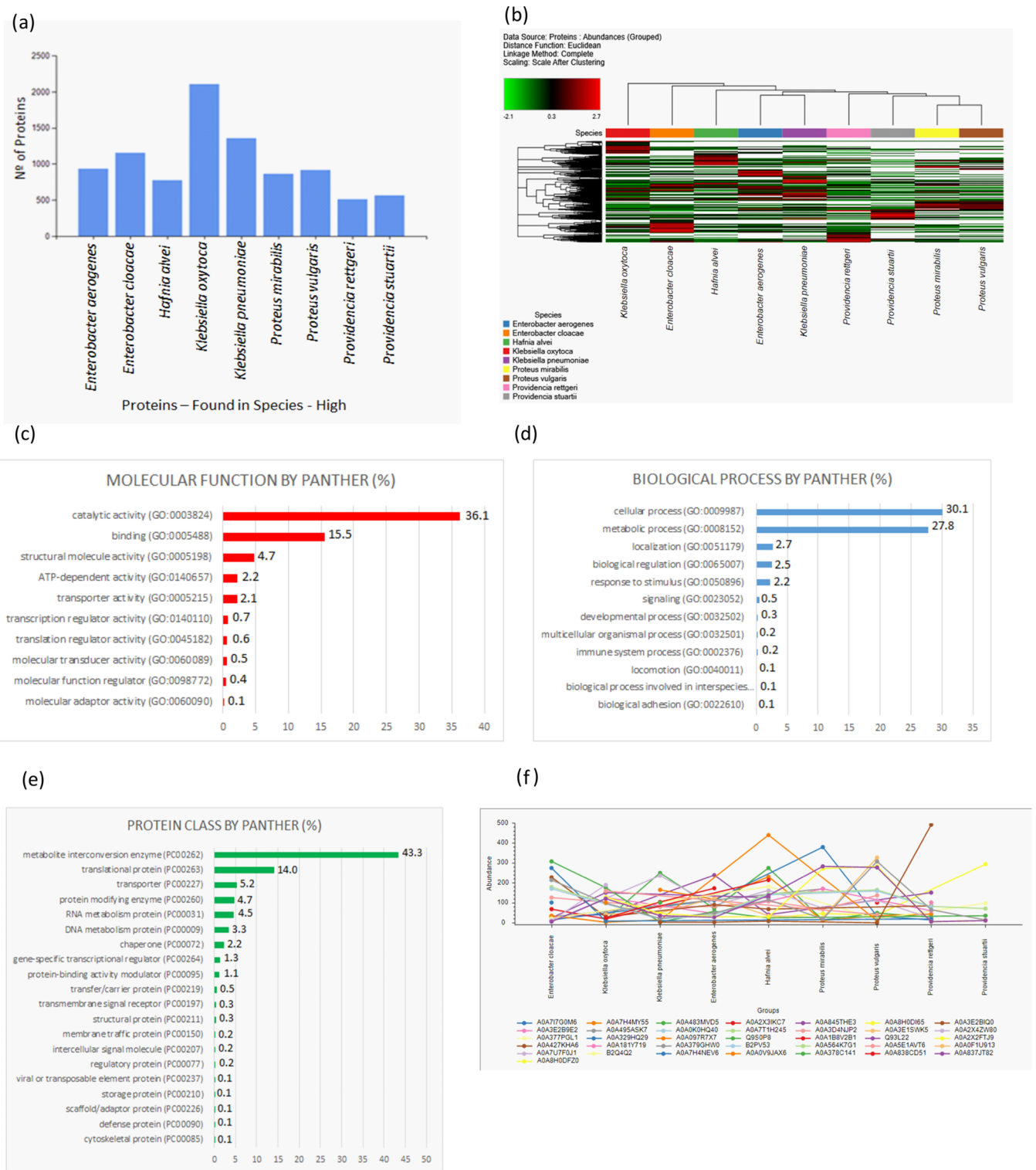

Figure 1. (a) Distribution of the high-abundance proteins for each species determined using LFQ; the *y*-axis represents the number of identified proteins. (b) Heatmap view of proteins identified by shotgun proteomics analyses of the 9 different foodborne bacterial strains that generate BAs. The bars indicate the presence or absence of a particular protein. Upregulated proteins are indicated in red; while green represents downregulated proteins. The species listed below (*E. aerogenes, E. cloacae, H. alvei, K. oxytoca, K. pneumoniae, P. mirabilis, P. vulgaris, P. rettgeri, Providencia stuartii*) were used to determine the Euclidean hierarchical distances. Five principal clusters were identified. (c) Molecular functions of the BAs produced by the bacteria, identified using shotgun proteomics and categorized according to the PANTHER classification system, using the gene names as inputs for the software; (d) biological processes carried out by the bacterial proteins identified by shotgun proteomics, and classified according to PANTHER; (e) biological processes carried out by the proteins identified by shotgun proteomics and categorized according to PANTHER; (f) distribution of the abundance of the proteins identified as decarboxylases in the 9 different bacterial species studied. The polypeptides are named according to their given accession numbers for each microbial species. The relative amounts of each protein were determined by the LFQ technique.

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bicarbonate pH 8.0, and exposed to enzymatic digestion with trypsin (ratio 1:100) (Promega, WI) at 37 °C overnight.

LC−MS/MS Analysis in an LTQ-Orbitrap Elite Equipment. Peptide digests were acidified with 5% formic acid (FA), to reach pH 2, and cleaned on a C_{18} MicroSpin column (The Nest Group, Southborough, MA). Then, the peptide samples were analyzed by LC−MS/MS, by means of a Proxeon EASY-nLC II liquid chromatography system (Thermo Scientific, San Jose, CA) combined with an LTQ-Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Peptides separation (2 μg) was executed on a reversed-phase (RP) column (EASY-Spray column, PepMap C18, 50 cm × 75 μm ID, 100 Å pore size, 2 μm particles, Thermo Fisher Scientific), containing a 10 mm precolumn (Accucore XL C18, Thermo Scientific), using either 0.1% FA in Milli-Q-water or 98% acetonitrile and 0.1% FA as mobile phases A and B, respectively. A flow rate of 300 nL/min during 240 min of linear gradient from 5 to 35% B was utilized. A temperature of 230 °C was applied and a spray voltage of 1.95 kV was utilized for ionization. Spectra were investigated in positive mode from 400 to 1600 amu (1 μscan), followed by 10 data-dependent higher-energy C-trap dissociation (HCD) tandem mass spectrometry (MS/MS) scans (1 μscans), with a normalized collision energy of 38% and an isolation width of 3 amu. MS/MS spectra were set in dynamic exclusion for 30 s once the second fragmentation event occurred. Unassigned charged ions were neglected from MS/MS explorations.

LC−MS/MS Data Processing. SEQUEST-HT (Proteome Discoverer 2.4 platform, Thermo Fisher Scientific) was utilized to analyze the spectra of MS/MS comparing against the Proteobacteria UniProt/TrEMBL database that contains all nine bacteria included in the present work (containing 2,627,375 protein sequence entries, August 2022). Spectra of MS/MS were searched by means of fully tryptic cleavage restrictions, and up to two missed cleavage sites were permitted. Tolerance windows were set at 10 ppm for precursor ions and 0.06 Da for the MS/MS fragment ions. The variable modifications permissible were acetylation of the N-terminus of the protein (N-Acyl), methionine oxidation (Mox), and carbamidomethylation of Cys (C*). Results were submitted to statistical analysis using the Percolator mode included in Proteome Discoverer 2.4 package.[14] The false discovery rate (FDR) was retained below 1%. The mass spectrometry proteomics data obtained are available in the ProteomeXchange Consortium, via the PRIDE Web site with the data set identifier PXD043997.

Label-Free Quantification (LFQ). LFQ of relative protein abundance, for all strains analyzed, was performed using the Minora Feature Detector mode as well as the analysis of variance (ANOVA) (individual proteins) approach incorporated in the Proteome Discover 2.4 program (Thermo Fisher Scientific). Peak areas of the ion feature, corresponding to different charge forms of the same peptide, were added together.

Euclidean Hierarchical Clustering. R (version (v) 4.1.1) for statistical analysis (http://www.r-project.org, retrieved on 25 May 2023) and the function heatmap.2 was employed to reach the Euclidean hierarchical clustering of the data set. Complete linkages for the agglomeration, Euclidean distance metric, and the Ggplots v.4.1.1 package were used as parameters.

Functional Pathways and Gene Ontology (GO) Analysis. PANTHER platform (http://www.pantherdb.org/) (accessed on 28 May 2023) was utilized to categorize the complete list of nonredundant protein IDs ("Gene name" column in Supporting Information, Data S1) on three principal classes of annotations: biological process, protein class, and molecular function. The complete *E. coli* genome was designated as a reference set, and the statistical probability was provided as a percentage. For this procedure, all of the orthologous gene ID entries were implemented as a reference set. The pathway analysis data were clustered, supporting an evaluation of the statistical significance of over- or under-representation, based on the GO terms of the proteins.

Protein−Protein Interaction Network Analysis. Protein−protein interaction network analysis was performed by submitting the orthologous gene IDs to the STRING software v.11.5 (http://string-db.org/, accessed on 15 May 2023).[15] Nodes were utilized to denote the proteins, and interactions are depicted with continuous lines. Completely the edges were supported by at least one reference from the publications or from official evidence stored in the STRING program. The confidence score was fixed at ≥0.7 (high confidence). Clusters were generated by using the Markov clustering system (MCL) software located in the STRING program, and a default value of 2 was designated for all analyses.

Virulence Factors. The Virulence Factors of Pathogenic Bacteria Database (VFDB) (http://www.mgc.ac.cn/VFs/, accessed on June 2023) was operated to identify virulence factors. Additionally, the analyses were expanded to comprise virulence factors registered in multiple scientific publications.[16−18]

Potential Peptide Biomarkers. The BLASTp system was managed to resolve the specificity on each of the peptides identified by LC−MS/MS, as well as to evaluate the protein homologies and uniqueness of the peptides, as compared to the proteins stored in the NCBI database.[19]

RESULTS AND DISCUSSION

Proteomics Data Repository. This study involved nine different bacterial species, found in seafood, that produce either histamine or other BAs (Table 1). The peptides analyzed for these bacteria were prepared by trypsin treatment and loaded in an LTQ-Orbitrap Elite instrument, as designated before.[7−9,20] This produced a total of 35,621 peptide spectrum matches (PSMs), identified as belonging to 20,792 non-redundant peptides; their sequences correlated with 4621 annotated proteins present in the Proteobacteria UniProt/TrEMBL database (August 2022) (Supporting Information, Data S1). The MS/MS-based proteomics results were submitted to the ProteomeXchange Consortium, through the Proteomics Identification Database (PRIDE depository) and were issued the data set identifier PXD043997.[21] To the best of our information, this data set corresponds to the biggest collection of peptides and proteins, so far identified, for bacteria present in seafood and capable of producing either histamine or other BAs. This protein repository constitutes a valuable new contribution to the study of biogenic amines that significantly furthers the knowledge in this field as well as supports and facilitates future research in the area.

Label-Free Quantification (LFQ) of Bacteria That Produce Biogenic Amines. The results achieved for LFQ of each of the nine bacterial species comprised in this study are described in Supporting Information, Data S2. The experiments were performed to determine the protein quantities present in each of the individual microbial species.

Figure 1a shows a comparison of the total levels of proteins estimated for each of the bacterial species. As seen in the figure, the species containing the highest protein quantities were *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, and *Enterobacter aerogenes*, in decreasing order. The determination of the high-abundance proteins, present in the different bacteria, was analyzed by LFQ and is demonstrated, as a heatmap diagram, in Figure 1b. Analysis of the Euclidean hierarchical distance allowed differentiation into five main clusters; Cluster A (*K. oxytoca*), Cluster B (*E. cloacae*), Cluster C (*H. alvei*), Cluster D (*K. pneumoniae* and *E. aerogenes*), and Cluster E (*P. rettgeri*, *P. stuartii*, *P. mirabilis*, and *P. vulgaris*). These clusters were divided into two groups according to their abundance levels; the upregulated proteins were depicted in Red (as determined via LFQ), while downregulated polypeptides appear in Green.

The peptides identified from the different bacterial strains were further analyzed by functional *in silico* analyses,

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Table 2. Histamine and Other Biogenic Amine-Related Proteins Detected by LC−MS/MS for the Bacterial Strains Studied[a]

Biogenic amine	Precursor	Proteins identified by LC-MS/MS	S1	S2	S3	S4	S5	S6	S7	S8	S9
Agmatine	Arginine	Arginine decarboxylase								■	
		Arginine ABC transporter substrate-binding protein		■	■	■	■	■	■	■	
		Arginine N-succinyltransferase		■							
		Arginine transporter		■							
		Arginine tRNA ligase	■	■	■	■	■	■	■		
		Biosynthetic arginine decarboxylase		■	■	■					■
		Histidine/lysine/arginine/ornithine ABC transporter		■							
		L-arginine ABC transporter membrane protein					■				
		Lysine/arginine/ornithine ABC transporter		■	■	■					
		Lysine-arginine-ornithine-binding periplasmic protein		■							
		N-succinylarginine dihydrolase		■		■					
Cadaverine	Lysine	Lysine decarboxylase			■						
		Lysine-arginine-ornithine-binding periplasmic protein		■							
		Lysine/arginine/ornithine ABC transporter membrane prot.					■				
		Lysine tRNA ligase	■		■	■	■	■	■		
Histamine	Histidine	Aminoacyl-histidine dipeptidase	■	■			■	■	■		
		Histidine ABC transporter substrate-binding protein		■		■	■				
		Histidine ammonia-lyase	■	■	■	■					■
		Histidine biosynthesis bifunctional protein					■				
		Histidine kinase	■	■	■	■	■	■	■	■	■
		Histidine phosphatase	■				■				
		Histidine-utilization repressor	■	■							
		Histidine/lysine/arginine/ornithine ABC transporter		■							
		Histidine tRNA ligase	■	■	■	■	■	■	■	■	■
		Hybrid sensor histidine kinase/response regulator	■								
		Phosphohistidine phosphate			■	■					
		Signal transduction histidine kinase				■					
Putrescine	Arginine	Arginine decarboxylase								■	
		Arginine ABC transporter substrate-binding protein		■	■	■	■	■	■	■	
		Arginine N-succinyltransferase		■							
		Arginine transporter		■							
		Arginine tRNA ligase	■	■	■	■	■	■	■		
		Biosynthetic arginine decarboxylase		■	■	■					■
		Histidine/lysine/arginine/ornithine ABC transporter		■							
		L-arginine ABC transporter membrane protein					■				
		Lysine/arginine/ornithine ABC transporter		■	■	■					
		Lysine-arginine-ornithine-binding periplasmic protein		■							
		N-succinylarginine dihydrolase		■		■					
	Glutamine	Bifunctional glutamine synthetase adenylytransferase		■						■	
		Glutamine ABC transporter periplasmic protein		■	■	■	■				
		Glutamine synthetase	■	■			■	■	■	■	■
		Glutamine-binding periplasmic protein	■				■				
		Glutamine-fructose-6-phosphate aminotransferase	■	■		■	■	■	■		■
		Glutamine tRNA ligase	■	■	■	■	■	■	■		
		Protein-glutamate methylesterase/protein-glutaminase	■								
		Type 1 glutamine amidotransferase domain-containing prot.	■			■					
	Methionine	Adenosylmethionine-8-amino-oxononanoate aminotransferase				■			■		
		DL-methionine transporter		■							
		D-methionine ABC transporter		■							
		L-methionine gamma lyase									■
		Methionine aminopeptidase	■	■		■		■	■		■
		Methionine synthase		■		■	■				
		Methionine tRNA ligase	■	■	■	■	■	■	■	■	
		Methionine sulfoxide reductase	■	■	■	■	■	■	■	■	
		S-adenosylmethionine decarboxylase proenzyme	■				■				
		S-adenosylmethionine synthase		■		■	■	■	■		■
	Ornithine	S-adenosylmethionine tRNA ribosyltransferase-isomerase	■			■	■				
		Lysine/arginine/ornithine ABC transporter substrate-binding		■	■	■					
		Lysine-arginine-ornithine-binding periplasmic protein		■							

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Table 2. continued

Biogenic amine	Precursor	Proteins identified by LC-MS/MS	S1	S2	S3	S4	S5	S6	S7	S8	S9
		Ornithine carbamoyltransferase				■					
		Ornithine cyclodeaminase			■						
		Succinylornithine transaminase				■					
Spermidine	Agmatine	Agmantine deiminase			■						
	Methionine	Adenosylmethionine-8-amino-oxononanoate aminotransferase				■			■		
		DL-methionine transporter		■							
		D-methionine ABC transporter		■							
		L-methionine gamma lyase									■
		Methionine aminopeptidase	■	■		■		■	■		■
		Methionine synthase		■		■	■				
		Methionine tRNA ligase	■	■	■	■	■	■	■	■	
		Methionine sulfoxide reductase	■	■	■	■	■	■	■	■	
		S-adenosylmethionine decarboxylase proenzyme	■				■				
		S-adenosylmethionine synthase		■		■	■	■	■		■
		S-adenosylmethionine tRNA ribosyltransferase-isomerase	■			■	■				
	Putrescine	Gamma-glutamylputrescine oxidoreductase				■					
		Putative spermidine/putrescine transport system			■						
		Putrescine aminotransferase	■								
		Putrescine-binding periplasmic protein			■	■	■	■	■		■
	Spermine	Bifunctional glutathionylspermidine amidase/synthase						■	■		
		Putative spermidine/putrescine transport system			■						
		Spermidine synthase		■		■					
		Spermidine/putrescine ABC transporter PotD				■	■				
		Spermidine/putrescine import ATP-binding protein PotA									■
		Spermidine-binding periplasmic protein SpuE						■	■		
Spermine	Agmatine	Agmatine deiminase			■						
	Methionine	Adenosylmethionine-8-amino-oxononanoate aminotransferase				■		■			
		DL-methionine transporter		■							
		D-methionine ABC transporter		■							
		L-methionine gamma lyase									■
		Methionine aminopeptidase	■	■		■		■	■		■
		Methionine synthase		■		■	■				
		Methionine tRNA ligase	■	■	■	■	■	■	■	■	
		Methionine sulfoxide reductase	■	■	■	■	■	■	■	■	
		S-adenosylmethionine decarboxylase proenzyme	■				■				
		S-adenosylmethionine synthase		■		■	■	■	■		■
		S-adenosylmethionine tRNA ribosyltransferase-isomerase	■			■	■				
	Spermidine	Bifunctional glutathionylspermidine amidase/synthase						■	■		
		Putative spermidine/putrescine transport system			■						
		Spermidine synthase		■		■					
		Spermidine/putrescine ABC transporter PotD				■	■				
		Spermidine/putrescine import ATP-binding protein PotA									■
		Spermidine-binding periplasmic protein SpuE						■	■		

[a]S1 (*E. aerogenes*); S2 (*E. cloacae*); S3 (*H. alvei*); S4 (*K. oxytoca*); S5 (*K. pneumoniae*); S6 (*P. mirabilis*); S7 (*P. vulgaris*); S8 (*P. rettgeri*); S9 (*P. stuartii*).

comprising (i) GO enrichment and functional pathways, hierarchical clustering examination, (ii) functional networks exploration, (iii) presence of virulence factors and (iv) selection for putative species-specific peptide biomarkers.

Functional Pathways and GO. The global protein repository obtained for foodborne strains of biogenic-amine-producing bacteria was individually examined, by means of functional bioinformatics programs, such as functional pathway analysis and GO term enrichment; thus, PANTHER analyses were performed using the gene names (including all non-redundant proteins). These analyses represented the categories most significant in terms of percentage content displaying 10 different molecular functions (Figure 1c), involved in 12 different biological processes (Figure 1d), and representing 20 different protein classes (Figure 1e).

A closer view of the molecular function classification (Figure 1c) indicates that catalytic activity is the most commonly shared characteristic (36.1%), with a lower percentage of proteins involved in binding (15.5%) and structural molecular activity (4.7%). The catalytic activity group included a variety of enzymes, such as reductases, decarboxylases, proteases, hydrolases, and dehydrogenases. On the other hand, the polypeptides identified as displaying a binding function encompassed reductases, transferases, lyases, and protein kinases. Meanwhile, the third most abundant group corresponds to proteins displaying a structural molecular activity,

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such as ribosomal proteins and tubulin- and actin-binding molecules.

Classification of the proteins identified according to their biological processes (Figure 1d) revealed that almost a third of the polypeptides were involved in cellular processes (30.1%), while a slightly lower proportion participated in metabolic processes (27.8%). The rest of the proteins were distributed into smaller groups, including localization (2.7%), biological regulation (2.5%), response to stimulus (2.2%), and signaling (0.5%). The proteins playing a role in cellular processes encompassed molecules displaying functions, such as amino-acyl-tRNA synthetases, ligases, decarboxylases, transferases, and transporters. The proteins included in the metabolic process group ranged from ligases to decarboxylases, isomerases, and transferases. Conversely, the localization group polypeptides incorporated adenosine triphosphate (ATP) synthases, transporters, oxidoreductases, chaperones, dehydrogenases, and storage proteins.

Classification of the polypeptides into protein classes (Figure 1e) revealed a predominance of metabolite interconversion enzymes (43.3%), followed by translational proteins (14.0%) and transporters (5.2%). The biological catalysts included in the metabolite interconversion enzymes group included dehydrogenases, transferases, esterases, oxidoreductases, and isomerases. Accordingly, the translational protein group displayed aminoacyl-tRNA synthetases and ribosomal proteins, while the transporter group encompassed amino acid transporters, ATP synthases, ion channels, and ATP-binding (ABC) cassettes.

The existence of high concentrations of decarboxylases among the proteins identified indicates the importance of these enzymes in BAs generation by bacteria.[10] The abundance of decarboxylases in the 9 bacterial species studied by LFQ can be clearly observed in Figure 1f, with the proteins labeled according to their accession name. Protein quantification was carried out by the LFQ technique. *H. alvei*, *P. mirabilis*, *P. vulgaris*, and *P. rettgeri* were the bacterial species that contained the highest amount of proteins exhibiting decarboxylase activity. During fish spoilage, the occurrence of bacterial strains displaying high proteolytic enzyme activity accelerates the process of deterioration, increasing the breakdown of proteins; polypeptides cleavage releases small peptides and amino acids, making them accessible to the action of decarboxylases, that convert them into BAs.[22] The main BAs studied in fish are cadaverine (resulting from lysine), histamine (resulting from histidine), tyramine (resulting from tyrosine), putrescine (resulting from arginine, glutamine, methionine, and ornithine), spermidine (resulting from methionine, agmatine, putrescine, and spermine), and spermine (resulting from methionine, agmatine, putrescine, and spermidine).[23]

Biogenic Amine-Related Proteins Identified by LC−MS/MS. Table 2 recapitulates the biogenic amine-related proteins recognized in this study, by LC−MS/MS, for the 9 bacterial strains studied.

Agmatine is an aliphatic BA resulting from the amino acid arginine.[23] Ten different agmatine-related proteins were also detected, by shotgun proteomics analysis of the different bacterial strains, as shown in Table 2. The first step in the bacterial synthesis of agmatine needs the transport and presence of arginine into the periplasmic space of the microorganism. In Gram-negative bacteria, both nitrogen and amine transports are carried out by solute-binding proteins localized in the periplasmic space. Arginine decarboxylase catalyzes the transformation of L-arginine into agmatine and carbon dioxide; a mechanism similar to the deamination and decarboxylation carried out by pyridoxal-5′-phosphate-dependent enzymes (PLP enzymes) in the formation of a Schiff base intermediate.[23]

Cadaverine is an aliphatic biogenic polyamine resulting from the amino acid lysine.[23] Four different cadaverine-related proteins were also identified from the different bacterial strains by shotgun proteomics (Table 2). The enzyme lysine decarboxylase converts lysine to cadaverine. In bacteria, this enzyme is often associated with the fermentation of amino acids required to produce particular foods and beverages, such as cheese and wine. A variety of bacteria contain lysine-arginine-ornithine-binding periplasmic proteins and these binding proteins are implicated in the transportation and uptake of amino acids, such as lysine, arginine, and ornithine, across the bacterial cell membrane.[24]

Histamine is a BA resulting from the amino acid histidine;[25] this BA is existent in a lot of foods, but particularly abundant in fish products.[26] Histamine is the major cause behind what was denominated “histamine fish poisoning” or “scombroid poisoning”.[27] Although histamine is usually present in fish, poisoning occurs when fish are not stored properly, at low temperatures; under those conditions, histamine-forming bacteria can rapidly multiply and convert the amino acid histidine into histamine.[28] When people consume fish that comprises high levels of histamine, they can develop scombroid poisoning,[27] with symptoms that typically appear in a short time after ingestion as flushing of the upper body, headaches, abdominal cramps, dizziness, and rapid heartbeat. In severe cases, symptoms can resemble an allergic reaction and could even result in anaphylaxis.[27] A total of 10 different histamine-related proteins were identified via shotgun proteomics (Table 2). Aminoacyl-histidine dipeptidase is an enzyme that catalyzes the hydrolysis of aminoacyl-histidine dipeptides. Histidine ABC transporter is an enzyme that specifically binds to histidine and acts as a receptor for histidine. Histidine ammonia-lyase is an enzyme that catalyzes the transformation of histidine into urocanic acid and ammonia. In microorganisms, the enzyme histidine ammonia-lyase allows utilization of histidine as a nitrogen source for growth and energy production. The histidine biosynthesis bifunctional protein is an enzyme implicated in the biosynthesis of the amino acid histidine and also offers potential signatures for the development of antimicrobial agents. It is well recognized that histidine kinase/phosphatase controls signal transduction and histamine synthesis, by activating histidine decarboxylase through phosphorylation/dephosphorylation.[29] On the other hand, the histidine-utilization repressor is a regulatory protein that controls the expression of genes concerned in the utilization of histidine as a nutrient source in bacteria. The histidine/lysine/arginine/ornithine transported belongs to the ABC type of transporters and utilizes ATP hydrolysis to drive the translocation of a variety of substances across membranes. These molecules are also essential in bacterial pathogenesis and could represent a vital target in the development of antimicrobial strategies. Histidine tRNA ligase plays a vital role in the accurate incorporation of histidine into proteins during translation, contributing to the proper functioning and structure of proteins in the cell. The hybrid sensor histidine kinase/response regulator is a type of signaling protein that is implicated in the regulation of virulence factors and adaptation to stress conditions. Hence, the mechanisms

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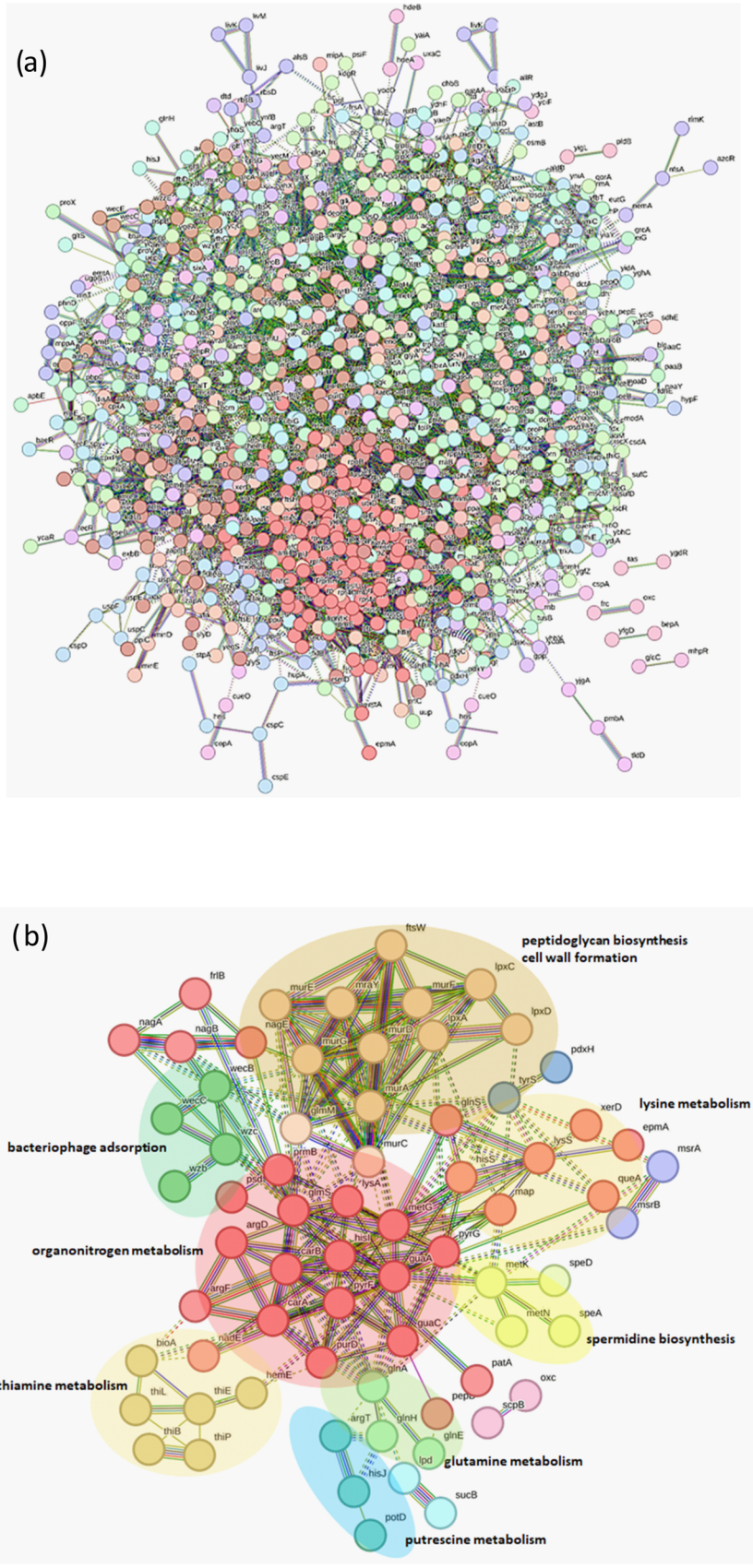

Figure 2. (a) Protein interactome network for the global protein repository of foodborne bacterial strains that produce either histamine or other BAs. The complete network for the global protein repository consists of 1160 nodes (proteins) and 10,318 edges (interactions); (b) subnetwork corresponding to specific biogenic amine-related proteins. The complete subnetwork consists of 75 nodes (proteins) and 217 edges (interactions). Protein interactions were analyzed using the STRING v.11.5 software. Only high-confidence interactions (≥0.7), as determined by the STRING

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Figure 2. continued

software, were selected for this study. The circles represent the proteins, while the interactions between proteins are represented by either continuous lines, for direct interactions (physical), or dotted lines, for indirect interactions (functional).

involved in the signal transduction of histidine kinases could represent a major target for the development of antimicrobial strategies.

Putrescine is a BA formed by the decarboxylation of arginine and ornithine; this process requires a two-step reaction for arginine but only one for ornithine. Putrescine can react with nitrite to create *N*-nitrosamines, which are carcinogenic mediators.[30] Moreover, the presence of putrescine can potentiate the toxic effects produced by other BAs, in particular histamine and tyramine.[31] Putrescines are the main BAs found in seafood, such as fish, squid, and octopus.[32] Our study identified a total of 35 different putrescine-related proteins (Table 2). Arginine decarboxylase is an enzyme that catalyzes the transformation of arginine into the BA agmatine. Agmatine is also a precursor in the synthesis of polyamines, such as spermidine and spermine. As is the case for agmatine, the metabolism of putrescine also needs the primary occurrence and transport of arginine in the periplasmic space of the cells, but, in this case, it can be substituted for ornithine. This shotgun proteomics study also identified a number of proteins responsible for the transport of glutamine and methionine, as well as amino/amido transferases; these polypeptides include glutamine ABC transporter periplasmic protein and glutamine-fructose-6-phosphate aminotransferase, among others.[33] A number of additional peptides were identified as corresponding to the *S*-adenosylmethionine decarboxylase enzyme. This enzyme is part of the polyamine biosynthetic pathway, generating the n-propylamine residue required for the generation of spermidine and spermine from putrescine.[33]

Spermidine is a polyamine resulting from agmantine, methionine, putrescine, or spermine;[34] this molecule is also a precursor to other polyamines, like spermine.[23] The presence of spermidine in fish can increase the toxic effect of histamine by impeding human gut histamine-catabolic enzymes. Twenty-two spermidine-related proteins were detected by shotgun proteomics, including spermidine synthase, an enzyme that catalyzes the last step in the synthesis of spermidine. This enzyme is present in a diversity of organisms, including bacteria, plants, and animals and plays an essential role in the conversion of putrescine into spermidine. Spermidine synthase is a PLP-dependent enzyme. The spermidine/putrescine ABC transporter PotD is a membrane protein implicated in the transport of spermidine and putrescine across the bacterial cell membrane; it belongs to the ATP-binding cassette (ABC) transporter superfamily. Conversely, the spermidine/putrescine import ATP-binding protein PotA, also a member of the ABC transporter superfamily, is responsible for the uptake of spermidine and putrescine in bacteria. This protein functions as the ATP-binding domain of the transporter complex and provides the energy required for substrate transport. In addition, the spermidine-binding periplasmic protein SpuE, a bacterial polypeptide responsible for binding to and transporting spermidine, is a periplasmic protein that cooperates with the spermidine substrate and facilitates its transport across the bacterial cell membrane.

Spermine-related proteins were identified via shotgun proteomics by LC−MS/MS (Table 2).[25] Agmatine deiminase is an enzyme that catalyzes the transformation of agmatine to *N*-carbamoylputrescine; this protein is present in certain bacteria and archaea and is involved in the catabolism of agmatine, a naturally occurring compound. The metabolic product obtained, *N*-carbamoylputrescine, can be further catabolized by additional enzymes to produce compounds such as putrescine and urea. As its name indicates, bifunctional glutathionylspermidine amidase/synthase is an enzyme intricate in the metabolism of glutathionylspermidine, a compound derived from the conjugation of glutathione and spermidine.[35] This enzyme, present in a variety of bacteria and archaea, is involved in both the breakdown and synthesis of glutathionylspermidine, a compound that plays a role in several cellular processes including the stress response, detoxification, and regulation of cellular redox balance. Glutathionylspermidine is also implicated in bacterial pathogenesis and antibiotic resistance. *S*-Adenosylmethionine decarboxylase is an enzyme involved in the synthesis of polyamines, including spermine and spermidine; it catalyzes the decarboxylation of *S*-adenosylmethionine into decarboxylated *S*-adenosylmethionine, a crucial precursor in the polyamine biosynthetic pathway. Spermine is believed to work as a free radical scavenger, keeping DNA from oxidative stress.[36] In fact, it appears that an increase in the cationic charge of a polyamine potentiates its effect on DNA−protein binding; this renders spermine a stronger effector than either spermidine or putrescine.

The last groups of enzymes identified correspond to additional decarboxylases (i.e., 2,4-diaminobutyrate decarboxylase, 4-carboxymuconolactone decarboxylase, α-keto acid decarboxylase, phosphatidylserine decarboxylase, orotidine 5-phosphate decarboxylase, uroporphyrinogen decarboxylase) and deaminases (i.e., 2-iminobutanoate/2- iminopropanoate deaminase, glucosamine-6-phosphate deaminase, cytidine deaminase, guanine deaminase, porphobilinogen deaminase). The roles of these proteins were identified by shotgun proteomics (Supporting Information, Data S3) but, as determined by both literature searches and PANTHER analyses, these enzymes are involved in additional metabolic pathways.

Network Analysis of Protein Interactions. Network analyses of protein interactions were executed operating the STRING v.11.5 program (https://string-db.org/), accessed on 7 May 2023;[15] all of the proteins recognized in this study were related to the genome of the model organism *E. coli* K12 MG1655, as the genetically closet group (Figure 2a). Each protein−protein interaction was consigned to the network regarding the confidence score obtained. To diminish false positives and false negatives, only the predicted interactions classified by the STRING software as "high confidence" ($\geq$0.7) were designated for this investigation.

The final protein interactions network (interactome) for the global protein data set is depicted in Figure 2a; it involves 1160 nodes (proteins) and 10,318 edges (interactions). The protein network presented here constitutes the major comprehensive interactomics map currently available for histamine- and amine-biogenic-producing bacteria that colonize seafood. Cluster networks were created using the MCL (inflation

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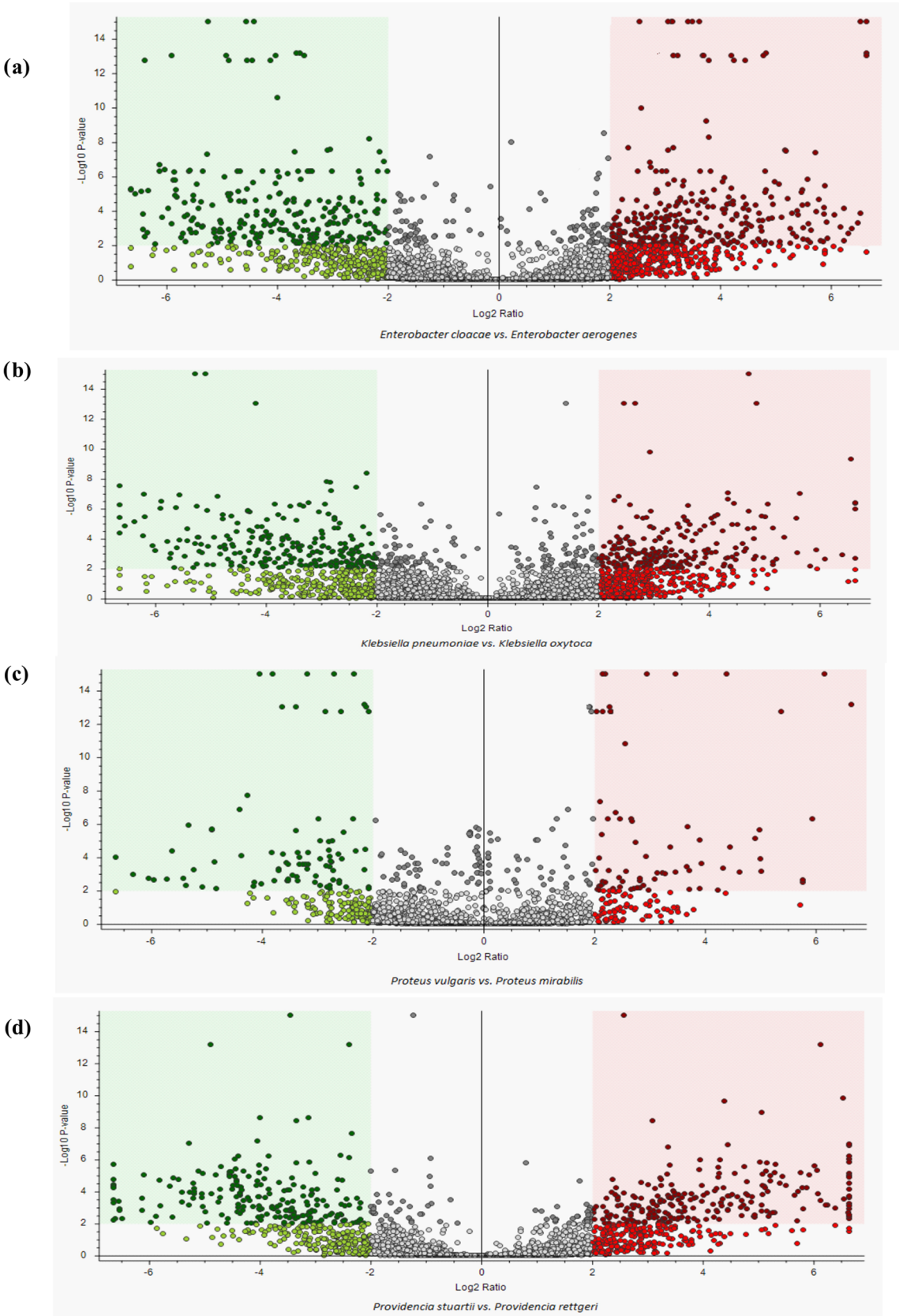

Figure 3. Volcano plot representation of different protein groups. The comparisons between bacterial groups were as follows: (a) *E. cloacae* vs *E. aerogenes*, (b) *K. pneumoniae* vs *K. oxytoca*, (c) *P. vulgaris* vs *P. mirabilis* (d) *P. stuartii* vs *P. rettgeri*. Fold charge (FC $\geq$ 2, *p*-value $\leq$ 0.01).

clustering) software from the STRING program, and a default value of 2 was designated for all analyses. The cluster analyses identified 133 unique major clusters of interactions between the proteins (nodes) identified (Supporting Information, Data

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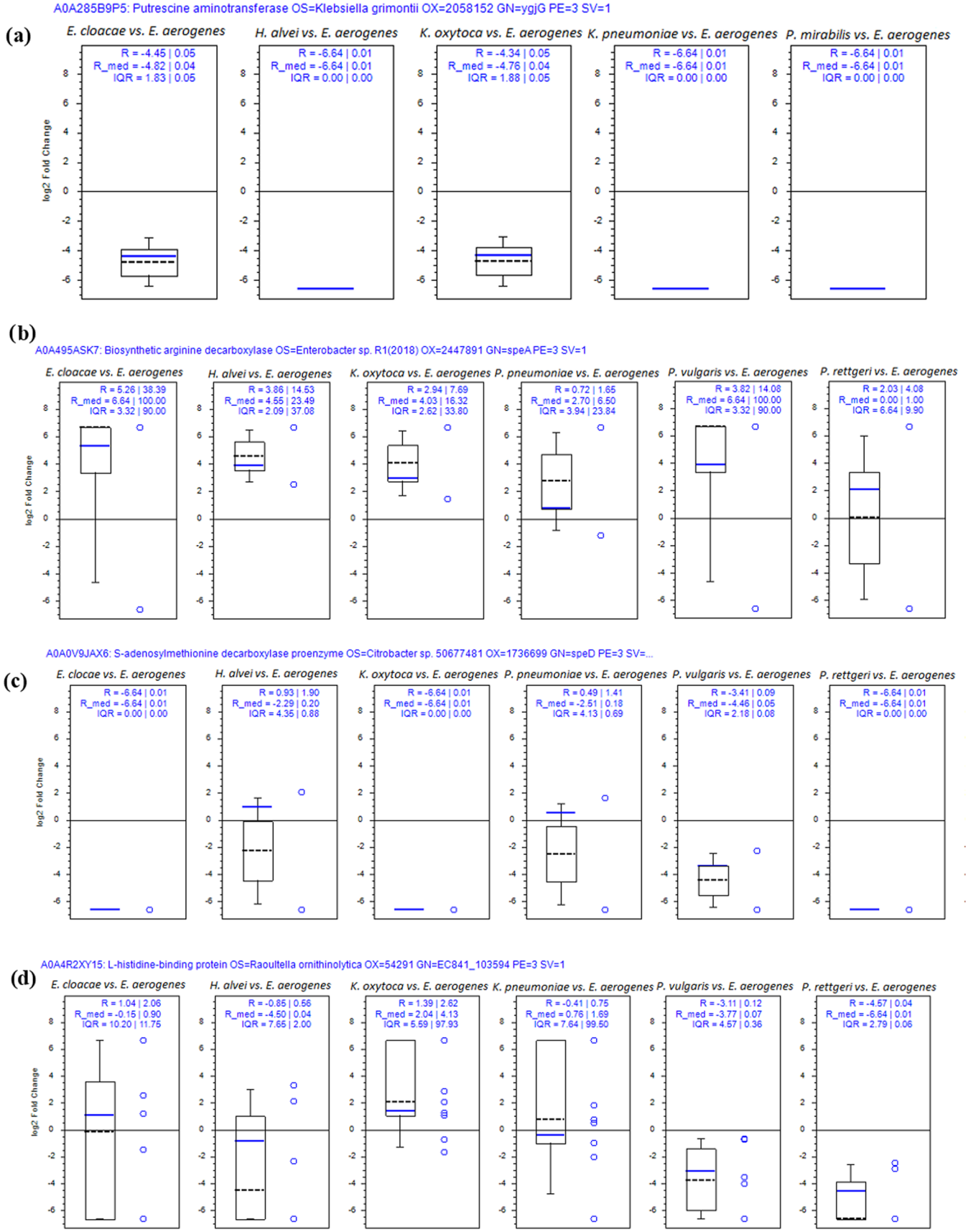

Figure 4. Box plots representations of specific biogenic amine-related proteins: (a) putrescine aminotransferase, (b) biosynthetic arginine decarboxylase, (c) *S*-adenosylmethionine decarboxylase, (d) L-histidine-binding protein.

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S4). The major clusters obtained in the study included 119 nodes related to ribosomal metabolism (depicted in red), 44 nodes concerning glycolytic metabolism (in pink), 37 nodes involving DNA metabolic processes (in dark pink), and 30 nodes corresponding to bacterial membrane assembly (in brown).

A detailed diagram of the biogenic polyamine subnetwork is included in Figure 2b (see zoom) and in Supporting Information, Data S4. This subnetwork consists of 75 nodes (proteins) and 217 edges (interactions); they include 11 major clusters, classified as such according to either the number of nodes involved or the biological relevance of the proteins involved. The information on these 11 major clusters can be found on Supporting Information, Data S4, including protein names, and reports of the corresponding name codes. In summary, the major clusters include 29 nodes involved in organonitrogen compound metabolism (depicted in red), 12 nodes concerning peptidoglycan biosynthesis-cell wall formation (in orange), 6 nodes implicated in thiamine metabolism (in brown), 4 nodes involved in spermidine biosynthesis (in yellow), and 4 nodes participating in bacteriophage adsorption (in green), with a final 3 nodes dealing with putrescine metabolism (in blue). Expanded study of the subnetworks and protein−protein interactions mentioned above could provide essential information for the development of new therapeutic treatments in the fight against pathogenic bacteria; this area of research is also important for comprehending the mechanisms involved in bacterial dispersion and antibiotic resistance as well as providing new approaches to combat food intoxication by BAs.

Identification of Differentially Regulated Proteins (DRPs). After relative protein quantification by LFQ, the proteins were further analyzed to identify differentially regulated proteins (DRPs). The filters applied were: (a) a minimal requirement of a Log_2 Fold Change (FC) ≥ 2 in the abundance rate in normalized ratios, (b) ANOVA on ranks posthoc test (p-value ≤ 0.01) (Supporting Information, Data S6). The volcano plot illustrations of DRPs are presented in Figure 3A−D.

The first comparison was carried out between species belonging to the same genus, as shown in Figure S1A−D in Supporting Information, Data S5 and S6. The proteins produced by the two *Enterobacter* species (*E. cloacae* and *E. aerogenes*) were compared and subjected to statistical analyses (Log_2 FC ≥ 2; p-value ≤ 0.01), revealing that 533 proteins were differentially regulated by the two bacteria. A total of 239 of those proteins were upregulated in *E. cloacae*, as compared to *E. aerogenes*, while the opposite was true for the remaining 295 proteins (Figure 3A). The proteins found in increased quantities in *E. cloacae* included histidine ABC transporter substrate-binding protein, which participates in the synthesis of histamine and other biogenic amines, while the cohort of proteins prevalently abundant by *E. aerogenes* encompassed arginine decarboxylase, lysine-arginine-ornithine-binding periplasmic protein, and putrescine aminotransferase, which are implicated in the transport and maintenance of optimal levels of polyamines in bacterial cells.

The comparison between *K. pneumoniae* and *K. oxytoca* revealed a total of 650 proteins that were differentially regulated by the two *Klebsiella* species; a total of 318 proteins were upregulated in *K. pneumoniae*, while the remaining 332 proteins were more abundant in *K. oxytoca* (Figure 3B). Both *K. pneumoniae* and *K. oxytoca* preferentially produced a variety of histamine and other biogenic amine-related proteins; these proteins included acetylornithine/succinyldiaminopimelate aminotransferase, pyruvate decarboxylase, and two periplasmic proteins (thiamine-binding periplasmic protein and putrescine-binding periplasmic protein) for *K. pneumoniae*. The proteins more abundant in *K. oxytoca* corresponded to the amino acid ABC transporter substrate-binding protein, histidine ABC transporter substrate-binding protein, histidine ammonia-lyase, and lysine-arginine-ornithine-binding periplasmic protein.

A comparison between the two *Proteus* species (*P. vulgaris* and *P. mirabilis*) revealed a total of 252 DRPs, 127 of which were upregulated in *P. vulgaris*, while 125 were more abundant in *P. mirabilis* (Figure 3C). *P. vulgaris* displayed an abundance of histidine kinase, histidine ABC transporter substrate-binding protein, aminoacyl-histidine dipeptidase, and lysine/arginine/ornithine ABC transporter substrate-binding protein, while aminotransferase and penicillin-binding protein activator LpoB were upregulated in *P. mirabilis*.

A total of 598 proteins were differentially regulated by *P. stuartii*, as compared to *P. rettgeri*, with 309 proteins upregulated in *P. stuartii* and 289 more abundant in *P. rettgeri* (Figure 3D). The proteins more abundant in *P. stuartii* included lysine/arginine/ornithine ABC transporter substrate-binding protein, penicillin-binding protein activator LpoB, histidine ammonia-lyase, adenylate kinase, and *S*-adenosylmethionine synthase. On the other hand, the proteins more abundant in *P. rettgeri* we identified ABC transporter substrate-binding protein, penicillin-binding protein activator LpoB, aspartate ammonia-lyase, and aminoacyl-histidine dipeptidase.

A further analysis was carried out to compare enzymes directly associated with the synthesis of either histamine or other biogenic amine-related proteins (Figure 4A−D). Statistical analysis showed that putrescine aminotransferase protein was more abundant in *E. aerogenes* than in any of the other bacterial species studied compared with all of the different ratios (Figure 4A).

The enzyme arginine decarboxylase plays a significant role in the biosynthesis of polyamines, such as the production of agmatine from arginine; our analyses demonstrated that this protein is upregulated in species such as *H. alvei*, *E. cloacae*, *P. pneumoniae*, *K. oxytoca*, *P. vulgaris*, and *P. rettgeri* compared with all of the different ratios (Figure 4B).

On the other hand, the proenzyme *S*-adenosylmethionine decarboxylase was more abundant in *E. aerogenes*, as compared to the other bacteria studied (Figure 4C).

Finally, the L-histidine-binding protein, a component of the bacterial histidine transport system also known as histidine permease or histidine-binding protein, is involved in the uptake of the amino acid histidine from the external environment into the bacterial cell. This protein is located in the periplasmic space of the bacterial cell between the inner and outer membranes in Gram-negative bacteria. The function of this protein is to specifically bind to the L-histidine, which is the histidine isomer utilized in protein synthesis, present in the extracellular environment. L-Histidine-binding protein is more abundant in *E. cloacae*, as compared to *E. aerogenes*, although *E. aerogenes* produces more of the protein than *H. alvei*. Similarly, the abundance of this protein is higher in both *K. oxytoca* and *P. pneumoniae* than in *E. aerogenes*, although L-histidine-binding protein production is higher in *E. aerogenes* than in either *P. vulgaris* or *P. rettgeri* (Figure 4D).

Virulence Factors. Many pathogenic bacteria capable of synthesizing biogenic amines like histamine are commonly

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found in fish and seafood byproducts. This is of particular concern since it was reported that some members of the Enterobacteriaceae family, such as species belonging to the genus *Enterobacter*, are rapidly acquiring multidrug resistance phenotypes. Furthermore, some bacterial species, like *K. oxytoca*, are not only developing multidrug resistance but can harbor a variety of virulence factors, including fimbriae and capsules.[37] This is further complex by the fact that bacteria, such as *S. pneumoniae*, are surrounded by a capsule that helps the bacterium evade phagocytosis, the capsule contains polysaccharides (often including uronic acids) that provide mucoviscosity, lipopolysaccharides (LPS), adhesins, and siderophores, systems that allow the bacterium to uptake iron from the environment.[18] In addition, some of the bacterial species included in this study, such as *P. stuartii*, *P. mirabilis*, *P. vulgaris*, *P. rettgeri*, and *H. alvei*, are known to contain virulence factors.[9,38]

The present study identified 1170 nonredundant peptides corresponding to 123 different virulence factors, including proteins described to perform a role in bacterial antibiotic resistance mechanisms, aid immune evasion, or constitute toxins (Supporting Information, Data S7). The results summarize the most relevant proteins so far identified as virulence factors.

Supporting Information, Data S7 also includes several peptides associated with the development of an antimicrobial state as well as proteins involved in their synthesis of the polypeptides responsible for it, such as pyocin/colicin proteins monooxygenases, PmbA metalloproteases, and the microcin ABC transporter. Some Gram-negative bacteria compete with other microorganisms sharing the same ecological niche, by secreting ABC system class III bacteriocins, such as colicin and microcins.[39] To deploy these bacteriocins, or antibiotics secreted by particular bacteria to attack related species, the bacterium requires an array of auxiliary proteins; for example, *E. coli* was described to require PmbA/TldD proteins for the maturation and secretion of microcins.[40]

Supporting Information, Data S7 also provides a summary of proteins identified in this study that are concerned with bacterial toxicity. These polypeptides include type II toxin-antitoxin (TA) system RelB/DinJ family antitoxin, type V toxin-antitoxin system, endoribonuclease antitoxin GhoS, lipoprotein toxin entericidin B, type VI secretion system tip protein VgrG, type II toxin-antitoxin system RelE/ParE family toxin, and cytotoxins. Entericidin B is a bacteriolytic lipoprotein produced by some strains of *Enterobacter* spp.; this microcin was used, as a probiotic treatment, to protect trout from infection by *Flavobacterium psychrophilum*, the causative agent of cold water disease (CWD).[41] Toxin-antitoxin (TA) systems are extensively spread in bacteria; they are constituted by two elements: a toxic protein that specifically inhibits an essential cellular event, and an antitoxin that can be an RNA (type I and III) or a protein (type II) that counteracts its effect. These bacterial TA systems have been recently associated with a variety of functions, including the control of responses against stress, antimicrobial persistence, interactions with the host, plasmid stability, and mobilization of genetic elements.[42]

Supporting Information, Data S7 also includes a number of proteins identified as involved in niche adaptation; these encompass Glycine betaine/L-proline transporter ProP, involved in osmoregulatory responses in bacteria, and playing a role both in osmoregulation and as an osmosensor.[17] Supporting Information, Data S7 also includes several heat shock proteins, Hsp20/α-Crystallin family protein, OsmC family protein, protein GrpE, putative biofilm stress and motility protein A, and small heat shock protein IbpA and IbpB; there is also a membrane protein PspB involved in the stress response, acid stress chaperones, and additional chaperones involved in environmental universal stress. Bacteria infecting human epithelial cells produce two chaperones, DnaK and GroESL, involved in stress response, while DnaK, GrpE, and DnaJ induce the synthesis of ClpB when bacterial heat damage is detected. There are two ClpB isoforms that, together, contribute to the survival of *E. coli* under thermal stress conditions.[43] In addition, the above-mentioned OsmC protein could participate in the bacterial protection from osmotic damage.[44]

As reflected in Supporting Information, Data S7, the present study identified numerous proteins that protect bacteria from either antibiotics or toxic substances, including TetR regulators (TFRs). The latter control the expression of genes encoding low-molecular-weight exporters, as well as being involved in antibiotic resistance; moreover, TFRs also appear to contribute to the development of "quorum sensing", a complex type of bacterial communication. VanZ is another interesting peptide identified; it confers resistance to the glycopeptide teicoplanin, a natural lipoglycopeptide antibiotic. Additional proteins, involved in antimicrobial resistance, identified include β-lactamases, penicillin-binding protein activators LpoA and LpoB, and a penicillin amidase, also known as peptidase S45, and MarR, a regulator of several proteins involved in antibiotic resistance.[20,44] Further polypeptides identified include multidrug resistance proteins and multidrug efflux systems, such as the multidrug ABC transporter, multidrug DMT transporter permease, modulator of drug activity B, and RND family efflux transporter MFP. Additional proteins, comprising TerD, TerY, TehB, TerB, TerD, and Ter, were identified as implicated in the resistance to toxic substances, such as tellurite, cadmium, copper, zinc, magnesium, and mercury.

A further group of identified peptides corresponds to proteins that play a role in immune evasion and bacterial colonization; these include several adhesins, such as the HecA family protein, and fimbrial proteins, namely, type 1 fimbria chaperone FimC and major fimbrial subunit. An additional group of polypeptides characterized correlates with chemotaxis-related proteins such as methyl-accepting chemotaxis protein CtpH, lysins, and capsular formation proteins. A final group of polypeptides identified corresponds to siderophores and related proteins, including achromobactin biosynthesis protein AcsF, yersiniabactin synthetase, and enterobactin (3-oxoacyl-ACP reductase FabG).[45]

The identification of virulence factors is a critical step in various aspects of microbiology and public health. It will not only help in understanding the mechanisms of infectious diseases but also contribute to the development of novel targeted and effective strategies for diagnosis, treatment, and prevention.

Identification of Potential Species-Specific Peptide Biomarkers. A considerable comparison of the proteomics data, proteins, and peptides, comprised in databases, was carried out in order to choose potential peptide biomarkers for the 9 bacterial species included in this study. Suitable peptides, identified by LC−MS/MS in only one of the microbial species, were further analyzed for sequence homology using the BLASTp software in order to verify their specificity to particular bacteria.[19]

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Supporting Information, Data S8 summarizes the analyses undergone by the 299 species-specific tryptic peptide biomarkers, corresponding to 233 different proteins, to achieve unequivocal identification of the 9 different bacterial species. The peptide biomarkers obtained for each of the microorganisms studied are as follows: 57 for *E. aerogenes*, 16 for *E. cloacae*, 35 for *H. alvei*, 85 for *K. oxytoca*, 16 for *K. pneumoniae*, 26 for *P. mirabilis*, 1 for *P. vulgaris*, 22 for *P. rettgeri*, and 41 for *P. stuartii*. These peptide biomarkers are unique and are present in only a specific species because the results were compared with all of the proteins included in the universal protein database by Blastp.

These peptide biomarker results could be applied by targeted proteomics for the rapid detection of biogenic-amine-producing bacteria contamination during seafood processing and processing, which is of great interest to the industry to preserve food quality and safety throughout the food production chain. Targeted proteomics methods will be faster (<1 h) than the currently available methods for biogenic-amine-producing bacteria detection as the real-time PCR (>24 h).[3]

Final Statements. The present article represents the first label-free quantitative proteomics study of 9 different foodborne bacterial strains that can produce BAs. The results obtained identified a number of protein data sets, which were further analyzed to determine the functional pathways that the polypeptides were involved in as well as to discriminate the bacterial strains into Euclidean hierarchical clusters. The results also include a predicted protein interactome network for foodborne strains. Most of the bacterial proteins identified were members of either pathways or networks associated with spermidine biosynthesis, bacteriophage adsorption, and putrescine metabolism. LFQ-based proteomics allowed identification of relevant DRPs as putrescine aminotransferase, arginine decarboxylase, *S*-adenosylmethionine decarboxylase, and L-histidine-binding protein. An additional 1170 non-redundant peptides corresponding to 123 different virulence factors were recognized; their main functions were associated with toxicity, antimicrobial resistance, antimicrobial compound production, immune evasion, host colonization, and phage proteins. In fact, 299 species-specific peptide biomarkers, belonging to 233 different proteins, were identified as specific to one of the nine bacterial species studied. At this stage, these results represent the major quantitative data set of peptides and proteins identified from foodborne bacterial species that produce BAs. This repository provides a substantial amount of data and provides a platform for the future development of novel therapies to combat the toxic effects of histamine and other BAs, as these compounds often contaminate food destined for human consumption. The results obtained here also provide relevant information to assess microbial contamination in foodstuffs as well as identify 9 pathogenic bacterial strains that could be applied for the detection of bacterial contamination by the food industry in order to guarantee quality and safety to the consumers. Particularly, the present investigation is relevant to the fish industry, as several peptide biomarkers for important biogenic-amine-producing bacteria in seafood are identified.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c06607.

Complete list of peptide spectrum matches (PSMs), peptides, and proteins identified by shotgun proteomics for each strain (Data S1) (ZIP)

Label-free quantification (LFQ) data for proteins and peptides for each of the nine bacterial species (Data S2) (ZIP)

List of decarboxylases identified in the present study (Data S3) (XLSX)

Major clusters created by MCL (inflation clustering) software from the STRING program (Data S4) (XLS)

Venn diagram of the proteins identified in this study, a comparison of the bacterial species belonging to the same genus (Data S5) (PDF)

Differentially regulated proteins (DRPs) after relative protein quantification by LFQ (Data S6) (XLSX)

Summary of the most relevant proteins identified as virulence factors (Data S7) (ZIP)

Analyses and list of the species-specific tryptic peptide biomarkers (Data S8) (XLSX)

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All authors registered did a considerable, complete, and academic contribution to the work and approved it for publication.

Notes

The authors declare no competing financial interest.

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