Extensive Shotgun Proteomic Analysis of *Salmonella enterica* subsp. *enterica* Serotypes from Chicken Meat: Insights into Pathogenicity and Food Spoilage

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ABSTRACT

Salmonella is a genus of bacteria that causes foodborne diseases, known as salmonellosis, in both humans and animals. This study presents a comprehensive proteomic analysis of 15 *S. enterica* subsp. *enterica* serotypes isolated from chicken meat. It identifies 13,117 peptide spectrum matches from 4,469 unique peptides, corresponding to 3,618 proteins. The analysis, performed using shotgun proteomics, reveals a complex interaction network consisting of 543 nodes and 1,480 edges. This network highlights roles in energy metabolism, peptidoglycan biosynthesis, infection, and antibiotic resistance. Functional bioinformatics classified the proteins, highlighting predominant catalytic activity essential for pathogenicity and food spoilage. The study identifies 941 virulence factors and 4 specific peptide biomarkers for *S. enterica* subsp. *enterica* serovar Typhimurium. These findings provide valuable insights into the pathogenicity and spoilage mechanisms of foodborne *Salmonella*, offering a significant proteomic repository for the development of targeted interventions to prevent food contamination and enhance food safety.

KEYWORDS: *Salmonella*, foodborne bacteria, chicken meat, LC-MS/MS, shotgun proteomics, foodomics, virulence factors, bacterial detection

INTRODUCTION

Salmonella is one of the classic, and best studied, bacterial genera; it belongs to the family *Enterobacteriaceae*, named by Salmon & Smith, who isolated the bacterium in 1884, from pig intestines.[1] The species included in this genus are non-sporulating Gram-negative rods, with a diameter of 0.7-1.5 μm. These microorganisms are facultative anaerobes that display a fermentative metabolism, cannot use lactose; as a general rule, they produce SH2, hence generating black sulfides when grown in Kligler differential medium.

Salmonella species are typically mobile, due to the presence of multiple peritrichous flagella; this ability allows the bacteria to inhabit a variety of environments, including food products. The *Salmonella* genus incorporates two species, *Salmonella bongori* and *Salmonella enterica*, along with over 2600 serotypes. These serotypes are organized according to the structure of both their somatic O and flagellar H antigens. This classification was developed by Kauffman-White to differentiate individual serological varieties.[2,3] *Salmonella enterica* is divided into six subspecies: *arizonae*, *diarizonae*, *enterica*, *houtenae*, *indica*, and *salamae*. Undoubtedly, *S. enterica* is a major worldwide health threat as, according to the World Health Organization, it represents “1 of the 4 key global reasons of diarrheal diseases” in humans worldwide.

S. enterica subsp. *enterica* is a pathogenic bacterium that poses significant public health risks, particularly when found in chicken meat.[4] As one of the most common causes of foodborne illnesses worldwide, its presence in poultry products is a major concern for both consumers and the food industry. Understanding the characteristics, transmission, prevention, and control measures of this pathogen is crucial in reducing its impact.

Government agencies, such as the U.S. Department of Agriculture (USDA) and the Food and Drug Administration (FDA), play critical roles in regulating and monitoring the safety of chicken meat. These agencies establish standards for microbial limits, conduct inspections, and enforce compliance with food safety regulations. Surveillance programs track the incidence of salmonellosis and identify outbreak sources, facilitating rapid response to control and prevent further spread. In Europe, Regulation (EC) No 2073/2005 sets microbiological criteria for various food products, including poultry meat.[4] This regulation defines acceptable limits for *Salmonella* presence in chicken meat at different stages of production and mandates regular testing to ensure compliance.

In 2011, Jacobsen and colleagues determined the pan-genome of *S. enterica*, by comparing the sequences of 45 individual genomes.[5] Using standard gene prediction methods, the authors estimated that the core and pan-genome of *Salmonella* contain approximately 2,800 and 10,000 gene families, respectively. According to Jacobsen, all *Salmonella* strains possess a large stable core, apart from numerous additional genes.[5] These accessory genes encode pathogenicity islands, plasmids, transposable elements, and bacteriophages. Recently, Liu and colleagues (2020) updated the pan-genome for this bacterium, by analyzing 341 *S. enterica* specimens, isolated from Chinese retail meats of various origins; the authors reported a total of 13,931 microbial genes, with the core genome spanning 3,635 genes.[6] From a genetic perspective, it appears that the species included in *Salmonella* are not naturally capable of undergoing transformation by naked DNA, as opposed to the case of *Escherichia coli*. In contrast to certain Gram-positive bacteria, *Salmonella* requires chemical treatment to become competent for the uptake of heterologous DNA.[7] However, it is worth noting that bacteriophage-mediated horizontal gene transfer, known as transduction, occurs within this genus; in fact, this type of genetic transfer was originally described in *Salmonella*.[8] Moreover, *S. enterica* can carry out conjugative gene transfer, through F-like self-transmissible plasmids,

such as the virulence plasmid pSLT.[9] The genes necessary for conjugal transfer are grouped together, in a DNA fragment spanning approximately 34-kb, this is similar to *E. coli*, that contains a single transcriptional unit known as the tra operon.[10, 11]

Salmonella displays a phenomenon known as "phase variation", where two genes (fliC and fljB) alternatively express flagellin; this alternation is regulated by the inversion of a DNA fragment, which is facilitated by two DNA invertases.[12] The relevant DNA segment contains the promoter for the fljB gene.[13] As a consequence of this process, *Salmonella* strains that only express one flagellin can display either motile and non-motile traits. This occurs because the DNA inversion can result in the absence of flagellin production, rendering the bacteria non-motile. According to Kutsukake & Iino (1980), strains of *S. enterica* subsp. *enterica* serotype Typhimurium lacking phase variation can be reverted to display an active phase variation; this reversion requires trans-acting cytoplasmic factors, that are present in either P1 or Mu bacteriophages and can mediate DNA inversion.[14] This capability can have significant implications when typing the *Salmonella* present in food products; while flagellar phase variation may not impact the enteropathogenesis of *S. enterica*, it does enhance its virulence, as demonstrated in a murine model of typhoid infection.[15]

A noteworthy feature of *Salmonella* flagellin is its capability to stimulate tumor necrosis factor α (TNF-α) in certain human cell lines, such as the promonocytic cell line.[6, 16] This is significant because TNF-α antibodies are commonly used in therapy for people suffering from autoimmune diseases.[17] Furthermore, TNF-α not only regulates immune cells but also acts as a pyrogen, raising body temperature, but also is involved in cell signaling pathways that can lead to either apoptosis or necrosis.[18] Dysregulation of TNF-α production is associated with human illnesses such as Crohn's disease, rheumatoid arthritis, cancer, Alzheimer's, and inflammatory bowel disease.[19-21]

Pathogenic species of *Salmonella* represent a major health threat, as they infect one billion people worldwide each year, causing 3 million deaths.[22] The main pathogens included in this genus are *S. enterica* subsp. *enterica* serotype Typhimurium, that produces a self-limiting gastroenteritis in humans, and serotype Typhi, that produces typhoid fever, a commonly lethal systemic illness. Moreover, serotype Enterica is one of the most relevant zoonotic pathogens, it not only infects humans but also poultry, birds and reptiles; this microorganism is transmitted to people by either water or food ingestion. Both *S. enterica* subsp. *enterica* serotypes Enterica and Typhi can replicate intracellularly, inside macrophages, hence establishing a systemic disease. The intracellular bacteria inhabit a membrane-bound vacuole, generated by a mechanism requiring a type 3 secretion system.[23] In order to survive inside the vacuole, the bacterium must overcome harsh conditions, such as extreme pH and the scarcity of oxygen and nutrients available. One of the theories indicates that, glucose is the main carbon source for the microorganism inside the vacuole, metabolized through the Entner-Doudoroff and the Embden-Meyerhof-Parnas pathways; indeed, mutants with an altered glucose transport or utilization, display reduced virulence.[24] *Salmonella* can also use phosphatidyl serine as its sole carbon source, when glucose is not available; for this purpose, these bacteria possess a full set of β-oxidation proteins, allowing the microorganism to catabolize fatty acids. It was proposed that the bacterial phosphoinositide phosphatase SopB regulates the membrane surface charge of the vacuole enclosing *Salmonella*, as well as inhibiting fusion of the vacuole with the lysosome, hence favoring the persistence of intracellular bacteria.[25] In addition, both Enterica and Typhi serotypes can affect the production of the protein claudin-2, thus weakening the intestinal tight junctions, that become leaky, and facilitate invasion of the colon; the microorganism can then reach and enter Peyer´s patches, in the terminal ileum.[26] The latter requires expression of the bacterial lpfABCDE operon, encoding long polar fimbriae, that damage the epithelial M cells in Peyer's patches.[27, 28] These

patches, therefore, act as an entry point, for the serotype Typhi, into the blood system; this breach causes serious disease, that can even result in death.

Shotgun proteomics is a groundbreaking development that completely transformed the field of protein research; this advanced technique relies on the combination of liquid chromatography and tandem mass spectrometry (LC-MS/MS), permitting the identification and quantitation of proteins present in even the most intricate mixtures.[29] Shotgun proteomics has revolutionized protein research, it represents a powerful tool for comprehensive analyses of the proteomes present in biological samples, leading to a profounder understanding of cellular processes and disease mechanisms. Comparative proteomic analyses allowed protein studies in in Enteritidis and Dublin serotypes, under a variety of conditions concerning growth media, temperature and host cells.[30] Proteomics also contributed to the identification of drug targets and mechanisms of antimicrobial resistance in the serotype Typhimurium.[31] By analyzing the proteomes of drug-resistant strains, compared to susceptible strains, researchers can identify key proteins associated with resistance mechanisms, representing potential targets for novel antimicrobial treatments. Proteomics studies are essential for the development of strategies for the prevention, diagnosis and treating *Salmonella* infections.

The current article includes an extensive analysis of both the proteome and virulence factors (VFs) produced by 15 different *S. enterica* subsp. *enterica* serotypes isolated from chicken meat, through the use of expanded shotgun proteomics by high-resolution LC-MS/MS. This publication aimed to reveal unique protein expression patterns, specific to different bacterial serotypes, and identify key VFs that affect microbial pathogenicity and host adaptation. Understanding changes in the expression of the VFs, as well as their mechanism of action, is critical for unraveling *Salmonella* pathogenic processes, as well as developing successful targeted interventions against this pathogen. Moreover, this study identified a

variety of peptide biomarkers, specific to particular pathogenic *enterica* serotypes; these biomarkers represent a first crucial step in the accurate detection and identification of this foodborne pathogen, that contaminates foodstuffs destined for human consumption.

MATERIALS AND METHODS

Bacterial strains

Table 1 presents the 15 different *S. enterica* subsp. *enterica* serotypes studied in the current article; they were isolated from chicken meat and are available in the collection of the Central Veterinary Laboratory belonging to the Ministry of Agriculture, Fisheries and Food, (Spain). *Salmonella*, a Gram-negative bacillus, was grown in Brain-Heart-Infusion media for 24 hours at 31ºC. Subsequently, the bacteria were cultured on count agar (PCA; Oxoid, Hampshire, England) until individual colonies were visible, at which point they were carefully collected for further analyses. *S. enterica* subsp. *enterica* serotyping involved the Kauffman-White typing method, and slide agglutination with standard antisera to distinguish somatic (O) from flagellar (H) antigens. All samples were analyzed in triplicate.

Protein extraction

Protein extracts were prepared following the procedure outlined by Carrera et al. (2017).[32] Bacteria were treated with a lysis buffer comprising 60 mM Tris-HCl pH 7.5, 1% lauryl maltoside, 1% dithiothreitol (DTT), and 5 mM phenylmethanesulfonyl fluoride. Cell lysis was completed by the addition of glass beads, followed by several rounds of gentle mechanical breakage, for 10 minutes at 4 ºC. Samples were then centrifuged at 40,000 xg for 10 minutes, on a J221-M centrifuge (Beckman, CA, USA). The proteins, present in the supernatant, were quantified by the BCA assay (Sigma Chemical Co., MO, USA).

Peptide preparation

Proteins were digested with trypsin, as previously described by Carrera et al. (2013).[33] Samples, comprising 100 µg of protein, were dried in a SpeedVac, and then reconstituted in 25 mM ammonium bicarbonate pH 8.0, plus 25 µL of 8 M urea. Proteins were then subjected to sonication for 5 minutes, followed by addition of DTT to a final concentration of 10 mM; the polypeptide mixture was then maintained at 37 ºC for 1 hour. Following addition of

196 iodoacetamide, to a final concentration of 50 mM, the samples were maintained at room
197 temperature for 1 hour in the dark. The next step involved diluting the samples four-fold with
198 25 mM ammonium bicarbonate, at pH 8.0, followed by overnight digestion with trypsin at 37
199 ºC with a ratio of 1:100 (protease:protein) (Promega, WI, USA).

200 **LC-MS/MS analysis using a LTQ-Orbitrap XL**

201 The peptide mixture obtained above was gently acidified by addition of 5% formic acid
202 (FA), until the pH of the sample reached value of 2. The peptides were then purified through
203 a C18 MicroSpin™ column (The Nest Group, South-borough, MA); followed by LC-MS/MS
204 analyses, using a Proxeon EASY-nLC II LC system (Thermo Scientific, San Jose, CA, USA)
205 in conjunction with an LTQ-Orbitrap XL (Thermo Fisher Scientific). To achieve peptide
206 separation, samples (2 µg) were injected into RP columns (EASY-Spray column, PepMap
207 C18, 50 cm x 75 µm ID, 100 Å pore size, 2 µm particles, Thermo Fisher Scientific),
208 incorporating a 10-mm pre-column (Accucore XL C18, Thermo Fisher Scientific). Mobile
209 phase A contained 0.1% FA and 98% acetonitrile, with 0.1% FA as mobile phase B. The flow
210 rate was fixed at 300 nL/min for a duration of 240 min, with a gradual increase in the
211 percentage of solvent B from 5% to 35%. Ionization involved a spray voltage of 1.95 kV and
212 a temperature of 230 ºC. The spectra obtained were analyzed, in positive mode over a range
213 of 400 to 1600 amu (1 µscan), followed by 10 data-dependent CID MS/MS scans (1 µscans).
214 The normalized collision energy was set at 35%, with an isolation width of 3 amu. Dynamic
215 exclusion, for 30 seconds, followed the second fragmentation event; unassigned charged ions
216 were omitted from the analyses.

217 **LC-MS/MS data processing**

218 Proteome Discoverer 2.4 package, including the search engine SEQUEST-HT
219 (Thermo Fisher Scientific), was used to investigate the MS/MS spectra, and the results
220 obtained matched to the *Salmonella* UniProt/TrEMBL database (11,685,506 protein sequence

entries, March 2024). MS/MS searches were conducted taking into account tryptic cleavage constraints, allowing for a maximum of two missed cleavage sites. Tolerance was set at 10 ppm for precursor ions, and 0.06 Da for fragment ions. The permissible variable modifications included N-terminal protein acetylation, carbamidomethylation of cysteines (C*) and methionine oxidation (M*). The results thus achieved were subjected to statistical evaluation, via the Percolator node in the Proteome Discoverer 2.4, with the false discovery rate (FDR) threshold set below 1%. Data from this study can be accessed at the ProteomeXchange Consortium, PRIDE website, using the dataset code PXD051565.[34]

Label-free quantification (LFQ)

All *S. enterica* subsp. *enterica* serotypes identified underwent LFQ of relative protein abundance, utilizing the Minora Feature Detector node and the ANOVA (individual proteins) analysis integrated into the Proteome Discover 2.4 (Thermo Fisher Scientific). The areas in the MS chromatogram that corresponded to the same peptide, but different charge status, were added together.

Euclidean hierarchical clustering

Euclidean hierarchical clustering of the data was determined by the heatmap.2 function, statistical program R (version 4.1.1) (http://www.r-project.org, accessed on 28 March 2024). Graphics were created with the Ggplots package (version 4.1.1), the Euclidean distance measured, and the whole association for the agglomeration methodology were implemented as constraints.

Gene Ontology (GO) and functional pathways

All the unique proteins identified, included under the "Gene name" label in Supplementary Data 1, were characterized using PANTHER (http://www.pantherdb.org/ retrieved on 01 April 2024). The principal categories included biological processes, protein classes and molecular functions. The complete *E. coli* genome was used as reference, and the

246 statistical probability expressed as a percentage. The ID numbers of the orthologous genes
247 were used as standard sets. The pathway examination data were organized, to assess the
248 statistical significance of either over- or under-expressed polypeptides, in relation to the GO
249 of the proteins.

250 **Protein networks**

251 Networks of protein-protein interactions were stablished by analogy to orthologous
252 genes, using the STRING program v.12 (http://string-db.org/, accessed on 29 March 2024).[35]
253 In this algorithm, proteins are symbolized by nodes, and their interactions showed as
254 continuous lines (edges). The connections were validated by either data from the STRING
255 database or from published articles; the confidence score was set at ≥0.9. Clusters were
256 elucidated by the use of the Markov clustering (MCL) algorithm, in STRING, with the
257 standard value set at 2 for the analyses.

258 **Virulence factors (VFs)**

259 The resources available in The Virulence Factors of Pathogenic Bacteria Database
260 (VFDB) were essential to identify the bacterial proteins associated with VFs
261 (http://www.mgc.ac.cn/VFs/, retrieved on 14 April 2024). Furthermore, additional VFs were
262 identified by an examination of current scientific literature.[36-42]

263 **Peptide biomarkers**

264 The peptides recognized by LC-MS/MS were analyzed for homology with the
265 BLASTp program, to determine their specificity to a precise bacterial strain. The selected
266 peptides were further subjected to homology studies by comparison with relevant proteins in
267 the NCBI archive, to identify peptide sequences specific to a particular *Salmonella*
268 species/serotype.[43] These peptide biomarkers could be used to accurately identify relevant
269 *Salmonella* pathogens present in food.

270

RESULTS AND DISCUSSION

Repository of proteomics data

This article analyzes 15 different *S. enterica* subsp. *enterica* serotypes, isolated from chicken meat and that were obtained from the bacterial collection of the Central Veterinary Laboratory, an organization belonging to the Spanish Ministry of Agriculture, Fisheries and Food (Table 1). The bacteria were cultivated in the laboratory, and subjected to proteomic analyses; the bacterial proteins were subjected to trypsin digestion, and the peptides obtained evaluated by LC-MS/MS, in an LTQ-Orbitrap XL.[32, 44-47] The data obtained identified a total of 13,117 peptide spectrum matches (PSMs), with 4,469 representing unique *Salmonella* peptides, correlating with 3,618 individual proteins in the UniProt/TrEMBL database (March 2024) (Supplementary Data 1). The data was submitted to the ProteomeXchange Consortium, via PRIDE repository, identified by the code PXD051565.[48] To the best of our knowledge, this dataset constitutes the largest compendium of proteins and peptides identified, by shotgun proteomics, of *S. enterica* subsp. *enterica* serotypes from chicken meat samples.

LFQ of *S. enterica* subsp. *enterica* serotypes and hierarchical clustering

LFQ analyses identified the prevalence of high-abundance proteins in the bacterial serotypes (Supplementary Data 2). Figure 1 is a graphical representation of the total number of high-abundance proteins present in each of the *S. enterica* subsp. *enterica* serotypes analyzed. The Enteritidis serotype exhibited the greatest number of these proteins, followed by the serotypes Plymouth, Amsterdam, and Thompson.

Figure 2 is a heatmap illustration indicating the presence of high-abundance proteins in the *Salmonella* serotypes. Euclidean hierarchical distance allowed classification of the bacteria into three primary clusters: Cluster A (S1: serotype Typhimurium), Cluster B (S9, S10, S15, and S11: serotype Typhimurium monophasic variant, serotype Worthington, serotype Hadar, and serotype Liverpool), and Cluster C (S2, S14, S13, S7, S5, S8, and S4: serotype Virchow,

296 serotype Enteritidis, serotype Blockley, serotype Gloucester, serotype Plymouth, serotype
297 Amsterdam, and serotype Thompson). The protein clusters depicted in Figure 2 are organized
298 according to the number of proteins, present in each particular bacterial strain, that are either
299 up-regulated (Red) or down-regulated (Green), according to LFQ.

300 The repository was subjected to additional functional *in-silico* studies, to further
301 investigate the functional roles of the proteins. These analyses included: (*i*) investigating
302 functional pathways and GO enrichment, (*ii*) exploring functional networks, (*iii*) identifying
303 the incidence of VFs, and (*iv*) selecting potential specific peptide biomarkers.

304 **Functional pathways and GO**

305 The proteins identified in this work, isolated from 15 *S. enterica* subsp. *enterica*
306 serotypes in chicken meat, were subjected to further functional bioinformatic studies. Protein
307 classification, according to functionality, was determined by submitting the gene names,
308 encoding all non-redundant proteins identified, into the PANTHER classification approach.[49]
309 Figure 3 summarizes the PANTHER results; the identified proteins were classified as
310 concerned in 8 different molecular functions (Figure 3a), 8 diverse biological processes (Figure
311 3b), and belonging to 16 different protein classes (Figure 3c).

312 A meticulous examination of the molecular function category (Figure 3a) reveals
313 catalytic activity as the most common feature among the proteins produced by these pathogenic
314 bacteria (29.3%), followed by polypeptides displaying binding functions (11.2%) and
315 structural proteins (6.5%). *Salmonella* polypeptides with catalytic activity are vital for the
316 bacteria to synthesize amino acids and metabolize sugars, allowing them to obtain nutrients
317 and energy. These enzymes are also essential for the bacterium to display virulence and
318 pathogenicity. The diverse set of catalytic proteins includes kinases, ligases, hydrolases,
319 oxidoreductases, dehydrogenases, proteases, metalloproteases, and transferases. Additionally,
320 some bacterial enzymes break down lipids and proteins, releasing volatile compounds that can

affect the taste and smell of food.[50] Conversely, proteins exhibiting binding functions, such as DNA metabolism proteins, oxidases, reductases, transferases, lyases, ligases, decarboxylases, and oxidoreductases, play a crucial role in various cellular processes. Transporters are also important proteins (primary and secondary carrier transporters, transfer proteins and ATP-binding (ABC) cassette transporters), they move substances within cells.

The protein classification based on biological processes (Figure 3b) revealed a major group, including nearly one-third of the polypeptides analyzed, that are implicated in cellular processes (27%). A slightly smaller percentage of polypeptides (21.4%) plays a role in metabolic processes; while the remaining proteins are classified into groups that include cell localization (4.9%), biological regulation (3.2%), response to stimulus (3.1%), homeostatic processes (0.4%), locomotion (0.2%), immune system mechanisms (0.2%), and developmental processes (0.1%). The serovars included in *S. enterica* subsp. *enterica* are well known pathogens, they cause gastroenteritis, typhoid fever and other illnesses in both humans and animals. Infection with the subsp. *enterica* involves a complex interaction between bacterial proteins and host cells.[51] This subspecies has a type III secretion system that delivers bacterial proteins into host cells, controlling processes like cell structure, transport and immune response, to help the bacteria survive and multiply.[52] *S. enterica* subsp. *enterica* also produces adhesin and invasion proteins, that enable the adhesion and invasion of host cells.[53] Some bacterial strains produce toxins, such as the cytolethal distending toxin and enterotoxins, which damage host cells, producing diarrhea and inflammation.[54] The *enterica* subspecies also expresses proteins involved in acquiring essential nutrients from the host cell environment; they enable *Salmonella* to scavenge for nutrients, such as iron, and compete with the host for resources, thereby promoting bacterial growth and proliferation. This bacterium also produces proteins that help the pathogen evade the host immune response; they can interact with host immune pathways, inhibit immune cell stimulation and modulate the generation of

346 antimicrobial peptides (AMPs).[55] A proper understanding of the task of these proteins in the
347 pathogenesis of *S. enterica* subsp. *enterica* is crucial for developing targeted interventions to
348 both prevent and treat infections by this pathogen.

349 Classification of the identified proteins (Figure 3c) indicated a preponderance of
350 metabolite interconversion enzymes (33.7%), translational proteins (15.2%) and transporters
351 (9.6%). The biological catalysts group, involved in metabolite interconversion, contains
352 reductases, hydrolases, transferases, decarboxylases, ligases, transaminases, mutases, kinases,
353 and deacetylases.[56] The translational protein group incorporates ribosomal proteins and
354 aminoacyl-tRNA synthetases; while the transporter group contains transporter polypeptides,
355 primary active transporters, secondary carrier transporters, ATP-binding (ABC) cassettes, and
356 ATP synthases. Regulating the action of metabolite interconversion enzymes is essential to
357 reduce food spoilage. The presence of bacteria with high proteolytic activity can accelerate
358 food spoilage, by breaking down proteins and promoting food decomposition; this process
359 releases small peptides and amino acids, which become the substrate for decarboxylases.[57]
360 Decarboxylases are important enzymes, that remove carboxyl groups from amino acids,
361 converting them into amines and carbon dioxide; they are also involved in fermentation, that
362 can produce food spoilage. Furthermore, decarboxylases can convert peptides and amino acids
363 into biogenic amines (BAs), some of which represent a threat to human health.[46, 58] Figure 4
364 displays the distribution of the main decarboxylase enzymes identified in the different serovars
365 of *S. enterica* subsp. *enterica*. The serotypes Typhimurium and Virchow produce higher
366 decarboxylase levels, which can facilitate colonization and survival in the host, increasing their
367 pathogenicity. Some reports suggest that, decarboxylases expedite bacteria growth and survival
368 inside the host, by forming biofilms, increasing resistance to host defenses. In summary, these
369 enzymes increase the versatility of the bacteria, facilitating survival, adaptation and
370 pathogenesis in a variety of environments, including the human host.

Network analyses of protein interactions

Protein network analyses were performed with the STRING v.12 package (https://string-db.org/, retrieved on 29 March 2024); all the proteins identified in this study were compared to the genome of the bacterium *S. enterica* subsp. *enterica* Typhimurium, considered the genetically closest organism.[35] To prevent either false positive/negatives, the constraints in the STRING package were fixed at the highest confidence rate (≥0.9).

The complete protein network for the entire polypeptide dataset identified is displayed in Figure 5; the interactome network contains 543 nodes (proteins) and 1,480 edges (interactions). This network represents the most extensive interactome map currently available for *S. enterica* subsp. *enterica* pathogenic serotypes. Cluster networks were constructed using the inflation clustering MCL algorithm, integrated in STRING; the standard value was set at 2 for all evaluations. This study identified 81 exclusive main clusters (Supplementary Data 3). They comprise 57 nodes associated to ribosomal metabolism (dark red), 10 related to Gram-negative bacterium membrane assembly (pink), 10 connected to bacterial peptidoglycan membrane assemblage (brown), as well as 8 connected with flagellar metabolism (pink); there were also 7 nodes linked to antibiotic resistance (orange), 6 corresponding to virulence (brown) and 2 involved in biofilm formation (red). Peptidoglycan assembly is essential for bacteria to maintain their cell shape and integrity. This is particularly important for foodborne bacteria, exposed to environmental stresses during food storage and handling; disruptions in either peptidoglycan synthesis or assembly, can weaken the bacterial cell wall, leading to cell breakdown and release of intracellular contents into the foodstuff, hence modifying its appearance, flavor, odor, and texture.[59] Enzymes implicated in peptidoglycan metabolism (Supplementary Data 3) are essential for cell wall repair and maintenance, and crucial for bacterial growth and adaptation to different environmental situations.[60] It is worth noting that foodborne bacteria can have flagella, structures that facilitate movement; this allows the

bacteria to spread further within the food environment and colonize a larger area. Flagella not only aid bacterial movement and ability to find nutrients, but are also intricated in the formation of biofilms. Biofilms constitute a main problem in the food industry, as they are hard to detect and eliminate; they often cause persistent contaminations that result in food spoilage, and even outbreaks of foodborne diseases. Furthermore, flagella can increase the virulence and survival of certain spoilage bacteria.[61]

Examination of the protein interaction network identified here, could provide important insights to develop improved strategies to combat harmful foodborne bacteria. Understanding these interactions would also help elucidate the complex processes involved in bacterial metabolism and dissemination, facilitating the prevention of food contamination.

Proteins involved in the pathogenesis *S. enterica* subsp. *enterica* serotypes

S. enterica subsp. *enterica* pathogenesis involves an interplay between bacterial proteins and host cellular processes; with polypeptides facilitating bacterial invasion, survival, immune evasion, and systemic spread. Table 2 summarizes the bacterial enzymes, identified by LC-MS/MS, that are involved in pathogenesis.

Adhesin proteins facilitate bacterial adhesion to the host; they interact with host cell receptors and trigger the internalization process, allowing *Salmonella* to breach the intestinal barrier and establish infection.[53] As summarized in Table 2, the shotgun proteomic analyses identified a total of 14 adhesin proteins in *S. enterica*.

Invasion proteins enable the bacterium to enter host cells, as they trigger internalization processes within the host cells.[53] As reflected in Table 2, this study identified 4 invasion proteins.

Bacteria produce Type III secretion systems to inject effector proteins directly into host cells; these effectors control host cellular procedures, to facilitate bacterial invasion and intracellular survival.[51] They can modulate signaling pathways and cytoskeletal dynamics, as

well as vesicular trafficking, to create a niche favorable for bacterial replication. A total of 26 Type III secretion system proteins were identified, by shotgun proteomics, in *S. enterica* subsp. *enterica* (Table 2).

Bacterial toxins facilitate bacterial pathogenesis; for example, the cytolethal distending toxin causes DNA damage and cell cycle arrest in host cells, hence promoting tissue damage and inflammation.[54] Enterotoxins produced by certain *Salmonella* strains can disrupt intestinal epithelial barrier function and contribute to diarrhea. A total of 21 toxin proteins were identified in *S. enterica* subsp. *enterica* (Table 2).

Once inside host cells, bacterial effector proteins modulate various cellular processes, to promote bacterial survival and dissemination; they can interfere with host immune signaling pathways, inhibit apoptosis and manipulate vesicular trafficking, to evade host defenses and establish intracellular replication.[51] This study identified a total of 11 effector proteins in *S. enterica* subsp. *enterica* (Table 2).

S. enterica subsp. *enterica* expresses iron acquisition proteins, that permit the bacterium to obtain iron from the host environment; iron is elemental for bacterial growth, and *Salmonella* has evolved mechanisms to scavenge iron from host proteins. This ability contributes to bacterial proliferation and systemic spread within the host. A total of 9 iron acquisition/transport proteins were identified from the bacterial serotypes studied (Table 2).

As mentioned above, flagellar proteins allow *Salmonella* to move through the host environment and penetrate the intestinal mucosa.[61] Flagellar motility is essential for bacterial dissemination within the host, and contributes to the establishment of infection. A total of 9 flagellar proteins were identified, by shotgun proteomics, from the 15 different *S. enterica* subsp. *enterica* serotypes analyzed (Table 2).

Understanding the role of the proteins, reported here, in bacterial pathogenesis is crucial for developing targeted interventions, such as vaccines and antimicrobial therapies, not only to

446 effectively combat *Salmonella* infections, but also to eliminate, or at least limit, food
447 contamination by of these pathogenic bacteria.

448 **Bacterial proteins implicated in food spoilage, identified by LC-MS/MS**

449 Spoilage bacteria release enzymes, as part of their natural processes, that significantly
450 contribute to food spoilage.[62] Table 3 provides a list of these bacterial enzymes, identified
451 through LC-MS/MS analysis, along with the specific proteins present in the 15 different *S.*
452 *enterica* subsp. *enterica* serotypes.

453 Aminopeptidases are crucial for protein metabolism, as they remove amino acids from
454 the N-terminal of peptide chains. These enzymes can contribute to food spoilage by breaking
455 down proteins and peptides, in turn, affecting the taste and structure of food.[63] This shotgun
456 proteomics work identified three aminopeptidases (Table 3); the enzymes facilitate bacterial
457 adaptation, virulence, and survival within the host.[64] Inhibitors of aminopeptidases could
458 disrupt bacterial protein metabolism and reduce their ability to cause infections, rendering them
459 potential targets for new antibacterial agents against foodborne pathogens.

460 Amylases are enzymes that facilitate the breakdown of starch into sugars. When
461 spoilage bacteria containing amylases are present in food, they have the ability to degrade the
462 carbohydrates in the product, which can impact its texture and taste.[65] Alpha-amylase is one of
463 these proteins identified (Table 3). While this polypeptide does not constitute a primary enzyme
464 in *Salmonella*, the presence of alpha-amylase could enhance the bacterial ability to use a variety
465 of carbohydrate sources, present in different environments, potentially facilitating survival and
466 colonization.

467 Decarboxylases play important roles in various bacterial metabolic and regulatory
468 pathways, affecting the capability of the microorganism to subsist and cause disease. This study
469 used shotgun proteomic analyses to identify 13 different decarboxylases in *S. enterica* subsp.
470 *enterica* serotypes (Table 3). These enzymes break down amino acids and other organic

471 molecules in food, potentially creating unpleasant or harmful compounds, like the BAs
472 histamine and cadaverine.[66] Certain decarboxylases also help the bacteria respond to stress and
473 enhance their virulence; these include lysine decarboxylase and ornithine decarboxylase.[67] By
474 targeting the activity of these enzymes, it may be possible to disrupt *Salmonella's* ability to
475 survive in acidic environments and form biofilms, reducing its virulence and persistence of
476 infections.[68]

477 Lipases are enzymes that break down lipids into fatty acids and glycerol. In bacteria,
478 these enzymes can spoil food and affect its quality. This study identified two different lipases
479 (patatin-like phospholipase and GDSL family lipase) in *Salmonella* strains, believed to play a
480 role in bacterial virulence and persistence (Table 3). These enzymes help break down host cell
481 membranes, facilitating invasion and infection, as well as using host lipids for nutrition to
482 promote bacterial growth. Lipase activity also affects biofilm formation, helping pathogens
483 persist and resist antimicrobials.[69] Ultimately, by breaking down lipids in host cell membranes,
484 lipases can increase the pathogenicity of *S. enterica* subsp. *enterica*.

485 Proteases are enzymes that break down proteins in bacteria, playing a crucial role in
486 their capability to cause disease and survive. This study used LC-MS/MS to identified 10
487 proteases in *Salmonella* that are involved in various bacterial functions, such as tissue invasion,
488 immune evasion, and nutrient acquisition (Table 3). Some of these enzymes are considered
489 VFs, as they break down host proteins and facilitate tissue invasion.[70] Proteases also help
490 *Salmonella* elude the host immune system, and affect biofilm formation, which is important
491 for bacterial survival and resistance to antibiotics. Understanding how proteases function in
492 pathogenic *Salmonella* strains, could lead to targeted therapies to combat infections and protect
493 public health.

494 Sulfur-release enzymes are crucial for the survival, metabolism and pathogenicity of
495 bacteria; they assist the bacterium by producing essential sulfur-containing compounds,

managing oxidative stress, and surviving in different environments, particularly during host infection.[62] This study identified five sulfur-release enzymes, from *S. enterica* subsp. *enterica* serovars, that are implicated in important metabolic pathways, such as producing sulfur-containing amino acids and cofactors (Table 3). The enzymes are essential for *Salmonella* survival in different environments, such as inside their host, by helping it withstand stress, eliminate toxins, and effectively regulate its metabolism.

Virulence factors (VFs)

A total of 941 non-redundant VFs were identified in this study, using the VFDB database and relevant literature.[36-42] These proteins are implicated in immune evasion and eukaryotic cell colonization, while others represent toxins and polypeptides linked to antibiotic resistance. The 941 virulent peptides mentioned above (Supplementary Data 4) are classified according to the functions they perform: *i*) *Salmonella*-host interaction and surveillance within eukaryotic cells, *ii*) toxins, *iii*) mobile genetic elements; *iv*) production of antimicrobial compounds, *v*) antimicrobial and drug resistances, *vi*) other tolerance proteins implicated in resistance to toxic elements, *vii*) transporters, and *viii*) BA production.

S. enterica subsp. *enterica* is one the principal sources of foodborne diarrhea worldwide; with the Typhimurium serotype considered as the major pathogen involved in human foodborne diseases.[71] Many virulence genes, associated with adhesion, intracellular survival, invasion, iron acquisition, systemic infection, and toxin generation, have an impact on the pathogenicity of *Salmonella*.[37] The proteins detected as VFs in this study are summarized in Supplementary Data 4.

As a general observation, *Salmonella* typically possesses 5-10 flagella, distributed in a seemingly random pattern, facilitating its movement. Typhimurium serotype utilizes flagella-assisted swimming near cell surfaces to approach their target cells. A recent study highlighted the presence of the fliC gene in this subspecies, that encodes the flagellin protein, essential for

interacting with target cells. Furthermore, *Salmonella* employs various strategies to breach the intestinal mucosa and target absorptive intestinal epithelial cells (IECs), such as M cells or dendritic cells. These pathogenic bacteria can also colonize Peyer's patches, using actin as transporter to reach vital organs, like the liver, mesenteric lymph nodes and spleen.

The type III secretion system (TTSS), present in *Salmonella*, enables the transfer of effector proteins from the bacterial cytoplasm into the host cell, by direct contact. Two genes, spaN and sipB, are associated to the TTSS structure; the spaN gene facilitates the entrance of bacteria into non-phagocytic cells, while sipB induces macrophage death.[23, 39] Invasion of IECs and B cells is interceded by the *Salmonella* pathogenicity island 1 (SPI-1), which codes for the TTSS-1, as well as several effectors; the latter include a set of adhesins, that facilitates docking to the host cell and acts as an injection needle, followed by host cell pro-inflammatory responses.[71] SPI is also involved in additional virulence mechanisms of host colonization, such as capsule formation, toxin production, fimbriae, biofilm formation, invasiveness, flagella, secretion, and serotype conversion.[23]

The LC-MS/MS analyses, on *S. enterica* subsp. *enterica* serotypes, revealed the presence of key proteins, including fimA, faeC, faeE, lpf, and pilW, involved in host recognition, facilitating bacterial attachment to the intestinal epithelium and invasion of host cells. The fim operon, including genes fimA, C, D, H, I, and F, along with fae genes (faeC, D, E, F, H, and I), are crucial components of the K88 fimbrial adhesin, responsible for facilitating bacterial adhesion to eukaryotic cells.[41] The pilW protein plays a crucial task in bacterial adherence to host cells, while the long polar fimbriae (lpf) facilitate attachment to the Peyer's patches.[39, 41] Additionally, the MisL protein, involved in intestinal occupation, acts as an extracellular matrix adhesin; its passenger domain enables serotype Typhimurium to increase invasiveness, by binding fibronectin to human epithelial cells.[72] The two-component system QseBC is responsible for regulating biofilm formation and flagella activity. Specifically, the

546 QseC protein in subsp. *enterica* Typhimurium, was demonstrated to up-regulate the expression
547 of genes associated to colonization and motility.[40] The RatB protein, discovered by LC-
548 MS/MS, is a key factor in intestinal colonization, present in the 25-kb pathogenicity island
549 CS54; this island is unique to certain *S. enterica* subspecies, and plays a critical role in both
550 intestinal colonization and persistence.[73] The aptitude of the bacteria to persist in host intestines
551 is partly attributed to extracellular matrix binding, facilitated by the protein ShdA, also
552 identified in these studies. ShdA is an element of the autotransporter family of outer membrane
553 proteins, with a passenger domain that binds collagen I and fibronectin.[74, 75] Other proteins
554 identified include components of the SipBC translocon, and the TTSS-1 effector proteins SopB
555 and SopD. The translocation of TTSS-1 effectors into the host's cytosol starts with the addition
556 of the SipBC translocon into the host cell membrane.[71] SopB is a lipid phosphatase that
557 modifies phosphatidylinositol-phosphate in the plasma membrane, while genes like SopB/SigD
558 and SopE2 facilitate rapid bacterial internalization. The sopB gene in *Salmonella* strains
559 expedites bacterial infection and promotes diarrhea, facilitating pathogenic spread.

560 The key mechanism in the pathogenicity of *Salmonella* is its ability to remain enclosed
561 in a vacuole inside the host cell, unlike other pathogens, such as *Shigella* and *Listeria*, that
562 break out of the vacuole and multiply in cytoplasm of host cells. This unique trait allows
563 *Salmonella* to release effector proteins, encoded in the SPI-2 pathogenicity island, into the host
564 cytosol, creating a protective environment that enables the bacteria to elude the host immune
565 defenses.[76] The protein SsrA-ssrB, identified in this study, plays a critical role regulating the
566 SPI-2 gene, which is crucial for *Salmonella* survival and replication within host macrophages.[77]

567 This work identified the occurrence of the PhoQ protein in *S. enterica* strains. PhoPQ,
568 a two-component regulatory system, is one of the key genetic factors in *Salmonella* for
569 interaction with the host, while located inside eukaryotic cells. This system regulates the
570 production of *Salmonella* effector proteins, essential for bacterial survival inside macrophages;

PhoPQ is encoded within the *Salmonella* SPI-2.[76] Additional proteins that facilitate bacterial intracellular survival include the polypeptides coded by the msgA, and tolC genes.[23]

Host production of AMPs triggers the activation of the bacterial protein PbgA, crucial for maintaining the PhoPQ system in *S. enterica* subsp. *enterica* Typhimurium. This process promotes restructuring of the bacterial outer membrane, enhancing resistance to host innate immune AMPs. The LC-MS/MS studies also identified the roles of PbgA and the PmrA/Pmrb system in LPS assembly, influencing variable Type I IFN responses, and facilitating bacterial survival. Furthermore, the protein SpoD helps evade lysosomal degradation.[78] *Salmonella* is resilient to oxidative stress, through the actions of catalase, antioxidant proteins, and superoxide dismutase (sodCI), encoded by the lysogenic phage Gifsy-2.[79] These findings shed light into the mechanisms used by *S. enterica* subsp. *enterica* serotypes to survive oxidative processes, with superoxide dismutase playing a major role.

LC-MS/MS facilitated the identification of 425 peptides, that facilitate *Salmonella*-host interaction and surveillance of eukaryotic cells.

The capability of *S. enterica* serovars to produce both endotoxins and exotoxins is also linked to their pathogenicity. Endotoxins can trigger a diverse array of biological reactions, whereas exotoxins, that include cytotoxins and enterotoxins, are associated with mammalian cell death.[80] Of the 941 peptides identified in this study, 28 are associated with bacterial toxicity. These 28 polypeptides comprise Type II toxin-antitoxin system VapB family antitoxin, addiction module toxin, GnsA/GnsB family, Type II toxin-antitoxin system ParD family antitoxin, and Type II toxin-antitoxin system RelE/ParE family toxin.

Plasmids represent the main source for *Salmonella* to spread its antibiotic resistance genes around the world. The LC-MS/MS work identified a total of 231 bacterial peptides related with mobile genetic elements. The proteins correspond to plasmids such as IncFIB (S), IncFII (S) and IncI1, that are not only virulence-related plasmids, but also carry multidrug

596 resistance genes.[41] Plasmids within the IncI1 family are believed to be virulence plasmids,
597 because they encode type IV pili, that enable bacterial adhesion to host cells. Other plasmid
598 virulence peptides are spvA and spvB, both from the spv locus; that contains five genes (spv
599 RABCD) and are strongly linked with bacterial strains that trigger non-typhoid bacteremia and
600 dispersion of infection in humans.[23, 42] Proteins encoded by traT genes, also encoded by
601 virulence plasmids, were also identified in this investigation, they could be involved in
602 *Salmonella* resistance to the bacteriolytic action of serum.[42]

603 *Salmonella*, a pathogenic bacterium, poses a significant global health threat, due to its
604 rapid development of multidrug resistance, including resistance to significant antimicrobials,
605 like fluoroquinolones and third-generation cephalosporins.[80] This study identified a range of
606 peptides linked to antimicrobial resistance, including penicillin-binding protein, parC, TetR
607 transcriptional regulator, streptomycin 3"-adenylyltransferase, acriflavine, and tetracycline
608 resistance proteins; they also encompass specific drug transporters and multidrug resistance
609 proteins.[45] For instance, mutations in the parC gene are known to reduce susceptibility to
610 ciprofloxacin, a key treatment for *Salmonella* infections in humans.[41] In fact, 17 peptides
611 associated with antimicrobial and drug resistance were identified.

612 Additional tolerance proteins play a role in protecting *Salmonella* from toxic
613 compounds. The LC-MS/MS study identified a total of 152 peptides, corresponding to a variety
614 of tolerance proteins associated with resistance to toxic elements, such as the magnesium
615 transporter MgtC, that is crucial for virulence and bacterial intracellular survival; as well as
616 MgtC, a copper resistance protein, and MerR, that binds mercury ions.[47, 81] The PhoPQ regulon,
617 mentioned above, is also involved in the transcriptional regulation of ferric iron uptake. This
618 study identified a diversity of transporters that play a role in the elimination of toxic
619 components; in addition to 63 peptides corresponding to transporters involved in bacterial
620 pathogenicity.

Numerous bacteria naturally produce BAs in their metabolic processes; these BAs, derived from amino acids, can pose potential health risks when consumed by individuals. A study by Abril et al. (2024) revealed the occurrence of 34 peptides linked to BAs in *S. enterica* subsp. *enterica*; they include key enzymes, such as acetylornithine aminotransferase, ornithine decarboxylase, lysine decarboxylase CadA, spermidine synthase, putrescine-binding periplasmic protein, and histidine kinase. Notably, lysine decarboxylase converts lysine to cadaverine, while ornithine decarboxylase facilitates the conversion of ornithine to putrescine - both of which are classified as BAs, alongside histamine and spermidine.[82]

Finally, 5 peptides were identified as antimicrobial compounds, while 3 peptides belong to the translocation domain superfamily Pyosin/cloacin, a bacteriocin and a colicin.

Selection of specific peptide biomarkers

The primary aim of these investigations was to select peptide biomarkers unique to each of the bacterial serotypes under study. Peptides exclusively associated with a single microbial serotype, as revealed by LC-MS/MS, were selected for further analyses, involving extensive sequence homology searches with the BLASTp package, to validate their specificity to a particular *Salmonella* serotype.[43] Any peptides with homology to more than one serotype were disregarded.

Supplementary Data 5 provides a detailed overview of the analyses and selection of four serotype-specific tryptic peptide biomarkers: RC*SNPATGM*WITARSR, TSGGNGSNLKIYRLGDILTAM*M*TM*PAVTGENGPNK, GMSLEAARAEMIGC*LDR, and QHPHVHAQATIV. The peptides correspond to 4 different proteins; *i*) predicted bacteriophage protein, *ii*) DUF1441 family protein, *iii*) Microcin C ABC transporter ATP-binding protein, and *iv*): Uncharacterized protein. Through rigorous testing and comparison against the NCBI protein database, by means of the BLASTp program, these biomarkers were confirmed to be specific for the *S. enterica* subsp. *enterica* Typhimurium serotype.

These peptide biomarkers are a valuable resource for the rapid identification and characterizing of the Typhimurium serotype present in food as chicken meat. This represents a novel tool to facilitate food quality, and also safety, at the diverse stages of food production for human consumption.

Final Statements

The presence of *Salmonella* in food products, particularly chicken meat, often triggers food alerts and recalls due to its potential to cause severe health issues such as gastroenteritis, typhoid fever, and septicemia. The pathogenicity of *Salmonella* is attributed to its virulence factors, including adhesins, invasins, and toxins, which facilitate bacterial invasion, survival, and immune evasion within the host. The rapid emergence of multidrug-resistant *Salmonella* strains exacerbates the public health threat, complicating treatment and control measures. Continuous monitoring and stringent food safety protocols are essential to mitigate the risks associated with *Salmonella* contamination. This study's proteomic analysis of *S. enterica* subsp. *enterica* serotypes from chicken meat provides critical insights into the bacterial proteins involved in pathogenicity and spoilage, aiding in the development of targeted interventions to enhance food safety and public health.

This manuscript provides a comprehensive analysis of the data collected, through shotgun proteomics, for 15 distinct foodborne *S. enterica* subsp. *enterica* serotypes from chicken meat. This investigation identified a total of 13,117 PSMs, including 4,469 unique peptides and corresponding to 3,618 annotated proteins from the *Salmonella* UniProt/TrEMBL database. By exploring the functional pathways and protein interactions, a complex protein interactome network was created, shedding light into the role of these bacterial strains in food pathogenicity and spoilage. The majority of the proteins identified are related with energy pathways, peptidoglycan biosynthesis, infection metabolism, and antibiotic resistance, hence, providing valuable insights for further research in this field. Moreover, this study successfully

identified 941 unique peptides associated with VFs, playing crucial roles in immune evasion, toxin production, antimicrobial compound production, host colonization, and bacterial tolerance to toxic substances. Furthermore, 4 peptide biomarkers, specific to *S. enterica* subsp. *enterica* Typhimurium serotype, were identified, representing a rapid and accurate method for the detection of this major pathogen. The data presented here constitutes a considerable dataset of peptides, and proteins, belonging to foodborne bacterial strains that cause pathogenicity and food spoilage. This massive body of knowledge could represent the basis for the development of improved treatments to counteract the harmful effects of foodborne bacteria. The findings also provide valuable insights for evaluating microbial contamination in food, as they facilitate the identification of harmful pathogenic bacterial strains. It is worth emphasizing that *Salmonella*, the central focus of this study, remains as the major bacterial source of foodborne diseases globally, resulting in elevated morbidity and mortality rates on a global scale. The data presented in this study can act as the foundations for future proteomic research into the detection of bacterial contamination in food processing, a critical element in maintaining quality standards and ensuring consumer safety, especially within the food industry.

In addition, the proteomic study presented here serves as a foundational resource for future multi-omics research. By providing a comprehensive dataset of proteins and peptides from various *S. enterica* subsp. *enterica* serotypes isolated from chicken meat, this study enables the integration of proteomic data with other omics approaches, such as genomics, transcriptomics, and metabolomics. This integrative approach may offer deeper insights into the molecular mechanisms underlying *Salmonella* pathogenicity, antibiotic resistance, and food spoilage. Furthermore, the extensive proteomic repository established in this study can facilitate the identification of novel biomarkers and therapeutic targets, ultimately contributing to the development of more effective strategies for preventing and controlling *Salmonella* contamination in the food supply chain.

AUTHOR CONTRIBUTIONS

The authors have all made significant contributions to the work presented in this article; it would not have been possible without their valuable participation. Additionally, all authors have given their approval for the manuscript to be published.

ACKNOWLEDGEMENTS

We would like to thank to Karola Böhme (Department of Analytical Chemistry, Nutrition and Food Science, Food Technology Division, University of Santiago de Compostela, School of Veterinary Sciences, Campus Lugo, Lugo, Spain) and Alexandre Lamas (Department of Analytical Chemistry, Nutrition and Bromatology, Food Hygiene, Inspection and Control Laboratory, University of Santiago de Compostela, Campus Terra, Lugo, Spain) for the providing, not only bacterial samples of the *S. enterica* subsp. *enterica* serotypes from chicken meat used in this work, but also the relevant information required for handling the bacteria.

SUPPORTING INFORMATION STATEMENTS

Supplementary Data 1: Complete list of peptide spectrum matches (PSMs), peptides and proteins identified by shotgun proteomics for each of the 15 different *S. enterica* subsp. *enterica* serotypes.

Supplementary Data 2: Label free quantification (LFQ) data for proteins and peptides for each of the 15 *S. enterica* subsp. *enterica* serotypes.

Supplementary Data 3: Major clusters created by MCL (inflation clustering) software from the STRING program.

Supplementary Data 4: Summary of the most relevant proteins identified as virulence factors.

Supplementary Data 5: Analyses and list of the serotype-specific tryptic peptide biomarkers.

FUNDING

This study received financial support from various sources including the Spanish AEI/EU-FEDER PID2019-103845RB-C21 project, and the Plan Complementario en Ciencias Marinas (PCCM), founded by the Ministry of Science and Innovation (3.6.B. NANOSEAOMICS). Added funding was provided by the European Union (European Social Fund–ESF), the Spanish Ministry of Economy and Competitiveness (AGL 2.013-48.244-R), and the European Regional Development Fund (ERDF). A.G.A. would like to extend her appreciation to the USC for her postdoctoral fellowship, the "Convocatoria de Recualificación do Sistema Universitario Español-Margarita Salas", which is part of the "Plan de Recuperación Transformación" and funded by the Spanish Ministry of Universities through the EU NextGeneration Fund.

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FIGURE CAPTIONS

Figure 1. Total number of high-abundance proteins in each of the bacterial *S. enterica* subsp. *enterica* serotypes analyzed, as determined by LFQ; the y-axis represents the number of identified proteins.

Figure 2. Heatmap diagram obtained from the shotgun proteomic analyses of 15 different *S. enterica* subsp. *enterica* serotypes. Every bar corresponds to a particular protein that can be either up-regulated or down-regulated. Red = up-regulated proteins; green = down-regulated proteins. Euclidean hierarchical distances were estimated for all the bacterial strains, and the principal clusters identified.

Figure 3. (a) Molecular functions of the proteins obtained from *S. enterica* subsp. *enterica* serotypes; the peptides were identified using shotgun proteomics, while the function was assigned by the PANTHER classification system, using the gene names as inputs in the software; (b) Biological processes carried out by the bacterial proteins identified by shotgun proteomics and classified according to PANTHER; (c) Determination of the protein classes the identified polypeptides belong to, according to the PANTHER classification system.

Figure 4. Decarboxylase enzymes identified for each of the *S. enterica* subsp. *enterica* serotypes analyzed. The y-axis represents the enzyme units. Group proteins in accession name.

Figure 5. Protein interactome network for all the proteins identified in this study, and deposited in the repository of foodborne *S. enterica* subsp. *enterica* serotypes. The complete network consists of 543 nodes (proteins) and 1,480 edges (interactions). Protein interactions were analyzed using the STRING v.12 software. Only the highest-confidence interactions (≥0.9), as determined by the STRING software, were selected for this study. The circles represent the proteins, while the interactions between proteins are depicted by either continuous lines, for direct interactions (physical), or dotted lines, for indirect interactions (functional).

Table 1. *S. enterica* subsp. *enterica* serotypes identified in this study.

Sample	Bacterial strain serotype	Source	O antigens	H antigens Phase 1	H antigens Phase 2
S1	*S. enterica* subsp. *enterica* serovar Typhimurium	Chicken meat	1,4,[5],12	i	1,2
S2	*S. enterica* subsp. *enterica* serovar Virchow	Chicken meat	6,7,14	r	1,2
S3	*S. enterica* subsp. *enterica* serovar Enteritidis	Chicken meat	1,9,12	g,m	-
S4	*S. enterica* subsp. *enterica* serovar Thompson	Chicken meat	6,7,14	k	1,5
S5	*S. enterica* subsp. *enterica* serovar Plymouth	Chicken meat	9,46	d	z_6
S6	*S. enterica* subsp. *enterica* serovar Enteritidis	Chicken meat	1,9,12	g,m	-
S7	*S. enterica* subsp. *enterica* serovar Gloucester	Chicken meat	1,4,12,27	i,l,w	-
S8	*S. enterica* subsp. *enterica* serovar Amsterdam	Chicken meat	3,{10},{15},{15,34}	g,m,s	-
S9	*S. enterica* subsp. *enterica* serovar Typhimurium monofasica	Chicken meat	1,4,[5],12	i	-
S10	*S. enterica* subsp. *enterica* serovar Worthington	Chicken meat	1,13,23	z,l,w	-
S11	*S. enterica* subsp. *enterica* serovar Liverpool	Chicken meat	1,3,19	d,e,n,	z_{15}
S12	*S. enterica* subsp. *enterica* serovar Typhimurium	Chicken meat	1,4,[5],12	i	1,2
S13	*S. enterica* subsp. *enterica* serovar Blockley	Chicken meat	6,8	k	1,5
S14	*S. enterica* subsp. *enterica* serovar Enteritidis	Chicken meat	1,9,12	g,m	-
S15	*S. enterica* subsp. *enterica* serovar Hadar	Chicken meat	6,8	e,n,x	z_{10}

Table 2. Bacterial enzymes, identified by LC-MS/MS analyses, that play a role in the pathogenesis of the 15 *S. enterica* subsp. *enterica* serotypes studied.

Enzyme Type	Proteins identified by LC-MS/MS	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Adhesin	Adhesin				■		■	■						■	■	■
Adhesin	Agglutinating adhesin						■	■							■	
Adhesin	Autotransporter adhesin Ag43							■								
Adhesin	Autotransporter adhesin BigA										■					
Adhesin	Fibronectin-binding autotransporter adhesin ShdA	■						■								
Adhesin	Fimbrial adhesin						■									
Adhesin	Intimin-like adhesin FdeC	■														
Adhesin	Fibronectin-binding autotransporter adhesin	■														
Adhesin	Prepilin pilus tip adhesin PilV	■														
Adhesin	Putative adhesin Stv domain-containing protein		■													
Adhesin	Putative fimbrial-like adhesin protein										■					
Adhesin	Shufflon syst plasmid conjugative transfer pilus tip adhesin PilV						■			■				■	■	
Adhesin	Trimeric autotransporter adhesin YadA-like C-terminal membrane anchor domain-containing prot.															■
Adhesin	Type 1 fimbrial prot. subunit FimA Fimbriae-like adhesin SfmA				■											
Invasion proteins	Invasion protein SipC							■								
Invasion proteins	Salmonella invasion prot. A N-terminal domain-containing prot			■										■		
Invasion proteins	Type III secretion system invasion protein IagB						■									
Invasion proteins	Type III secretion system outer membrane ring protein InvG											■				
Type III secret. prot.	EscI/YscI/HrpB family type III secretion system inner rod prot.	■	■													
Type III secret. prot.	EscN/YscN/HrcN family type III secretion system ATPase	■														
Type III secret. prot.	EscV/YscV/HrcV family type III secretion syst. export prot.						■									
Type III secret. prot.	Invasion protein SipC							■								
Type III secret. prot.	Pathogenicity island 1 effector protein SipB										■					
Type III secret. prot.	Secreted effector protein									■						

Type III secret. prot.	SPI-1 type III secret. syst. effector E3 ubiquitin transferase SlrP	
Type III secret. prot.	SPI-1 type III secretion system effector SipA	
Type III secret. prot.	SPI-1 type III secretion system effector SopD	
Type III secret. prot.	SPI-1 type III secretion system needle tip complex protein SipB	
Type III secret. prot.	SPI-1 type III secretion system needle tip complex protein SipC	
Type III secret. prot.	SPI-2 type III secretion system apparatus protein SsaQ	
Type III secret. prot.	SPI-2 type III secretion system apparatus protein SsaV	
Type III secret. prot.	SPI-2 type III secretion syst. effector E3 ubiquitin transf. SspH2	
Type III secret. prot.	SPI-2 type III secretion system effector SifA	
Type III secret. prot.	SPI-2 type III secretion system effector SifB	
Type III secret. prot.	Type III secretion system effector	
Type III secret. prot.	Type III secretion system effector PipB2	
Type III secret. prot.	Type III secretion system effector protein OrgC	
Type III secret. prot.	Type III secretion system invasion protein IagB	
Type III secret. prot.	Type III secretion system needle complex protein	
Type III secret. prot.	Type III secretion system needle tip complex protein IpaD	
Type III secret. prot.	Type III secretion system outer membrane ring protein InvG	
Type III secret. prot.	Type III secretion system protein SpaN	
Type III secret. prot.	Type III secretion system protein SsaI	
Type III secret. prot.	Type III secretion system translocon protein	
Toxin	Addiction module toxin, GnsA/GnsB family	
Toxin	Antitoxin of toxin-antitoxin stability system	
Toxin	Antitoxin ParD	
Toxin	Bacterial toxin 33 domain-containing protein	
Toxin	Cytolethal distending toxin subunit A	
Toxin	Putative antitoxin protein (CcdA-like)	
Toxin	Toxin CcdB	
Toxin	Toxin co-regulated pilus biosynthesis	
Toxin	Toxin YqcG C-terminal domain-containing protein	

Toxin	Toxin-antitoxin system, antitoxin component	
Toxin	Type II toxin-antitoxin system HicA family toxin	
Toxin	Type II toxin-antitoxin system HicB family antitoxin	
Toxin	Type II toxin-antitoxin system HigA family antitoxin	
Toxin	Type II toxin-antitoxin system HigB family toxin	
Toxin	Type II toxin-antitoxin system ParD family antitoxin	
Toxin	Type II toxin-antitoxin system RelB/DinJ family antitoxin	
Toxin	Type II toxin-antitoxin system RelE/ParE family toxin	
Toxin	Type II toxin-antitoxin system VapB family antitoxin	
Toxin	Type II toxin-antitoxin system VapC family toxin	
Toxin	Type V toxin-antitoxin system endoribonuclease antitoxin Gh	
Toxin	YhfG family protein	
Effector protein	DUF1471 domain-containing protein	
Effector protein	Pathogenicity island 1 effector protein	
Effector protein	Pathogenicity island 1 effector protein SipB	
Effector protein	Pathogenicity island 2 effector protein SseC	
Effector protein	Pathogenicity island 2 effector protein SseD	
Effector protein	Pathogenicity island 2 effector protein SseE	
Effector protein	Secreted effector kinase	
Effector protein	Secreted effector protein SifB	
Effector protein	SPI-2 type III secretion system effector SifA	
Effector protein	Type III secretion system effector	
Effector protein	Type III secretion system effector protein OrgC	
Iron acquisit./transp.	Fe(3+)-hydroxamate ABC transp. substrate-binding prot. FhuD	
Iron acquisit./transp.	Ferrous iron permease EfeU	
Iron acquisit./transp.	Ferrous iron transport protein B	
Iron acquisit./transp.	Formate dehydrogenase iron-sulfur subunit	
Iron acquisit./transp.	Iron transporter: fur regulated	
Iron acquisit./transp.	Iron-binding protein IscA	

Iron acquisit./transp.	Iron-containing redox enzyme family protein							■								
Iron acquisit./transp.	Iron-sulfur cluster-binding protein										■			■		
Iron acquisit./transp.	TonB-dependent receptor-like beta-barrel domain-contain. prot.				■	■	■									
Flagellar proteins	Flagellar biosynthesis protein FlhA	■				■			■							
Flagellar proteins	Flagellar hook length control protein FliK										■					
Flagellar proteins	Flagellar hook-associated protein 2							■								
Flagellar proteins	Flagellar protein FlhE					■										
Flagellar proteins	Flagellar protein FliT				■	■	■	■	■							
Flagellar proteins	Flagellin	■	■	■	■		■	■	■				■	■	■	
Flagellar proteins	Flagellin FliC									■						
Flagellar proteins	Negative regulator of flagellin synthesis	■								■					■	■
Flagellar proteins	Pili/flagellar-assembly chaperone											■				

1 *S. enterica* subsp. *enterica* serovar Typhimurium; 2 *S. enterica* subsp. *enterica* serovar Virchow; 3 *S. enterica* subsp. *enterica* serovar Enteritidis; 4 *S. enterica* subsp. *enterica* serovar Thompson; 5 *S. enterica* subsp. *enterica* serovar Plymouth; 6 *S. enterica* subsp. *enterica* serovar Enteritidis; 7 *S. enterica* subsp. *enterica* serovar Gloucester; 8 *S. enterica* subsp. *enterica* serovar Amsterdam; 9 *S. enterica* subsp. *enterica* serovar Typhimurium monofasica; 10 *S. enterica* subsp. *enterica* serovar Worthington; 11 *S. enterica* subsp. *enterica* serovar Liverpool; 12 *S. enterica* subsp. *enterica* serovar Typhimurium; 13 *S. enterica* subsp. *enterica* serovar Blockley; 14 *S. enterica* subsp. *enterica* serovar Enteritidis; 15 *S. enterica* subsp. *enterica* serovar Hadar. Prot: protein.

Table 3. Bacterial spoilage enzymes, identified by LC-MS/MS, in the 15 bacterial *S. enterica* subsp. *enterica* serotypes analyzed.

Enzyme Type	Proteins identified by LC-MS/MS	Bacterial samples
Aminopeptidases	Aminopeptidase	S6, S7, S8
Amylases	Alpha-amylase	S10
Decarboxylases	4-hydroxyphenylacetate decarboxylase	S1, S2
	Aspartate 1-decarboxylase	S3, S4, S5, S6, S9, S10, S13, S14, S15
	Carboxymuconolactone decarboxylase	S4
	Diaminopimelate decarboxylase	S1, S5, S8
	Glycine decarboxylase	S15
	Lysine decarboxylase	S9
	Malonate decarboxylase	S3, S6
	Ornithine decarboxylase	S2, S5, S6, S10, S11
	Oxaloacetate decarboxylase	S3, S5, S9, S13
	Phenolic acid decarboxylase	S14
	Phosphatidylserine decarboxylase	S9
	Sodium ion-translocating decarboxylase	S5
	UbiD family decarboxylase	S2, S14
Lipases	Patatin-like phospholipase RssA	S5
	GDSL family lipase	S6
Proteases	ATP-dependent Clp protease	S3, S4, S8, S11, S13,
	ClpXP protease specificity-enhancing factor	S8
	CPBP family intramembrane metalloprotease	S1, S10
	Metalloprotease PmbA	S9
	Periplasmic serine endoprotease DegP-like	S7, S13
	Protease FtsH-inhibitory lysogeny factor CIII	S6

	Omptin protease	S6
	Putative protease HtpX	S9, S10, S12
	Tail-specific protease	S7
	Zn-dependent protease	S13
Sulfur-release enzymes	Cysteine desulfurase	S3, S7, S8, S14
	DNA sulfur modification protein DndE	S6, S14
	Iron-sulfur cluster-binding protein	S11, S14
	Probable tRNA sulfurtransferase	S2, S7
	Sulfur carrier protein FdhD	S11, S14

S1 *S. enterica* subsp. *enterica* serovar Typhimurium; S2 *S. enterica* subsp. *enterica* serovar Virchow; S3 *S. enterica* subsp. *enterica* serovar Enteritidis; S4 *S. enterica* subsp. *enterica* serovar Thompson; S5 *S. enterica* subsp. *enterica* serovar Plymouth; S6 *S. enterica* subsp. *enterica* serovar Enteritidis; S7 *S. enterica* subsp. *enterica* serovar Gloucester; S8 *S. enterica* subsp. *enterica* serovar Amsterdam; S9 *S. enterica* subsp. *enterica* serovar Typhimurium monofasica; S10 *S. enterica* subsp. *enterica* serovar Worthington; S11 *S. enterica* subsp. *enterica* serovar Liverpool; S12 *S. enterica* subsp. *enterica* serovar Typhimurium; S13 *S. enterica* subsp. *enterica* serovar Blockley; S14 *S. enterica* subsp. *enterica* serovar Enteritidis; S15 *S. enterica* subsp. *enterica* serovar Hadar.

Figure 1

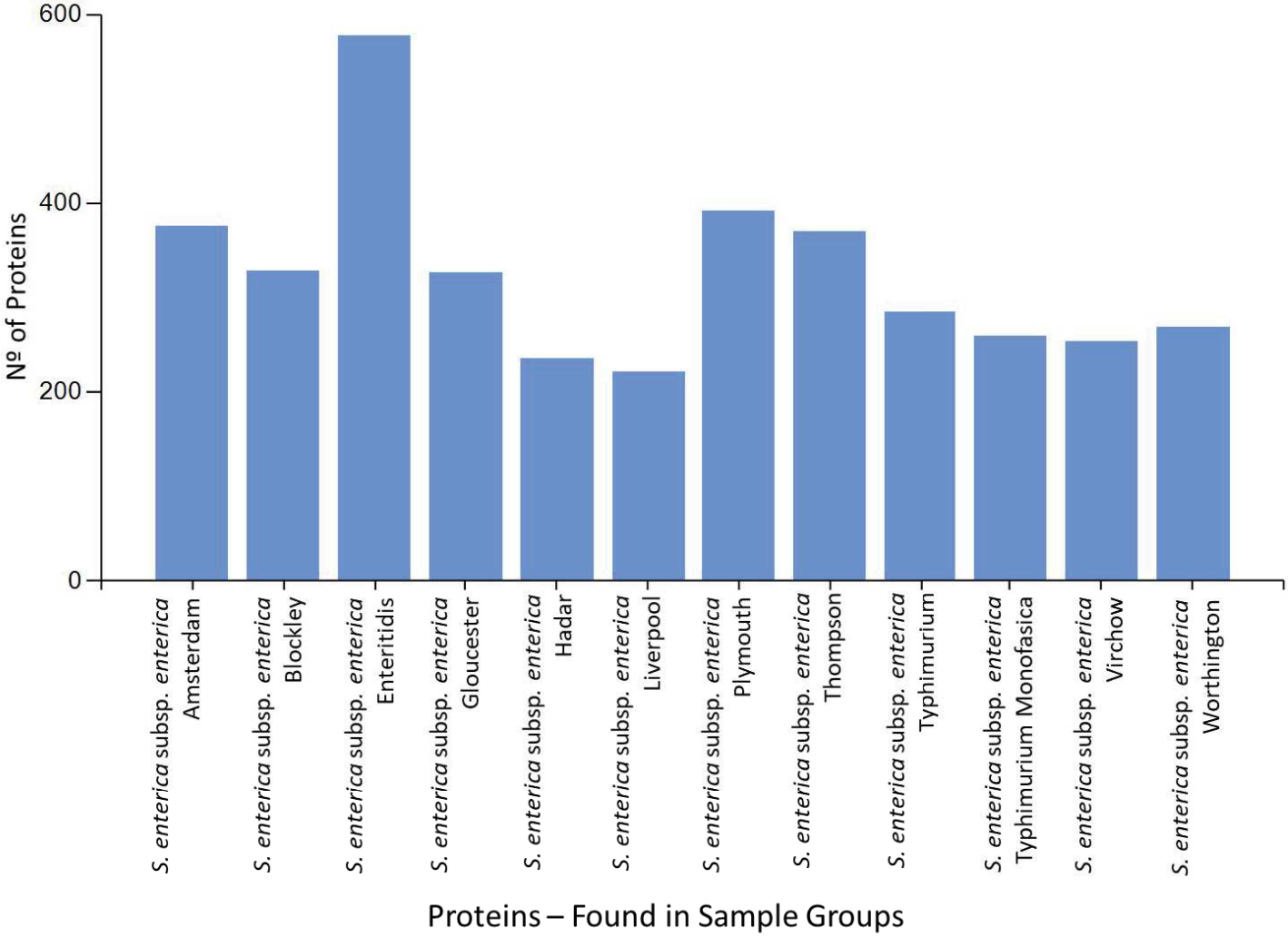

600
400
200
0
Nº of Proteins
S. enterica subsp. enterica Amsterdam
S. enterica subsp. enterica Blockley
S. enterica subsp. enterica Enteritidis
S. enterica subsp. enterica Gloucester
S. enterica subsp. enterica Hadar
S. enterica subsp. enterica Liverpool
S. enterica subsp. enterica Plymouth
S. enterica subsp. enterica Thompson
S. enterica subsp. enterica Typhimurium
S. enterica subsp. enterica Typhimurium Monofasica
S. enterica subsp. enterica Virchow
S. enterica subsp. enterica Worthington
Proteins – Found in Sample Groups

Figure 2

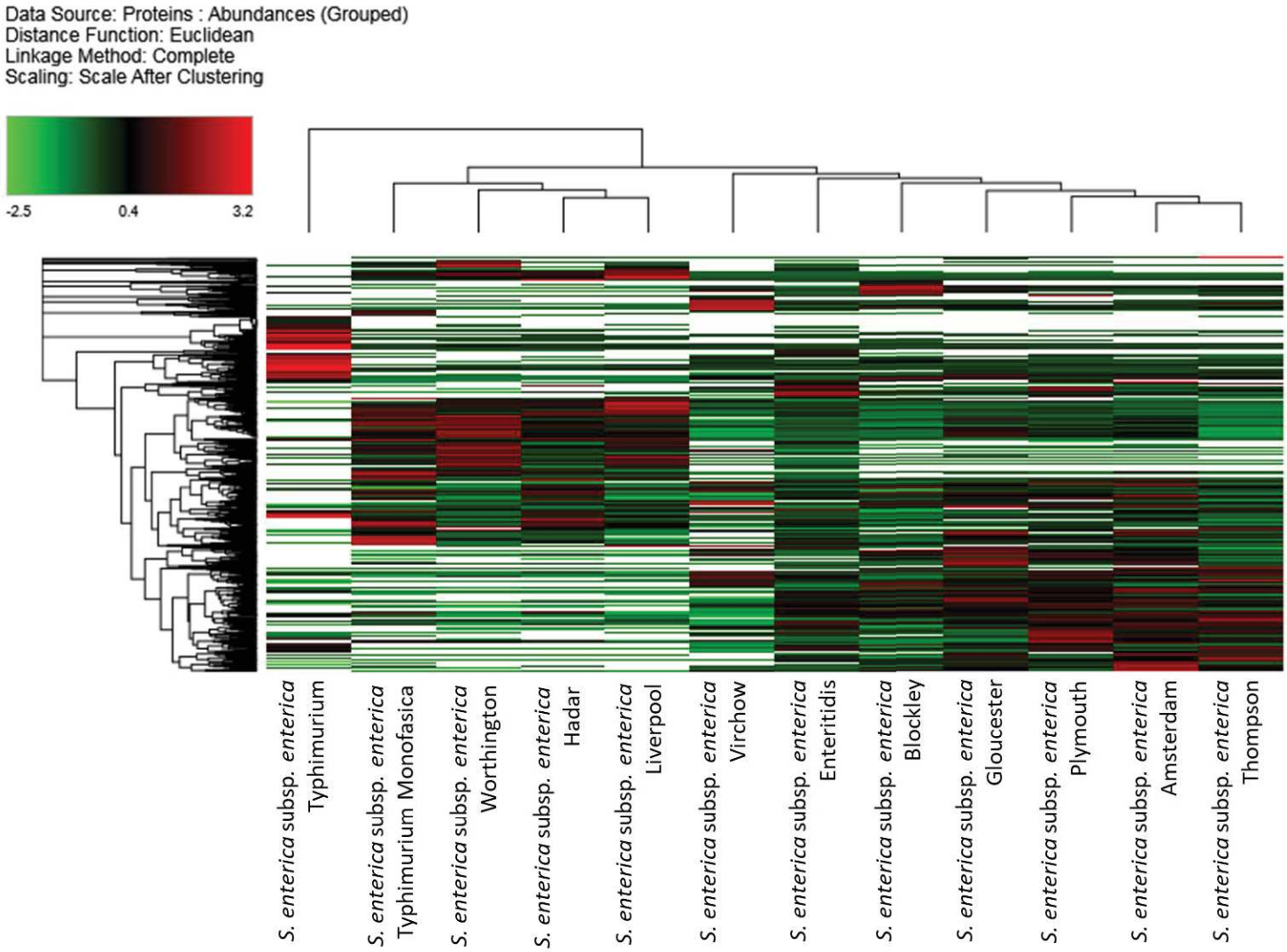
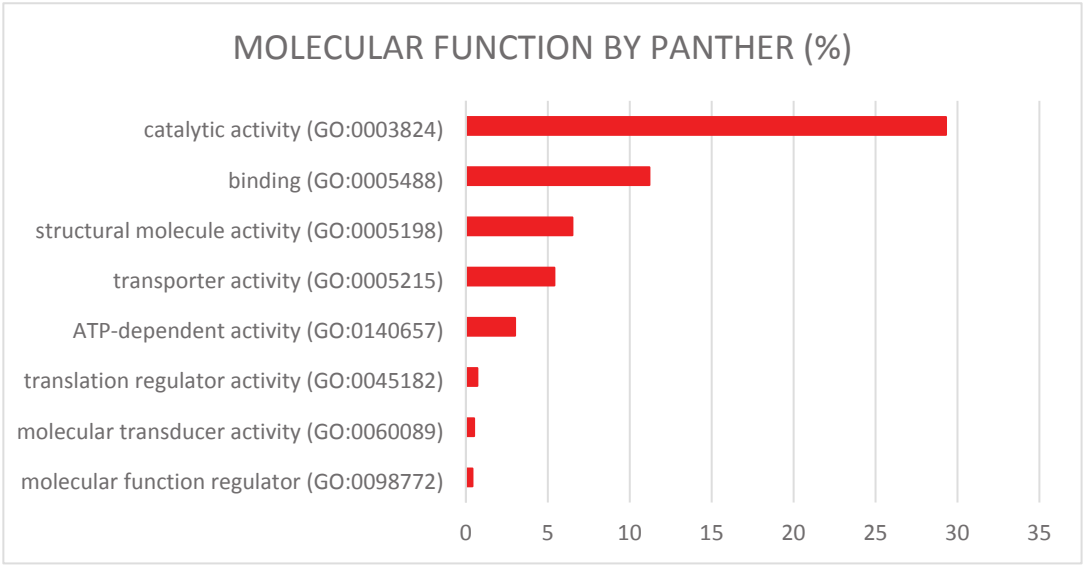
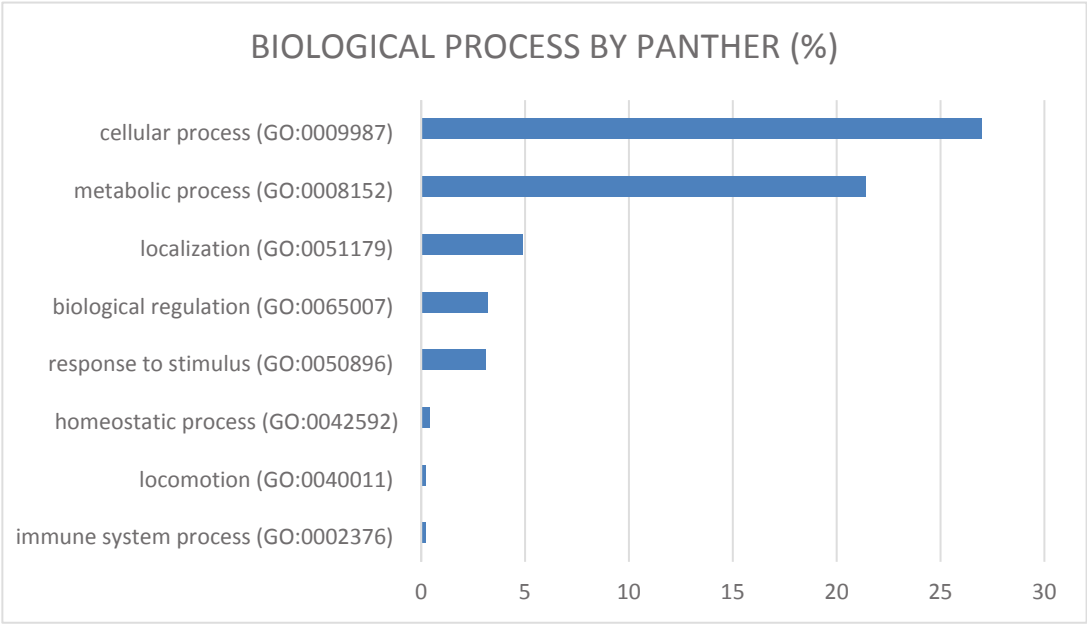

Data Source: Proteins : Abundances (Grouped)
Distance Function: Euclidean
Linkage Method: Complete
Scaling: Scale After Clustering
-2.5
0.4
3.2
S. enterica subsp. enterica Typhimurium
S. enterica subsp. enterica Typhimurium Monofasica
S. enterica subsp. enterica Worthington
S. enterica subsp. enterica Hadar
S. enterica subsp. enterica Liverpool
S. enterica subsp. enterica Virchow
S. enterica subsp. enterica Enteritidis
S. enterica subsp. enterica Blockley
S. enterica subsp. enterica Gloucester
S. enterica subsp. enterica Plymouth
S. enterica subsp. enterica Amsterdam
S. enterica subsp. enterica Thompson

Figure 3

a)


MOLECULAR FUNCTION BY PANTHER (%)
catalytic activity (GO:0003824)
binding (GO:0005488)
structural molecule activity (GO:0005198)
transporter activity (GO:0005215)
ATP-dependent activity (GO:0140657)
translation regulator activity (GO:0045182)
molecular transducer activity (GO:0060089)
molecular function regulator (GO:0098772)
0 5 10 15 20 25 30 35

b)


BIOLOGICAL PROCESS BY PANTHER (%)
cellular process (GO:0009987)
metabolic process (GO:0008152)
localization (GO:0051179)
biological regulation (GO:0065007)
response to stimulus (GO:0050896)
homeostatic process (GO:0042592)
locomotion (GO:0040011)
immune system process (GO:0002376)
0 5 10 15 20 25 30

c)

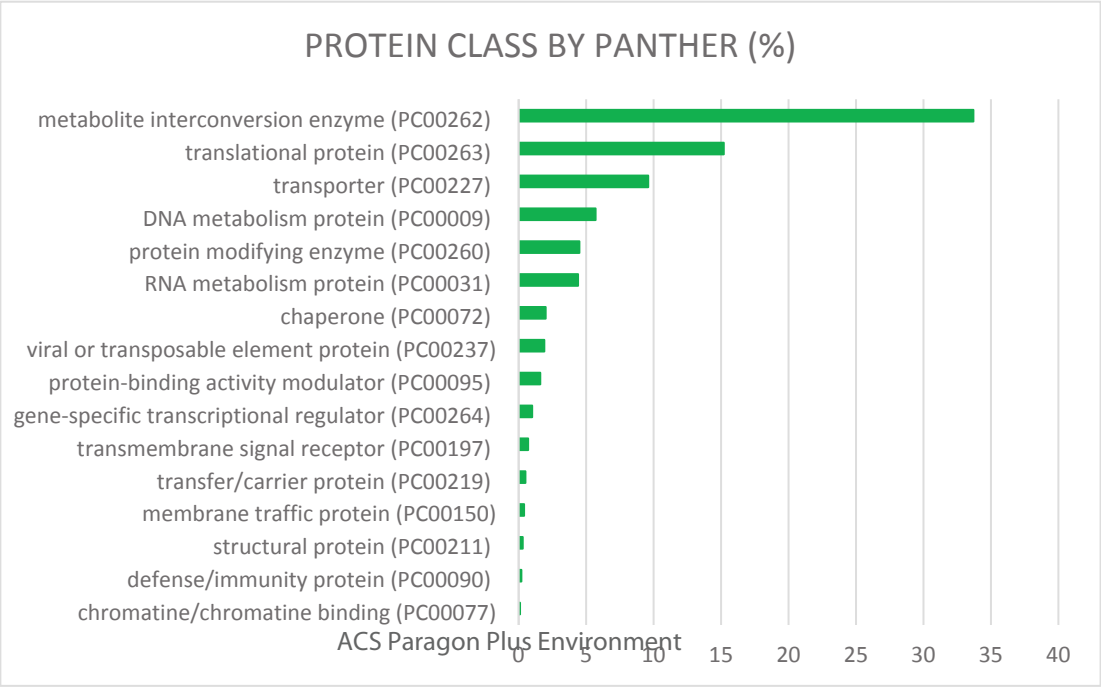

PROTEIN CLASS BY PANTHER (%)
metabolite interconversion enzyme (PC00262)
translational protein (PC00263)
transporter (PC00227)
DNA metabolism protein (PC00009)
protein modifying enzyme (PC00260)
RNA metabolism protein (PC00031)
chaperone (PC00072)
viral or transposable element protein (PC00237)
protein-binding activity modulator (PC00095)
gene-specific transcriptional regulator (PC00264)
transmembrane signal receptor (PC00197)
transfer/carrier protein (PC00219)
membrane traffic protein (PC00150)
structural protein (PC00211)
defense/immunity protein (PC00090)
chromatine/chromatine binding (PC00077)
0 5 10 15 20 25 30 35 40

Figure 4

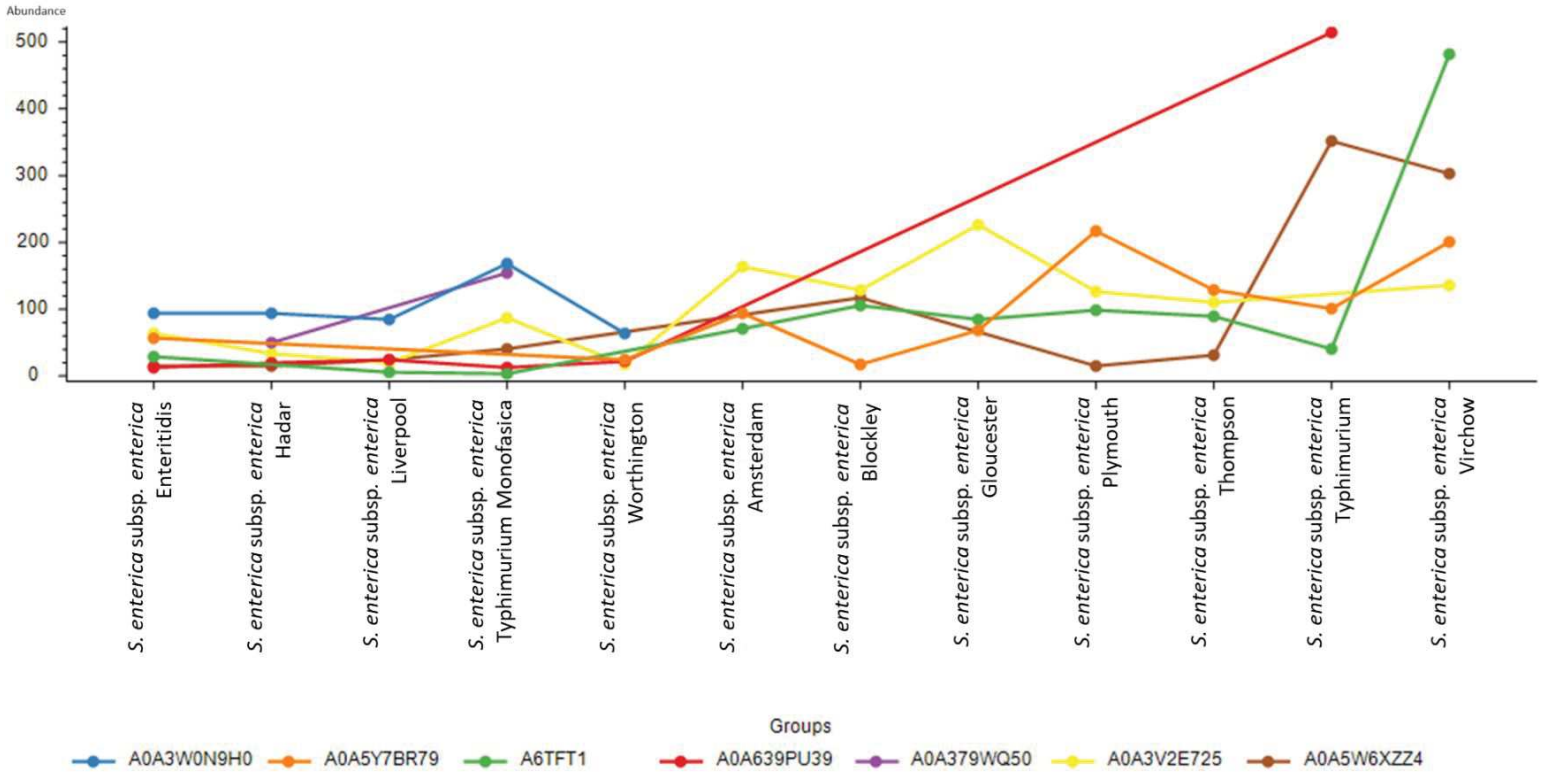
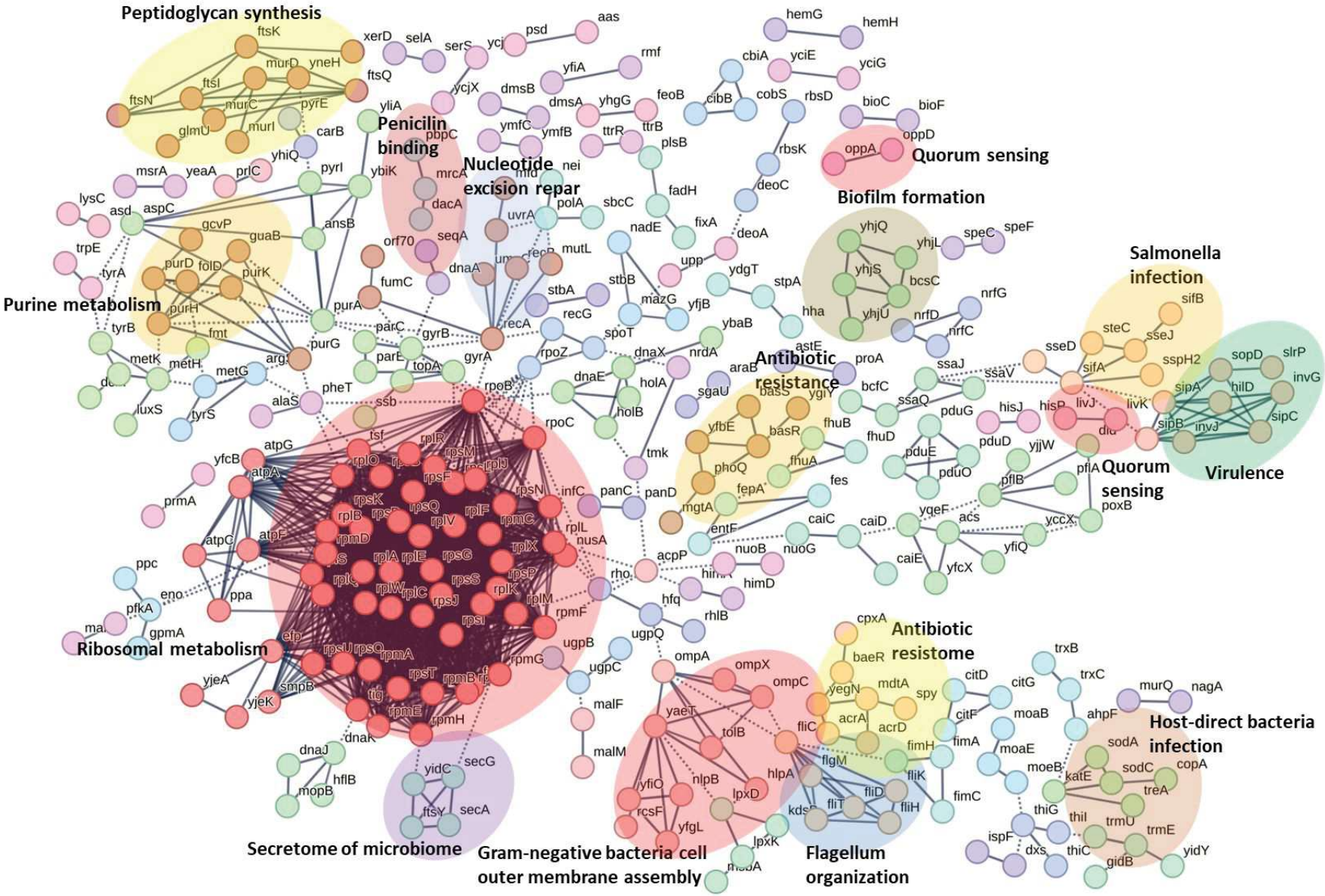
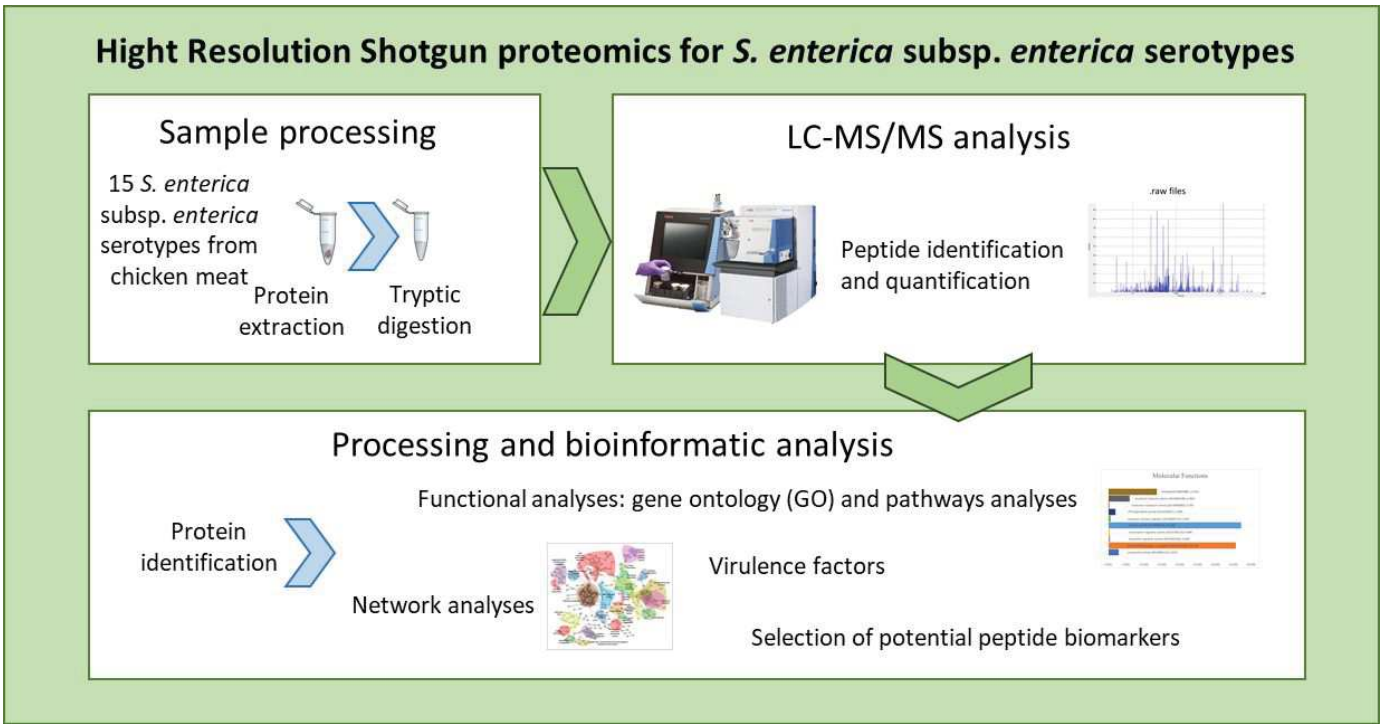

Abundance
500
400
300
200
100
0
S. enterica subsp. enterica Enteritidis
S. enterica subsp. enterica Hadar
S. enterica subsp. enterica Liverpool
S. enterica subsp. enterica Typhimurium Monofasica
S. enterica subsp. enterica Worthington
S. enterica subsp. enterica Amsterdam
S. enterica subsp. enterica Blockley
S. enterica subsp. enterica Gloucester
S. enterica subsp. enterica Plymouth
S. enterica subsp. enterica Thompson
S. enterica subsp. enterica Typhimurium
S. enterica subsp. enterica Virchow
Groups
A0A3W0N9H0
A0A5Y7BR79
A6TFT1
A0A639PU39
A0A379WQ50
A0A3V2E725
A0A5W6XZZ4

Figure 5


Peptidoglycan synthesis
Penicilin binding
Nucleotide excision repar
Quorum sensing
Biofilm formation
Purine metabolism
Salmonella infection
Antibiotic resistance
Quorum sensing
Virulence
Ribosomal metabolism
Antibiotic resistome
Host-direct bacteria infection
Secretome of microbiome
Gram-negative bacteria cell outer membrane assembly
Flagellum organization

GRAPHICAL ABSTRACT FOR COVER ART



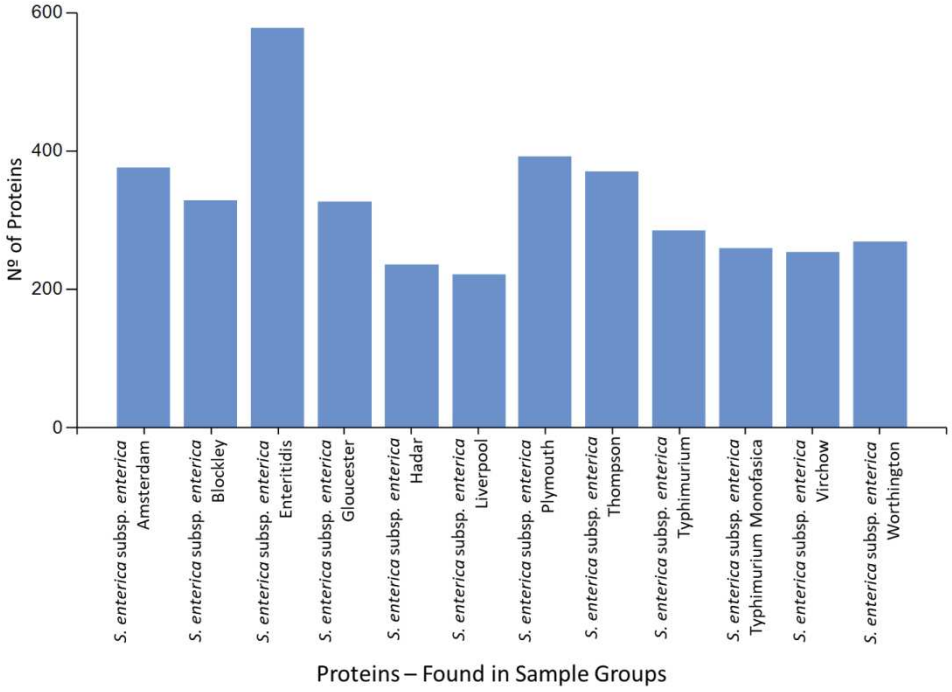


Figure 1. Total number of high-abundance proteins in each of the bacterial *S. enterica* subsp. *enterica* serotypes analyzed, as determined by LFQ; the y-axis represents the number of identified proteins.

322x233mm (120 x 120 DPI)

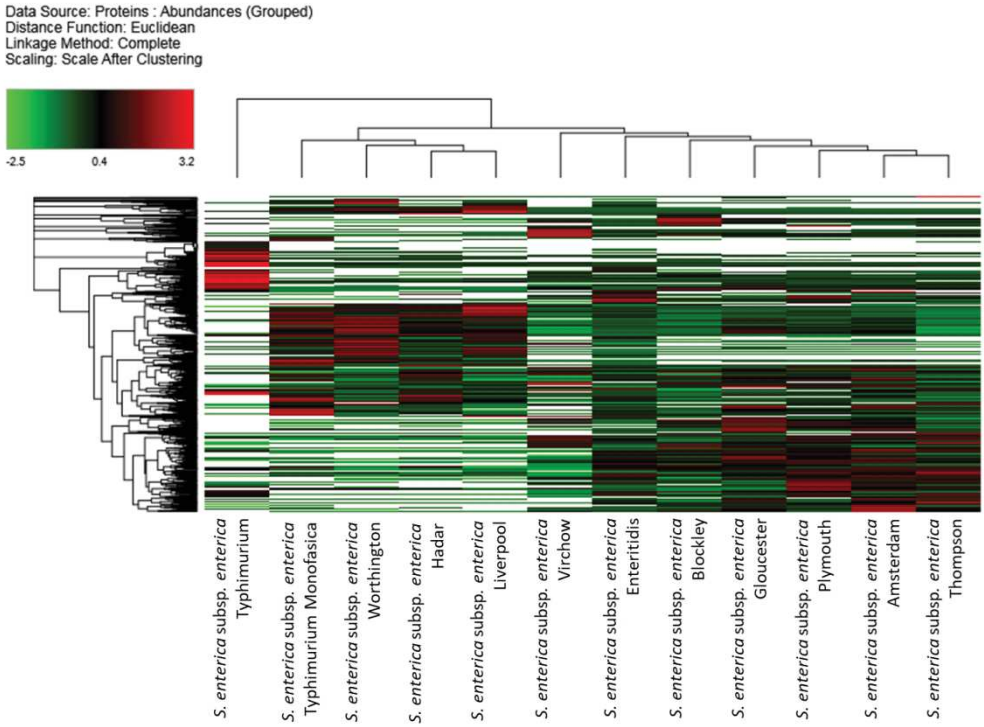


Figure 2. Heatmap diagram obtained from the shotgun proteomic analyses of 15 different *S. enterica* subsp. *enterica* serotypes. Every bar corresponds to a particular protein that can be either up-regulated or down-regulated. Red = up-regulated proteins; green = down-regulated proteins. Euclidean hierarchical distances were estimated for all the bacterial strains, and the principal clusters identified.

326x246mm (120 x 120 DPI)

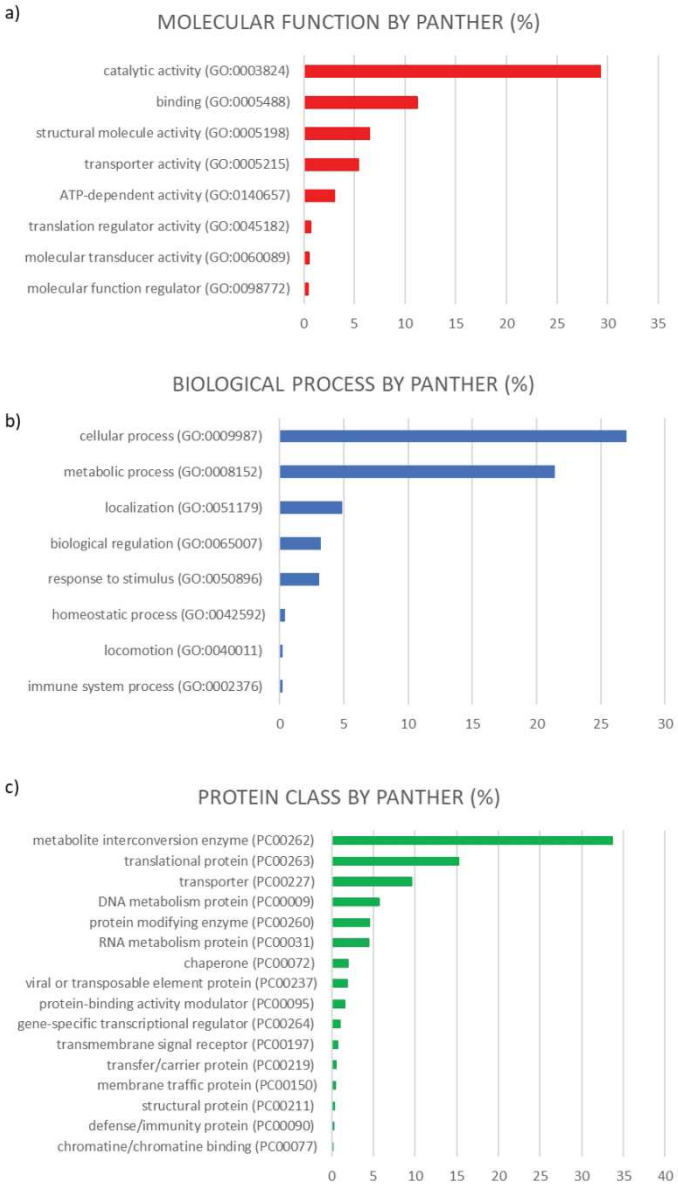


Figure 3. (a) Molecular functions of the proteins obtained from *S. enterica* subsp. *enterica* serotypes; the peptides were identified using shotgun proteomics, while the function was assigned by the PANTHER classification system, using the gene names as inputs in the software; (b) Biological processes carried out by the bacterial proteins identified by shotgun proteomics and classified according to PANTHER; (c) Determination of the protein classes the identified polypeptides belong to, according to the PANTHER classification system.

185x318mm (120 x 120 DPI)

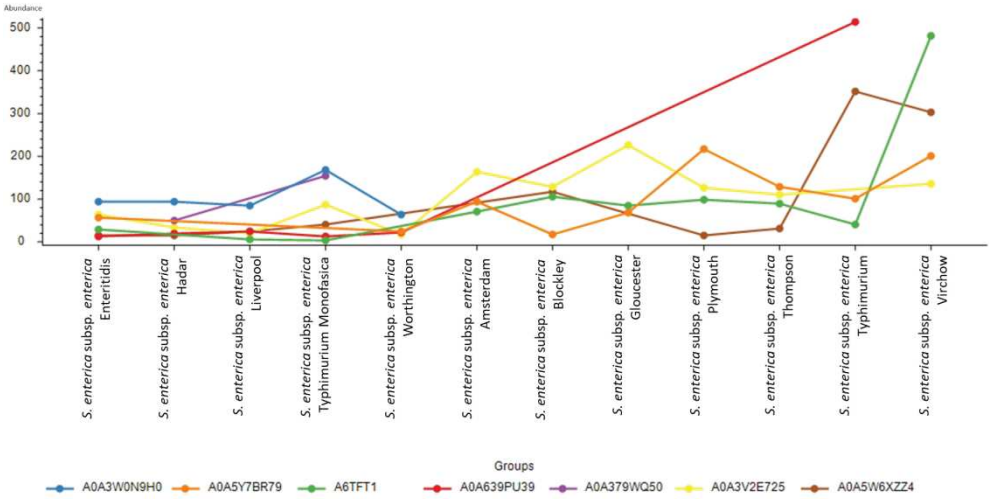


Figure 4. Decarboxylase enzymes identified for each of the *S. enterica* subsp. *enterica* serotypes analyzed. The y-axis represents the enzyme units. Group proteins in accession name.

407x207mm (120 x 120 DPI)

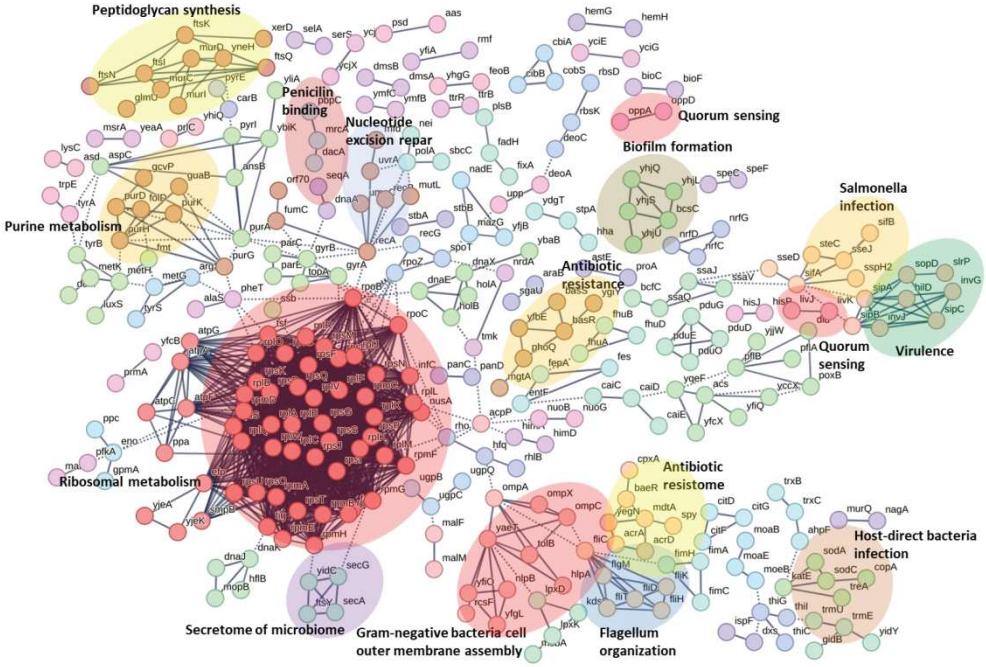


Figure 5. Protein interactome network for all the proteins identified in this study, and deposited in the repository of foodborne *S. enterica* subsp. *enterica* serotypes. The complete network consists of 543 nodes (proteins) and 1,480 edges (interactions). Protein interactions were analyzed using the STRING v.12 software. Only the highest-confidence interactions (≥0.9), as determined by the STRING software, were selected for this study. The circles represent the proteins, while the interactions between proteins are depicted by either continuous lines, for direct interactions (physical), or dotted lines, for indirect interactions (functional).

353x238mm (120 x 120 DPI)