Iran. J. Biotechnol. October 2023;21(4): e3640 DOI: 10.30498/ijb.2023.387696.3640

Research Article

Gene Networks Analysis of *Salmonella Typhimurium* Reveals New Insights on Key Genes Involved in Response to Low Water Activity

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Received: 2023/03/15 ; **Accepted:** 2023/08/29

Background: When *Salmonella enterica serovar Typhimurium*, a foodborne bacterium, is exposed to osmotic stress, cellular adaptations increase virulence severity and cellular survival.

Objectives: The aim of the gene network analysis of *S. Typhimurium* was to provide insights into the various interactions between the genes involved in cellular survival under low water activity (a_w).

Materials and Methods: We performed a gene network analysis to identify the gene clusters and hub genes of *S. Typhimurium* using Cytoscape in three food samples subjected to a_w stress after 72 hours.

Results: The identified hub genes of *S. Typhimurium* belonged to down-regulated genes and were related to translation, transcription, and ribosome structure in the food samples. The *rpsB* and *Tig* were identified as the most important of the hub genes. Enrichment analysis of the hub genes also revealed the importance of translation and cellular protein metabolic processes. Moreover, the biological process associated with organonitrogen metabolism in milk chocolate was identified. According to the KEGG pathway results of gene cluster analysis, cellular responses to stress were associated with RNA polymerase, ribosome, and oxidative phosphorylation. Genes encoding RNA polymerase activity, including *rpoA*, *rpoB*, and *rpoZ*, were also significantly identified in the KEGG pathways. The identified motifs of hub DEGs included EXPREG_00000850, EXPREG_00000b00, EXPREG_000008e0, and EXPREG_00000850.

Conclusion: Based on the results of the gene network analysis, the identified hub genes may contribute to adaptation to food compositions and be responsible for the development of low water stress tolerance in *Salmonella*. Among the food samples, the milk chocolate matrix leads to more adaptation pathways for *S. Typhimurium* survival, as more hub genes were down-regulated and more motifs were detected. The identified motifs were involved in carbohydrate metabolism, carbohydrate transport, electron transfer, and oxygen transfer.

Keywords: Hub genes, Low water activity, Network analysis, *Salmonella*.

1. Background

Salmonellosis, a disease caused by *Salmonella* infection, can be classified as typhoidal or non-typhoidal depending on the serotype of *Salmonella* associated with the infection. While *Salmonella Enterica* and *Salmonella Typhimurium* are the most commonly observed serovars in non-typhoidal salmonellosis cases (1, 2), recent reports have also linked the disease to foods with a low water activity (a_w) and high-fat content. Salmonellosis is typically associated with the consumption of raw or undercooked animal-derived foods, particularly poultry, shell eggs, and more recently, high-fat content and low a_w foods (3, 4). In low a_w foods, *Salmonella* survival and infection can occur even with a smaller number of bacterial cells due to their increased tolerance to sublethal osmotic stress (5, 6). Studies have demonstrated that the composition of food and the presence of solutes can influence the survival of *Salmonella* under severe conditions (7, 8). Furthermore, the increased resistance of *Salmonella* to thermal processing and its ability to adapt to various stress conditions, including low water stress, poses challenges for food safety practices (7, 9, 10, 11). When cells are exposed to sublethal osmotic stress, such as that found in dehydrated foods, cellular adaptations result in increased virulence severity and cellular survival at low metabolic activity (11). To develop effective control systems for food-borne diseases such as salmonellosis, it is crucial to understand the molecular mechanisms underlying *Salmonella* survival in low a_w environments. Previous studies have utilized RNA-Seq analysis to investigate the gene expression changes in *S. Typhimurium* under low a_w conditions and have identified key adaptation pathways involved in cellular survival (10). Exploring gene networks can provide insights into the interactions between genes and proteins associated with specific biochemical functions (12), facilitating a better understanding of the organism's physiological state (13). In this study, our objective was to analyze the gene interaction network in *S. Typhimurium* to identify gene clusters and hub genes with high connectivity in response to osmotic stress in three low a_w foods. We focused on the analysis of differentially expressed genes (DEGs) obtained from previous studies (10) to investigate the molecular changes associated with *Salmonella* survival under low a_w conditions.

2. Objectives

The primary objective of this study was to identify gene clusters and hub genes with high connectivity in the gene interaction network of *S. Typhimurium* in response to osmotic stress in three low-a_w foods. Specifically, we aimed to analyze the DEGs obtained from previous studies (10) to uncover potential molecular mechanisms associated with cellular adaptations and survival of *Salmonella* in low a_w environments.

3. Materials and Methods

3.1. Selection of Data Sets

The gene expression profiles with fold change (FC) of *S. Typhimurium* in the low a_w food samples, including dried black pepper, milk chocolate, and powdered skim milk **(Table 1)** were downloaded from the supplementary data (https://doi.org/10.1016/j.fm.2019.03.016) under BioProject ID: PRJNA490179 (SRR7815201, SRR7815202, SRR7815203, SRR7815204, SRR7815205, SRR7815206, SRR7815207, SRR7815208, SRR7815209) (10). In this study, the differentially expressed genes (DEGs) of *S. Typhimurium* were assessed by comparing the treatments (food samples) and overnight culture of bacteria at 37 ºC as a control; $|FC|>2$ ($p<0.05$) was selected for the overall significantly up-and down-regulated genes of each sample after 72 h storage at 25 ºC.

Table 1. The count of up- and down-regulated genes of *S. Typhimurium* under low a_w in different foods

	Black pepper	Milk chocolate	Powdered skim milk	Control
Up-regulated	515	763	837	An overnight culture of *S. Typhimurium* on LB agar plates was harvested and washed in sterile saline (NaCl 0.9%). The drained cells were used as a control sample.
Down-regulated	653	869	867	

Crucello *et al*. (2019) showed that the differential gene expression of *S. Typhimurium* seemed to be more related to the food matrix in the first 24 h and the desiccation response in the 72 h (10). However, other important genes involved in the desiccation-activated gene network, especially after 72 h, may also be important in the stress response that has not been identified. Therefore, only the 72 h-storage data were selected for network analysis to investigate the differential expression of hub genes in response to low a_w stress.

3.2. The Retrieval of Interacting Proteins

To investigate the interactions between the significantly up-and down-regulated genes of *Salmonella* obtained from the food samples, we constructed protein-protein interaction (PPI) networks using the STRING database (version 11.5) (https://string-db.org/). *Salmonella enterica subsp. enterica serovar Typhimurium* was selected as the reference organism, and interactions validated with a medium confidence score greater than 0.4 were considered significant (14). The interactions were analyzed separately for black pepper, milk chocolate, and powdered skim milk samples.

3.3. PPIs Networks Analysis

The PPI networks of *S. Typhimurium* obtained from the STRING database were further analyzed using Cytoscape-v3.9.1. Cytoscape is a powerful bioinformatics software platform used for visualizing and analyzing complex biological networks. The hub genes, which have the highest connectivity with other genes in the networks, were predicted and identified using the CytoHubba plugin. These hub genes play a crucial role in the overall network structure and may have significant functional implications. Four topological algorithms, including Degree, Maximum Neighborhood Component (MNC), Maximal Clique Centrality (MCC), and Density of Maximum Neighborhood Component (DMNC) were employed to evaluate the top genes in the PPI networks (15). To identify protein complex clusters within the PPI networks, cluster analysis was performed using the IPCA (Identifying Protein Complex Algorithm) of the Cytocluster plugin (Tin threshold=0.5, Complex size threshold=10, shortest path length=2). IPCA is a density-based clustering algorithm that can detect dense subgraphs in PPI networks (16). The clusters were ranked based on their connection scores, and the most important protein complex clusters (ranks 1-4) were identified and visualized within the network.

3.4. Pathway Enrichment and Gene Ontology Analyses

To gain insights into the biological processes and molecular functions associated with the hub genes and their interactions, pathway enrichment and gene ontology analyses were performed.

3.4.1. Pathway Enrichment Analysis

The networks containing the hub genes and their interactions were subjected to pathway enrichment analysis using the STRING database. This analysis allows for the identification of enriched pathways and biological processes that are statistically overrepresented among the hub genes. The results of the pathway enrichment analysis provide valuable information about the functional implications of the hub genes in the context of low a_w stress.

3.4.2. Gene Ontology Analysis

Gene ontology (GO) analysis was performed to categorize the hub genes based on their associated biological processes (BP), molecular functions (MF), and cellular components (CC) (17). This analysis provides a standardized vocabulary for describing gene products in a systematic and computable manner. By assigning GO terms to the hub genes, it becomes possible to uncover the functional annotations and characteristics of these genes with low a_w stress. The annotations obtained from the GO analysis enable the classification of genes into specific functional categories, providing valuable insights into their roles and interactions within cellular processes. The GO terms associated with the hub genes highlight the biological processes, molecular functions, and cellular components that are particularly relevant in the context of low a_w stress.

3.5. Cis-elements Analysis

By analyzing the promoter regions of differentially expressed hub genes, we aimed to identify potential regulatory elements involved in the response to osmotic stress. The 200 kbp upstream flanking regions of hub DEGs, which have been down-regulated, were extracted as promoter sequences (18) from Ensembl bacteria (http://bacteria.ensembl.org). Motif comparison tool (Tomtom) version 5.5.0 (https://meme-suite.org/meme/tools/tomtom) was applied to define the known motifs in *S. typhimurium* based on the database of prokaryote DNA for bacterial TF motif (19) with threshold E-value <10.

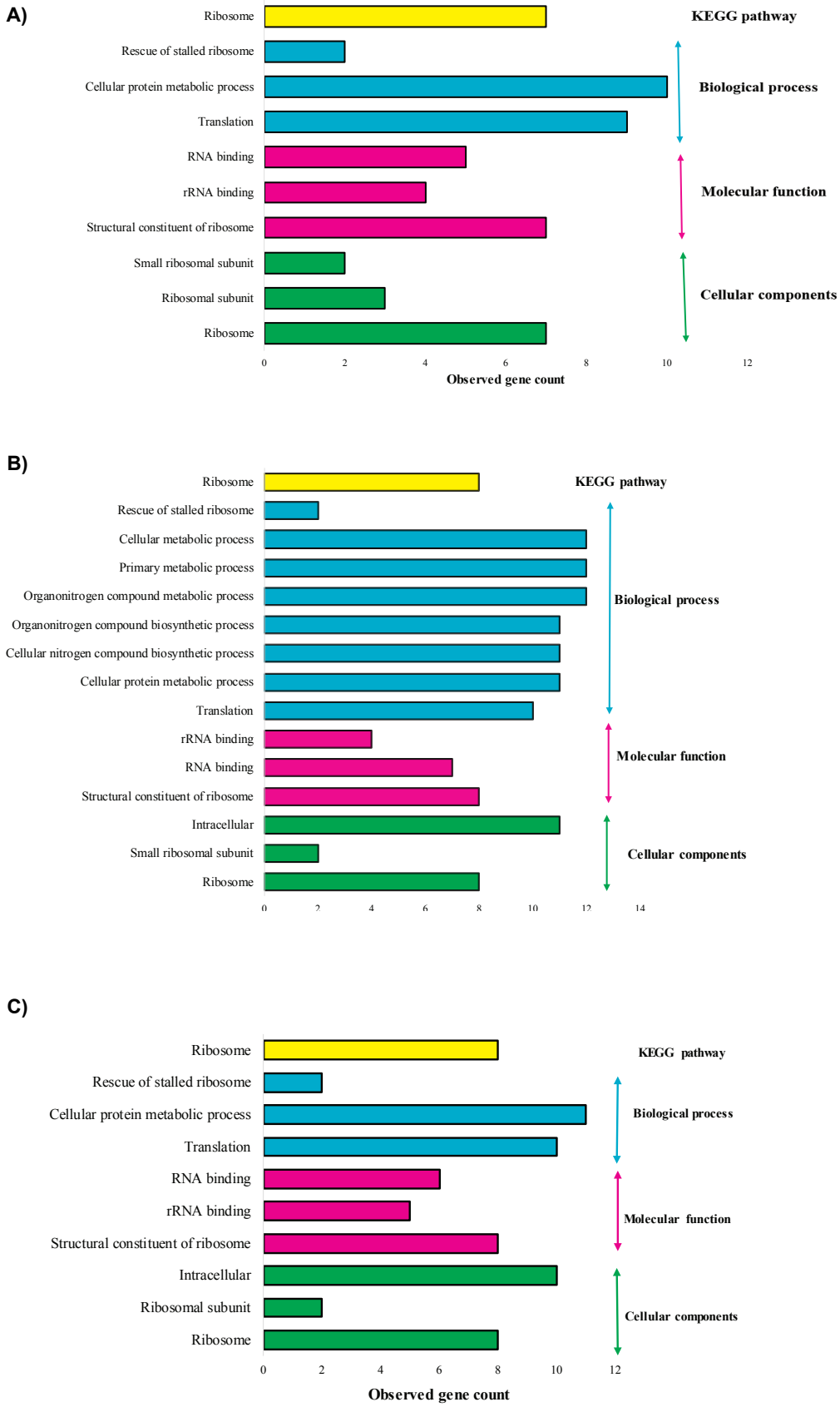


Figure 1. **Gene Ontology enrichment analysis of hub genes of *S. Typhimurium* in A)** black pepper, **B)** milk chocolate, **C)** powdered skim milk in response to a_w stress.

Then, the possible roles of found motifs were identified using gene ontology for motifs (GOMo) version 5.5.0 (https://meme-suite.org/meme/tools/gomo) (20).

4. Results

Low water activity (a_w) stress is a critical parameter that inhibits the growth of most pathogenic bacteria, but *Salmonella* has been shown to exhibit increased survival under low a_w conditions. In this study, network analysis was conducted to identify gene clusters and hub genes in *S. Typhimurium* that may contribute to cellular changes in response to low a_w stress.

4.1. Gene Network Analysis

Previous research by Crucello *et al*. (2019) highlighted the importance of induced genes in *S. Typhimurium* for the low a_w response in milk chocolate, powdered skim milk, and black pepper after 72 hours (10). However, there may be additional unrecognized genes involved in the desiccation-activated gene network that are important for this stress response. Therefore, network analysis was performed to identify other crucial genes involved in the response to low a_w stress after 72 hours. The STRING database was utilized to investigate possible protein-protein interactions among differentially expressed genes (DEGs) of *S. Typhimurium*. The interaction data extracted from this database revealed 719 genes with 8,115 interactions for black pepper, 1,087 genes with 14,441 interactions for milk chocolate, and 1,134 genes with 14,806 interactions for powdered skim milk.

4.2. Identification of Hub Genes

The topological parameters and summary statistics for the subnetworks analysis of hub genes of *S. Typhimurium* and their interactions in the food samples are provided in **Supplementary 1**. The identified hub genes were primarily associated with the differentially down-regulated genes at reduced a_w levels. **Supplementary 2** lists the top hub genes identified in the network analysis for each food sample. Hub genes, which have the highest number of connections, play essential roles in various molecular functions and biological processes. In this study, the top hub genes identified in the network analysis of *S. Typhimurium* were primarily down-regulated genes under reduced a_w conditions.

4.3. Functional Enrichment and GO Analysis

Functional gene enrichment analysis was performed to gain insights into the biological processes, molecular functions, and cellular components associated with the hub genes identified in *S. Typhimurium* under low a_w stress. The analysis was conducted using the STRING server, and the results are summarized in **Figure 1**. For each food sample (black pepper, milk chocolate and powdered skim milk), the functional enrichment analysis revealed several important functions associated with the hub genes involved in biological processes (BP), molecular functions (MF), and cellular components (CC). In general, the biological processes associated with the hub genes in *S. Typhimurium* under low a_w stress included translation, cellular protein metabolic processes, and the rescue of stalled ribosomes. These processes highlight the importance of protein synthesis and cellular protein maintenance under low a_w conditions. The molecular functions associated with the hub genes were mainly related to the structural constituents of the ribosome, RNA binding, and rRNA binding. This suggests that the ribosomal machinery and RNA-related processes play crucial roles in the cellular response to low a_w stress. In terms of cellular components, the hub genes were found to be associated with the ribosome, ribosomal subunits, and small ribosomal subunits. This further emphasizes the significance of ribosomal components in the cellular response to low a_w stress. Interestingly, there were shared gene ontology results between the 72-hour stored black pepper and powdered skim milk samples. These shared functions were primarily related to cellular protein metabolic processes, structural components of the ribosome, and translation. This indicates common adaptive mechanisms employed by *S. Typhimurium* under low a_w stress in these two food samples. In contrast, the gene ontology results for *Salmonella* inoculated in milk chocolate revealed different biological processes. The functions identified in milk chocolate included cellular nitrogen compound biosynthetic processes, organonitrogen compound metabolic processes, organonitrogen compound biosynthetic processes, primary metabolic processes, and cellular metabolic processes. These findings suggest that the cellular response to low a_w stress in milk chocolate involves specific metabolic pathways related to nitrogen compounds. Overall, the functional enrichment analysis provides valuable insights into the biological processes, molecular functions, and cellular components associated with the hub genes in

Table 2. The cluster analysis of PPIs networks of *S. Typhimurium* under low a_w in different foods using IPCA

	Nodes	Edges	Rank	KEGG pathways
Black pepper	91	3217	1	RNA polymerase, Ribosome, Oxidative phosphorylation
	90	3223	2	RNA polymerase, Ribosome
	90	3261	3	RNA polymerase, Ribosome, Oxidative phosphorylation
	90	3252	4	RNA polymerase, Ribosome, Oxidative phosphorylation
Milk chocolate	101	3833	1	RNA polymerase, Ribosome
	99	3919	2	RNA polymerase, Ribosome, Oxidative phosphorylation
	99	3909	3	RNA polymerase, Ribosome, Oxidative phosphorylation
	99	3941	4	RNA polymerase, Ribosome
Powdered skim milk	102	3934	1	
	101	3961	2	
	101	3942	3	
	100	3902	4	RNA polymerase, Ribosome
	11	53	2	

S. Typhimurium under low a_w stress. These findings contribute to a better understanding of the cellular adaptations and survival strategies employed by *Salmonella* in low a_w environments.

4.4. Clustering Analysis

To further explore the organization of the gene networks, clustering analysis was performed using the IPCA of the Cytocluster plugin (16). The IPCA algorithm identifies dense subgraphs in protein-protein interaction networks and helps identify the most important protein complex clusters. The IPCA analysis ranked the clusters based on their connection scores. This algorithm calculated the weight of each edge by calculating the common neighbor of two connecting nodes (16, 21), as shown in **Table 2**.

The rank 1 clusters of differentially expressed genes (DEGs) in *S. Typhimurium* under low a_w stress in black pepper (**Fig. 2A**), milk chocolate (**Fig. 2B**), powdered skim milk (**Fig. 2C**), and DEGs shared among three sample foods (**Fig. 2D**) were identified. The rank 1 clusters (represented inside a red square or ring) exhibited the highest scores and represented the most significant protein complex clusters. The identified clusters in rank 1 were visualized within the network. However, since the analysis results of the rank 1 cluster were similar to the other clusters (rank 2-4), only the rank 1 cluster was shown in the figures for each sample. Clustering analysis helps identify groups of genes that exhibit coordinated expression patterns and are likely involved in related biological processes or pathways. By clustering the hub genes, this analysis provides insights into the functional organization and potential interplay between different components of the cellular response to low a_w stress in *S. Typhimurium*.

4.5. Promoter Motif Analysis of Hub DEGs

To gain further insights into the regulatory mechanisms underlying the differential expression of hub genes in response to low a_w stress, we performed promoter motif analysis on the 200 bp upstream flanking regions of the hub differentially expressed genes (DEGs) in *S. Typhimurium*. This analysis aimed to identify conserved motifs that might be involved in the regulation of these genes. We used the MEME motif comparison tool (Tomtom) to search for known motifs in the *S. Typhimurium* promoter sequences based on the prokaryote DNA motif database for bacterial transcription factor motifs. We applied a threshold E-value of less than 10 to define significant matches. Additionally, we used the gene ontology for motifs (GOMo) tool to identify the potential roles of the identified motifs.

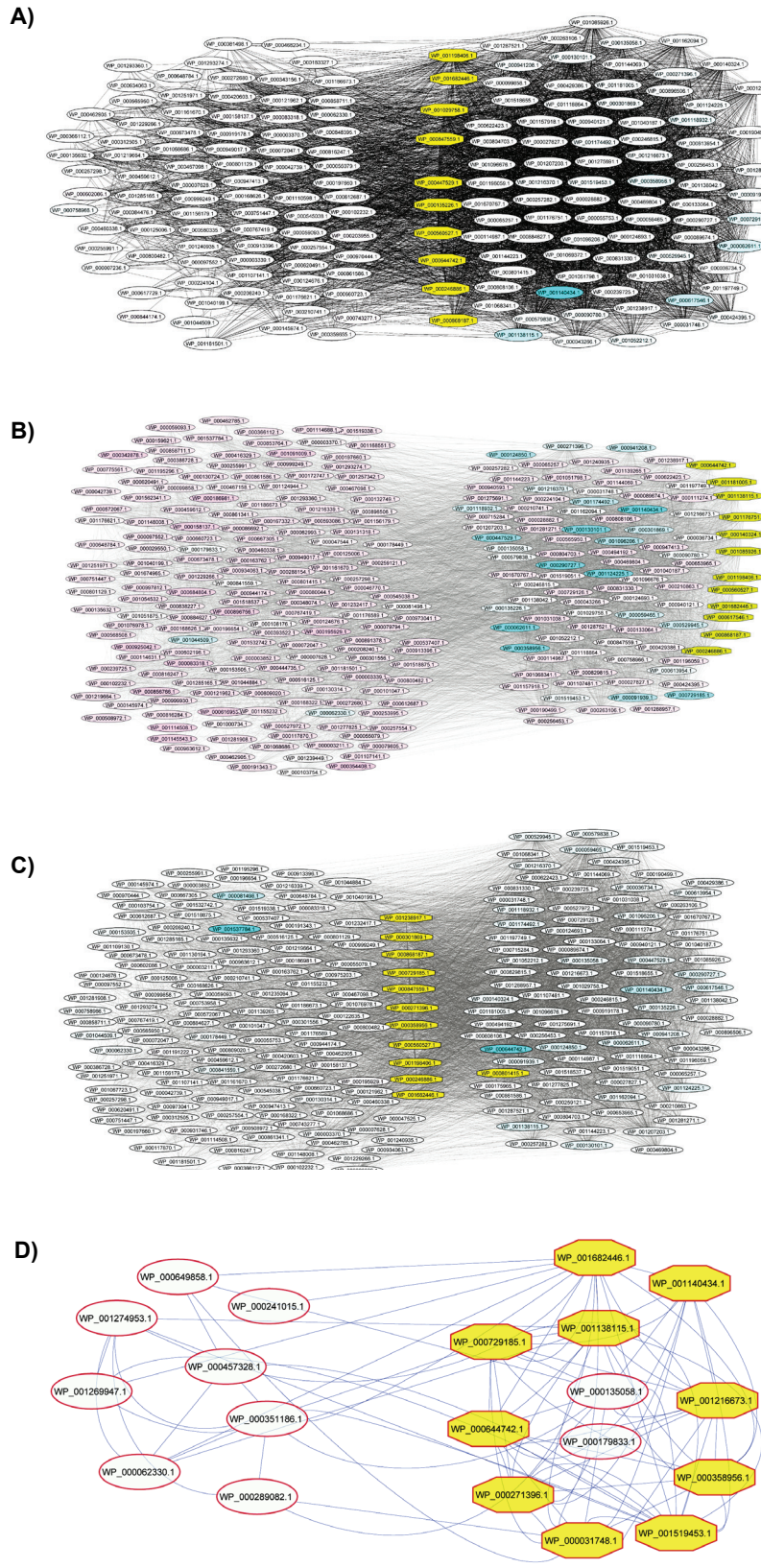
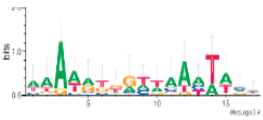
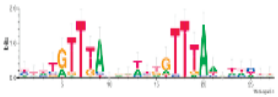
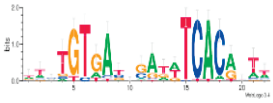
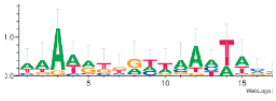
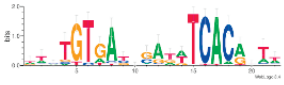
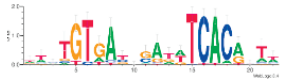
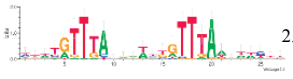


Figure 2. **Rank 1 cluster of the subnetwork containing hub genes of *S. Typhimurium* in A)** black pepper, **B)** milk chocolate, **C)** skim milk powdered; **D)** shared among milk chocolate, black pepper, and skim milk powdered. Hub genes are shown as Pentagon. The FC amounts were visualized based on the color intensity of the nodes (pink: high FC, turquoise blue: low FC)

Table 3. The conserved motifs in promoter of hub down-regulated genes of *S. Typhimurium* under low a_w by MEME analysis

Hub DEGs	Low a_w food: Black pepper	Milk chocolate	Powdered slim milk	Motif Logo	E-value	Width	Best match in Prokaryote DNA	GO term identified by GOMO
rplX			■	Motif 1 	7.01636	17	EXPREG_00000b00	**BP:** cellular homeostasis **BP:** chemical homeostasis
rpsS		■		Motif 2 	3.82e+00	27	EXPREG_000008e0	**MF**: active transmembrane transporter activity **MF**: ion transmembrane transporter activity
rpmC	■	■		Motif 3 	3.31e-01	22	EXPREG_00000850	**BP:** carbohydrate transport **BP:** pentose catabolic process **BP:** macromolecule catabolic process **MF:** sugar transmembrane transporter activity **MF:** intramolecular oxidoreductase activity, interconverting aldoses and ketoses
rplU			■	Motif 4 	7.96e+00	17	EXPREG_00000b00	**MF:** active transmembrane transporter activity **MF:** FAD binding **MF:** heme binding
rplM	■	■		Motif 5 	5.97e+00	22	EXPREG_00000850	**BP:** carbohydrate transport **BP:** pentose catabolic process **BP:** macromolecule catabolic process **MF:** sugar transmembrane transporter activity **MF:** intramolecular oxidoreductase activity, interconverting aldoses and ketoses
rpsJ		■		Motif 6 	5.44e+00	22	EXPREG_00000850	**BP:** carbohydrate transport **BP:** pentose catabolic process **BP:** macromolecule catabolic process **MF:** sugar transmembrane transporter activity **MF:** intramolecular oxidoreductase activity, interconverting aldoses and ketoses
rplW		■		Motif 7 	2.13e-01	27	EXPREG_000008e0	**MF:** active transmembrane transporter activity **MF:** ion transmembrane transporter activity

BP: Biological process **MF:** Molecular function

The analysis revealed the presence of seven motifs with known gene ontology (GO) annotations in the promoter regions of hub genes (**Table 3**). These motifs ranged in length from 17 to 27 base pairs. Interestingly, the identified motifs appeared to be somewhat dependent on the type of food sample analyzed. For example, motifs 3 and 5 (annotated as EXPREG_00000850) were found in the promoter sequences of hub genes from black pepper, while motifs 1 and 4 (annotated as EXPREG_00000b00) were identified in powdered skim milk. In milk chocolate, the motifs included EXPREG_000008e0 (motifs 2 and 7) and EXPREG_00000850 (motifs 3, 5, and 6). To better understand the potential roles of these motifs, we performed gene ontology analysis using the

GOMo tool (**Table 3**). The analysis provided insights into the biological and molecular functions associated with the identified motifs. For example, motifs 2, 3, 5, and 7 were found to be associated with processes such as regulation of transcription, DNA-templated, cellular response to stress, regulation of gene expression, and response to oxidative stress. Motif 6 was associated with processes such as response to antibiotics, DNA binding, and regulation of transcription. These findings suggest that the identified motifs in the promoter regions of hub DEGs may play crucial roles in the regulation of gene expression in response to low a_w stress. Further experimental validation and functional studies are needed to confirm the regulatory interactions between these motifs and the corresponding genes. Nevertheless, the promoter motif analysis provides valuable insights into potential regulatory mechanisms that contribute to the differential expression of hub genes under low a_w stress conditions. Overall, the analysis of promoter motifs adds a layer of understanding to the gene expression changes observed in response to low a_w stress. By identifying conserved regulatory motifs, we can begin to unravel the complex network of interactions that govern the cellular adaptations of *S. Typhimurium* in low a_w environments.

5. Discussion

The hub genes that have the most connections and potentially play crucial roles in various molecular functions and biological processes were identified. The activation of metabolic pathways in *Salmonella* cells under stress conditions may have led to a decrease in the expression of these genes. Among the hub genes, the *rpsB* gene and *Tig* gene were the most significant in all three food samples, based on their scores and identification methods. Additionally, the hub genes associated with the large subunit ribosomal protein L36 (*rpmJ*), a ribosome-associated protein with aminoacyl-tRNA hydrolase activity (*YaeJ*), and a putative cytoplasmic protein (STM3411) were identified in all three food samples. *YaeJ*, a novel ribosome-associated protein found in gram-negative bacteria like *Salmonella* and *E. coli,* possesses the ability to hydrolyze peptidyl-tRNA and rescue stalled ribosomes (22, 23). It was expected that a lower number of stalled ribosomes would be encountered by the cellular protein production machinery under low water stress. Consequently, the down-regulation of *YaeJ* as a hub gene may result in a reduction in stalled ribosomes. Similarly, the expression of genes related to translation (*YaeJ*) has been reported in *S. Typhimurium* affected by low a_w, as stated by Gruzdev and McClelland (24) and Maserati, Lourenco (25). A previous study (10) demonstrated that the *ArfA* gene was the most down-regulated ribosome alternative rescue factor. However, according to the current study's findings, the hub gene *YaeJ* appears to be more affected by low a_w stress. Moreover, in milk chocolate, the hub gene encoding the F-type H^+/Na^+-transporting Atpase subunit alpha, along with the small and large subunit ribosomal proteins, was affected by low a_w stress. While other large subunit ribosomal proteins such as *rplR* have been identified as hub genes in powdered skim milk. The *rplR* likely facilitates the connection of 5S RNA with the large ribosomal subunit. Given that bacterial growth rate is closely tied to protein synthesis and the number of ribosomal units, which dictate the intensity of protein production. The down-regulation of ribosomal proteins and subsequent decrease in ribosome production is expected under water stress, as it correlates with decreased protein synthesis and cell growth rate (26). In a previous study, Crucello, Furtado (10) reported that 25% of down-regulated genes in all food samples were associated with ribosomal proteins after 72 hours. Consequently, the production of ribosomal proteins decreases as anticipated, leading to a slowdown in cell growth under stressful conditions (27). Several reports have indicated that numerous genes involved in translation and transcription are either up-regulated (24) or down-regulated (28-30) during various stress conditions, such as desiccation and nutrient deprivation. These genes include *YaeJ*, Tufa, ribosomal proteins, and the protein export-associated gene *Tig*. *Tig* acts as a chaperone, functioning as a peptidyl-prolyl cis-trans isomerase, thereby maintaining newly produced proteins in an open conformation. Some of the genes identified in this study have previously been described in *Salmonella's* response to water stress, including STM3411 with an unknown function (31)ÿ ÿÿÿÿÿ ÿ-ÿÿ@, *rpsK, rpsD, rpsJ, rplO, rplB*, *rplX, rplW* (24), *rpmE2*, and *rplB* (25). Unlike the top up-regulated genes (309 genes) identified in the previous study (10), only significant down-regulated genes were recognized as hub genes in the 72-hour stored food samples, and the cold-shock proteins did not emerge as hub genes in the current gene network analysis. Based on the results of the gene network analysis, the identified hub genes

may contribute to adapting to food compositions and play a role in developing low water stress tolerance in *Salmonella*. In this study, water stress had a greater impact on the biological processes associated with organonitrogen metabolism in milk chocolate compared to other food samples. Previous research has demonstrated that both desiccation stress (32) and sodium hypochlorite stress (33) affect organonitrogen metabolism. Li and He (33) reported that proteins involved in the catabolic process of organonitrogen compounds play a role in oxidoreductase activity, metal ion binding, and cofactor binding under stressful conditions. The enrichment analysis of hub genes revealed significant gene ontology (GO) terms related to translation, rRNA binding, and the 30S and 50S ribosomal subunit proteins in the low water activity (a_w) samples. These findings suggest that the efficiency of post-transcription and translation processes may decrease under low water stress, consistent with previous studies (10, 34). Moreover, the analysis of KEGG pathways indicated that water stress significantly impacted the ribosome, indicating cellular adaptation and changes in cell metabolism. In general, microbial cell survival under environmental stress conditions relies on conserving energy by reducing cellular activities, including ion pumps and macromolecular syntheses, such as proteins and macromolecular turnover. Consequently, the decrease in metabolic processes leads to reduced rates of transcription and translation, resulting in a slowdown in protein synthesis. This metabolic depression and reduction in protein biosynthesis result in significant bioenergetic savings and ultimately promote cellular survival under stress conditions. The down-regulation of genes leading to metabolic down-regulation has been observed in various species in response to unfavorable environmental changes such as osmosis, starvation, low temperatures, and anoxia stress (35). The networks observed in black pepper and milk chocolate samples were biologically relevant, and their cellular responses were associated with RNA polymerase, ribosomes, and oxidative phosphorylation. Oxidative phosphorylation is a crucial metabolic pathway wherein cellular enzymes oxidize nutrients, leading to the release of energy (ATP) necessary for cellular growth, metabolism, and ultimately, survival. RNA polymerase and ribosomes also play active roles in transcription and translation, respectively, contributing to cell growth and vitality. As mentioned earlier, cells exposed to environmental stresses achieve survival by reducing essential activities and conserving energy. Consequently, the oxidative phosphorylation process, as well as the rate of gene transcription and translation, are diminished. Hence, the aforementioned KEGG pathways were expected to be affected in the food samples. Consequently, factors associated with these pathways could impact the cellular survival of *S. Typhimurium* under low a_w conditions. For instance, cluster analysis revealed significant identification of genes encoding RNA polymerase activity, such as *rpoA*, *rpoB*, and *rpoZ*, within the KEGG pathways. Similarly, the transcription-associated factor *RpoS* has been linked to the cellular tolerance of *E. coli* and *S. Enterica* serovar *Enteritidis* under osmotic stress (5, 36). *RpoS* is recognized as a critical regulator in the survival of enteric pathogenic bacteria, including *S. enterica*, under stressful conditions. It encodes the sigma factor σs (37). According to Zhang, Zhu (37), several genes of *ΔRpoS*, such as *nrfA* encoding the Cytochrome c552 precursor and *yaaI* encoding the Hypothetical protein t0011, were down-regulated under hyperosmotic conditions. They reported that most of these genes are associated with enzymes involved in metabolic pathways. Bacteria employ proline as an essential organic compatible solute for cellular survival under osmotic stress (38, 39). In the context of osmoadaptation, it is well established that changes in RNA polymerase activity regulate the expression of genes involved in osmoregulation, including those encoding compatible solutes like proline. These changes are mediated by the binding of specific transcription factors to the promoter regions of these genes. In summary, ribosomes play a critical role in translating the RNA molecules produced by RNA polymerase. The translation process is essen-tial for producing the enzymes and other proteins re-quired for osmoregulation. On the other hand, oxidative phosphorylation is responsible for generating ATP, which is essential for the energy required in osmo-regulation processes. Additionally, maintaining a proton gradient across the inner membrane of *Salmonella* is crucial for ATP production through oxidative phospho-rylation. In conclusion, RNA polymerase, ribosomes, and oxidative phosphorylation are all interconnected in the process of osmoregulation in gram-negative bacteria, such as *Salmonella*, under low water activity stress or osmo-adaptation. Gene ontology analysis indicated that several motifs may have participated in the regulation

of cellular and chemical homeostasis, active trans-membrane trans-porter activity, FAD binding, and heme binding in the powdered skim milk sample. Consequently, trans-cription factors involved in electron and oxygen transfer may have participated in the down-regulation of hub DEGs of *S. Typhimurium* in this sample. Meanwhile, the regulatory functions of motifs in black pepper and milk chocolate samples were related to carbohydrate metabolism and transport. The primary response of bacteria affected by severe osmotic conditions is the accumulation of compatible solutes, including proline, glutamate, glycine betaine, trehalose, and K^+, at concentrations proportional to the osmotic pressure of the environment (40). According to Kappes, Kempf (41), glycine betaine uptake in bacteria is driven by both single-component ion-dependent secondary systems and multicomponent ATP-dependent transporters. Therefore, motifs regulating active trans-membrane transporter activity were expected to be identified under water stress. *ProU* and *ProP*, as osmo-regulated permeases, mediate the uptake of osmo-protectants such as proline and glycine betaine in *S. Typhimurium* (39, 42). Higgins, Dorman (43) indicated that DNA supercoiling in bacteria may induce the trans-cription of *proU* under osmotic stress. The high extra-cellular osmolarity leads to DNA supercoiling, which highly affects the expression of *proU*.

6. Conclusion

Our study aimed to understand how *Salmonella* adapts and survives in low a_w conditions at the molecular level. Based on the results, it appears that the down-regulated hub genes are more related to the low a_w stress responses. These genes mainly belonged to ribosome proteins and factors related to replication, transcription, and translation, indicating a set of changes in the cellular metabolism of *S. Typhimurium* affected by low a_w. The energy savings by microorganisms may be achieved through the reduction of protein biosynthesis due to reducing the rate of transcription and translation under environmental stresses. Consequently, the gene down-regulation leads to metabolic down-regulation in response to osmotic stress. Among the food samples, the milk chocolate matrix leads to more adaptation pathways for *S. Typhimurium* survival because more hub genes were downregulated, additional bacterial biological processes were affected, and the discovered motifs of *S. Typhimurium* were more in this food sample than in other food samples. Our findings shed light on the cellular changes and molecular mechanisms that enable *Salmonella* to survive in low a_w environments. The identification of hub genes and gene clusters provides valuable insights into the mechanisms underlying *Salmonella* survival in low a_w foods. In conclusion, our study highlights the importance of understanding the genetic networks and molecular responses of *S. Typhimurium* to low a_w stress. However, future research should aim to define a specific research objective and pursue targeted goals to enhance the significance and relevance of such studies in the field of food safety and public health.

Acknowledgments

We gratefully appreciate the cooperation of all the current study participants.

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