Article

Insights into the Oxidative Stress Response of Salmonella enterica serovar Enteritidis Revealed by the Next Generation Sequencing Approach

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Abstract: As a facultative intracellular pathogen, Salmonella Enteritidis must develop an effective oxidative stress response to survive exposure to reactive oxygen species within the host. To study this defense mechanism, we carried out a series of oxidative stress assays in parallel with a comparative transcriptome analyses using a next generation sequencing approach. It was shown that the expression of 45% of the genome was significantly altered upon exposure to H$_2$O$_2$. Quantitatively the most significant (≥100 fold) gene expression alterations were observed among genes encoding the sulfur utilization factor of Fe-S cluster formation and iron homeostasis. Our data point out the multifaceted nature of the oxidative stress response. It includes not only numerous mechanisms of DNA and protein repair and redox homeostasis, but also the key genes associated with osmotic stress, multidrug efflux, stringent stress, decrease influx of small molecules, manganese and phosphate starvation stress responses. Importantly, this study revealed that oxidatively stressed S. Enteritidis cells simultaneously repressed key motility encoding genes and induced a wide range of adhesin- and salmonellae-essential virulence-encoding genes, that are critical for the biofilm formation and intracellular survival, respectively. This finding indicates a potential intrinsic link between oxidative stress and pathogenicity in non-typhoidal Salmonella that needs to be empirically evaluated.

Keywords: non-typhoidal Salmonella; oxidative stress response; next generation sequencing; biofilm formation; salmonellae-essential virulence genes

1. Introduction

Non-typhoidal Salmonella is a leading cause of foodborne gastroenteritis on the global scale. On the global scale, this foodborne pathogen is responsible for 80 million cases of gastroenteritis annually [1]. The situation in the USA is very similar to the global epidemiological picture of salmonellosis caused by non-typhoidal Salmonella. It has been estimated that from 2000 to 2008, non-typhoidal Salmonella serovars accounted for 1.2 million laboratory-confirmed illnesses, 19,000 hospitalizations, and 380 deaths each year in the USA [2]. The high incidence rate is made more significant by the fact that there have been no signs of decline in the incidence of salmonellosis over the past 15 years in the US. Intriguingly, infections caused by other five major food-borne pathogens (i.e., Escherichia coli O157:H7, Campylobacter spp., Listeria monocytogenes, Yersinia enterocolitica, and Shigella spp.) declined substantially over the same time [2].
Infections of humans with non-typhoidal *Salmonella* serovars occur mostly via the human food chain [3,4]. The important human food chain transmission route of non-typhoidal *Salmonella* is associated with ready-to-eat products such as vegetables [5,6], leafy green horticulture products [7,8], and fruits [9,10]. Traditionally, aqueous chlorine and ozone solutions are widely used in the food industry for sanitization of food-processing surfaces as well as vegetables and fruits prior to their distribution to the market [11,12]. Chlorine and ozone, potent oxidizing agents, impose an oxidative stress on cells, damaging nucleic acids, both DNA and RNA, proteins, lipids, and further causing membrane disorganization and genome breaks which result in cellular death. To survive oxidative stress assault in the food matrix and food processing facilities and successfully invade the host and survive intracellularly, these pathogens must acquire a robust oxidative stress protective mechanism.

A number of studies investigated the roles of transcription factors [13], RNA repair systems [14], sigma factors [15], and novel stress response proteins [16,17] in the oxidative stress response of non-typhoidal *Salmonella*. Although these studies gave us extremely valuable information on the functions of specific oxidative response mechanisms employed by these pathogens, they could not provide insights into the global impact of oxidative stress on the non-typhoidal *Salmonella* physiology. Recently, Fu et al. [18] by employing global comparative proteomics, identified 116 proteins in the proteome of *Salmonella Typhimurium*, treated with H$_2$O$_2$ that were significantly altered compared to the control. Among the altered proteome, iron acquisition systems were most significantly induced. Also, several phage-coding proteins with DNA repair capabilities were induced, whereas the apparatus of the *Salmonella* type III secretion system was repressed.

To gain insight into the expression dynamics of genes associated with distinct biological functions during an oxidative stress, expression of ten genes, including *iroN*, *sitA*, *rpoS*, *rpoH*, *ycfR*, *dps*, *nrdM*, *pocR*, and *ompF*, were tested after 30 min of 1 mM, 2 mM, and 4 mM H$_2$O$_2$ treatments. The 30 min of H$_2$O$_2$ treatment was selected as *Salmonella enterica* subspecies *enterica* serovar Enteritidis, hereinafter *Salmonella* Enteritidis cultures were already adapted at that point to different H$_2$O$_2$ treatments, showing only a slight decline in the viability. Finally, to determine the global impact of oxidative stress on the non-typhoidal *Salmonella* physiology, we carried out comparative transcriptome analyses using the next generation sequencing approach.

2. Materials and Methods

2.1. Bacterial Culture

*Salmonella* Enteritidis ATCC 13076 (American Type Culture Collection, Manassas, VA, USA) was used as the model organism. For each experiment, fresh culture was prepared by streaking frozen culture onto tryptic soy agar (EMD Chemicals Inc., Darmstadt, Germany), followed by incubation at 37 °C for 24 h. Three well-isolated colonies were used for subsequent inoculations.

2.2. Oxidative Stress Killing Assay

This assay was carried out using overnight cultures of the wild type *S. Enteritidis* strain grown in LB at 37 °C with continuous shaking at 180 rpm. These overnight cultures were diluted 1/100 in LB followed by growth to 0.4 using an optical density at 600 nm. The wild type cultures were exposed at their mid-exponential growth phase (0.4) to three incrementally increasing concentrations of H$_2$O$_2$, including: 1 mM, 2 mM, and 4 mM. Treatment was carried out for 90 min under the same incubation conditions. Wild type culture with no oxidative treatment was included as a control. Colony forming units (CFU) counts were carried out by 10-fold dilutions using sterile saline at time zero (before H$_2$O$_2$ exposure) and then at every 15 min of treatment. Aliquots of 0.1 mL were plated in triplicate followed by incubation at 37 °C for 24 h.
2.3. Incremental Oxidative Stress Gene Expression Assay

Cultures of S. Enteritidis were prepared as described above and after 30 min of oxidative treatment samples were taken and processed. Total RNA was isolated as described below followed by cDNA synthesis using Superscript™ III reverse transcriptase (RT) (Invitrogen by Life Technologies, Carlsbad, CA, USA). Quantitative PCR was carried out on a MiniOpticon™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with iQTM SYBR Green Supermix kit (Bio-Rad Laboratories) as described previously [19]. Genes rtcR and yjjA, encoding for transcriptional regulatory protein RtcR and DUF2501 domain-containing protein, were selected as the internal reference genes to normalize the expression of the tested genes. Primers were designed to target expression of the following genes: \textit{rpoS}, \textit{rpoH}, \textit{iroN}, \textit{sitA}, \textit{dps}, \textit{nrdM}, \textit{trxC}, \textit{ycfR}, \textit{ompF}, and \textit{pocR} (Table 1). These ten genes were selected based on their distinct gene ontologies (GO) associated with the oxidative stress response. Selected the GO include, iron homeostasis, alternative sigma factors, multiple stress response, DNA protection, antioxidants, DNA replication, uptake of small solutes and anabolic processes. Melting curves were analyzed to check the product specificity. The level of gene expression for each target gene was compared with the internal reference control gene and the fold changes in expression were calculated by the comparative \textit{CT} method [20].

| Gene Names | Forward Sequence (5′–3′) | Reverse Sequence (5′–3′) |
|------------|--------------------------|--------------------------|
| \textit{rpoS} | CGA AAA AGC GTT GCT GGA CA | ATC GTC ATC TTG CGT GGT GT |
| \textit{rpoH} | CCT TCG CCC TAC ACT GGA TT | GCT TTC GTG GTC GAG ACT TT |
| \textit{iroN} | TCA TGG AAA ATG ACC CCG CA | ACC GCG TTC GAA GTA CTG TT |
| \textit{sitA} | CGC CAA AAC AGG TGC GTA AA | ATC GGA AAC GTT ACT CTC GC |
| \textit{dps} | AAA AGC GAC GGT TGA GTT GC | TAC GGA AGC CAT CCA GCA TC |
| \textit{nrdH} | TGG TGA ACG TCG ATC TGG TG | CGG GTG CAG AGC GGT TT AAT CA |
| \textit{trxC} | AGC GGT AAA GTC CGT TTC GT | GAA AGG CTT TTG AGG CAC TG |
| \textit{ycfR} | CCG TTG AAG TCC AGG CAA CG | CCC ATC TCC TCC GCT TTT TG |
| \textit{ompF} | CAG GGT ACA GCA ACA GCA AG | TCA GCA TAT ACG GCA GGC AG |
| \textit{pocR} | GTG GTG AAA CCG CCA GAG AA | AGG TCT GGC GGA AGA CTG TG |

Table 1. Primers used in this study for the oxidative stress response gene expression assay and validation of RNA-seq data.

2.4. Preparation of Samples for Transcriptomics

Untreated and 30-min H\textsubscript{2}O\textsubscript{2} (3 mM) treated cultures (0.4 at OD<sub>600</sub>) of S. Typhimurium were centrifuged at 4000 rpm for 5 min. Supernatants were discard and the cell pellets were washed two times followed by RNA extraction using the RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer’s instructions.

2.5. Global Transcriptomic Analysis

Sample quality preparation was done using capillary electrophoresis (Agilent Bio Analyzer 2100, Santa Clara, CA, USA) as previously described [17]. Only samples that had RNA integrity number 8
or greater were considered for further analysis. Illumina sequencing libraries were prepared using Illumina’s TruSeq Stranded Total RNA Library Prep Human/Mouse/Rat Sample Preparation Kit (Cat. # 20020597) at the University of Minnesota Genomics Center (Saint Paul, MN, USA). For rRNA depletion, 500 ng of total amount of RNA was used in combination with Ribozero capture probes. After this step, the mRNA was fragmented followed by reverse transcription into complementary DNA (cDNA). The resulting cDNA fragments were coded with indexed adaptors followed by amplification using 15 PCR cycles. Again capillary electrophoresis was used to validate library size distribution, while the library quantification was carried out using fluorimetry. Libraries normalization was carried out followed by their hybridization to a single read flow cell. Once hybridization completed, the flow cell was loaded on the HiSeq 2500 and sequenced using Illumina’s SBS chemistry. The primary analysis and index de-multiplexing were performed as previously described [17]. Read mapping was carried out using publicly available genome of S. Enteritidis P125109 strain. Quantification of gene expression was completed using Feature Counts. Significantly differentially expressed genes were identified using the edgeR feature in CLCGWB (Qiagen, Valencia, CA, USA) based on a minimum 2 times absolute fold change difference and p < 0.05 false discovery rate (FDR).

2.6. Validation of RNA-Seq Data by Real-Time PCR

Cultures of S. Enteritidis were prepared as described above and after 30 min of oxidative treatment samples were taken and processed. Total RNA was isolated as described above. Quantitative PCR assay was carried out as described above. Genes rtcR and yjjA were selected as the internal reference genes to normalize the expression of the tested genes. Primers were designed to target expression of the following genes: dinP, dnaJ, eutC, mdlA, osmY, pagO, spaR, malK, and nrdD (Table 1).

2.7. Experimental Replication and Gene Ontology Analysis

The data of all experiments represent the average of three biological replications. The cell viability during the oxidative treatments were analyzed by CoStat version 6.4 software (Co-Hort Software, Monterey, CA, USA) using the homogeneity of linear regression slopes approach. The gene ontology (GO) analysis was carried out using the Database for Annotation, Visualization and Integrated Discovery (DAVID) database [21] as well as the National Center for Biotechnology Information (NCBI).

2.8. RN-Seq Accession Numbers

RNA sequencing data for all three biological replications and two different treatments were deposited in the NCBI under accession number GSE 155479.

3. Results

3.1. Effect of Increasing H₂O₂ Concentrations on the Viability of S. Enteritidis

To evaluate the ability of S. Enteritidis to survive exposure to oxidative stress, we performed oxidative stress killing assays with increasing concentrations of H₂O₂ over a short time. The concentration of 1 mM of H₂O₂ caused a death rate of 0.37 log₁₀ CFU for the first 15 min of exposure (Figure 1 and Figure S1). After this initial lethal period, the culture of S. Enteritidis exposed to the same concentration of H₂O₂ showed a slight decline in the number of viable cells, resulting in only 0.1 log₁₀ CFU of reduction during the entire experiment (Figure 1). The exposure of S. Enteritidis to 1 mM of H₂O₂ resulted in two distinct survival phases, an initial and brief lethal phase followed by a long bacteriostatic phase (Figure 1 and Figure S1). Similarly to 1 mM, the concentration of 2 mM of H₂O₂ caused an initial lethal phase resulting in 0.53 log₁₀ CFU decline (Figure 1). However, the survival pattern associated with the 2 mM H₂O₂ treatment was bactericidal not bacteriostatic (Figure 1). After an initial lethal period for the first 15 min of H₂O₂ exposure, the 2 mM H₂O₂ treatment continued to cause cell death, albeit at a slower rate compared to that of the initial 15-min period. This bactericidal phase was characterized by repetitive fluctuations of fast die-off periods (e.g., 0.23, 0.24 and 0.22 log₁₀
CFU declines at the 30-min, 60-min, and 90-min measurements, respectively) and slight growth periods (e.g., $0.06 \log_{10}$ CFU increase at the 45-min measurement and $0.03 \log_{10}$ CFU increase at the 75-min measurement) (Figure 1). The exposure of S. Enteritidis to the greatest concentration of H$_2$O$_2$, 4 mM, caused again an initial lethal phase, characterized by a fast die-off rate, $0.72 \log_{10}$ CFU decline over the first 15 min of treatment (Figure 1). Interestingly, this initial fast die-off period was followed by moderate die-off periods (e.g., $0.18$ and $0.17 \log_{10}$ CFU decline) at the next two measurements (Figure 1). After this moderate die-off period, the culture of S. Enteritidis entered progressively fast mortality rates resulting in a $0.38 \log_{10}$ CFU decline at the 60-min measurement and $1.19 \log_{10}$ CFU decline at the 75-min measurement (Figure 1). At the last, 90-min, measurement, the viable cell count could not be detected at $10^3$ CFU per 1 mL, indicating a continuation of this progressive and fast mortality rate. The oxidative treatments with 1 mM, 2 mM, and 4 mM of H$_2$O$_2$ caused significantly higher ($p < 0.01$) mortality rates during the exponential growth phase of S. Enteritidis compared with no treatment of this pathogen under the same growth conditions. The average mortality rates of S. Enteritidis for every 15 min of H$_2$O$_2$ treatment were $0.08 \log_{10}$ CFU decline for 1 mM, followed by $0.188 \log_{10}$ CFU decline for 2 mM treatment and $0.468 \log_{10}$ CFU decline for 4 mM. Although all three H$_2$O$_2$ treatments caused significantly higher mortality rates compared to that of no treatment, the survival patterns of H$_2$O$_2$ treated S. Enteritidis were different (Figure 1).

![Figure 1](image.png)

**Figure 1.** Oxidative stress killing assay. Mortality of S. Enteritidis, expressed by reduced $\log_{10}$/CFU for every 15 min of the H$_2$O$_2$ treatment. The data correspond to the mean value of three biological replications. Each measurement value was composed of nine replications (each biological replication was made up of three technical replications).

3.2. Expression Levels of the Genes Associated with Oxidative Stress and Anabolic Processes during Incremental Increase of H$_2$O$_2$ Concentration

To determine the relationship between a death rate of S. Enteritidis and expression of genes that are associated with the most important oxidative stress adaptive processes, we carried out a qRT-PCR assay. The gene expression assay was carried out after 30 min of H$_2$O$_2$ treatment when *Salmonella* cells have already been adapted to the oxidative stressor (Figure 1). The targeted genes were selected from the following stress response and anabolism associated processes, iron homeostasis (*iroN, sitA*), alternative sigma factors (*rpoS, rpoH*), multiple stress responses (*ycfR*), DNA protection (*dps*), antioxidants (*trxC*), DNA replication (*yrdM*), uptake of small solutes (*ompF*) and anabolic process (*pocR*).

No significant changes in expression of *rpoS* were observed during 1 mM and 2 mM H$_2$O$_2$ treatment, whereas 4 mM H$_2$O$_2$ treatment caused a significant (2.5-fold) downregulation of the *rpoS* gene (Figure 2). Similarly to the *rpoS* gene, the oxidative treatments of S. Enteritidis caused minor
alterations in the expression of the gene that encodes another stress response sigma factor, RpoH (Figure 2). The 1 mM and 4 mM treatments caused 2-fold and 2.9-fold upregulation of the rpoH gene, while 2 mM treatment caused no significant (1.4-fold) upregulation of the same gene.

![Gene expression](image)

Figure 2. Gene expression. mRNA expression levels of iroN, sitA (iron receptor), rpoS, rpoH (alternative sigma factors), ycfR, dps (stress response), nrdM, trxC (antioxidant), ompF (nonselective uptake), and pocR (anabolism) during no H$_2$O$_2$ treatment, 1 mM, 2 mM, and 4 mM H$_2$O$_2$ treatments. Values on the y-axis are relative expression levels (fold change) normalized against the level in the no H$_2$O$_2$ treatment group. The data correspond to the mean values of three biological replications. Error bars correspond to the standard deviation.

In sharp contrast to the genes that encode stress response sigma factors, the genes associated with iron homeostasis, in particular genes that encode the iron receptors, IroN (out membrane receptor) and SitA (periplasmic receptor) showed the greatest gene expression alterations (Figure 2). Most notably, the expression of iroN exhibited a profound upregulation with the 1 mM H$_2$O$_2$ treatment (532-fold) and with 2 mM H$_2$O$_2$ treatment, the upregulation of this gene was extraordinarily high (75664-fold) compared to that of the H$_2$O$_2$ untreated control. Interestingly, with the greatest concentration of oxidative stressor, 4 mM, the upregulation of the iroN gene was lower (292-fold) than that with 1 mM (532 fold) and 2 mM (75664 fold) treatment (Figure 2). Similarly, other genes involved in the iron homeostasis, sitsA, showed large upregulation upon the oxidative stress treatment with 1 mM (298-fold), 2mM (316-fold) and 4 mM (675-fold) H$_2$O$_2$ treatment. Although both genes encode for iron receptors, their expression (stress response) patterns were different. The upregulation of the sitsA gene was proportional to incremental increases of H$_2$O$_2$ concentration, whereas the upregulation of the iroN gene did not follow the level of H$_2$O$_2$ concentration (Figure 2).

The ycfR gene, which encodes a multiple stress response protein YcfR, was upregulated throughout the H$_2$O$_2$ treatments. The upregulation of the ycfR gene ranged from 283-fold and 273-fold to 103-fold in the Salmonella cultures exposed to 1 mM, 2 mM, and 4 mM H$_2$O$_2$, respectively (Figure 2). Similarly to the iroN gene, the greatest concentration of H$_2$O$_2$, 4 mM, caused the lowest upregulation of the ycfR gene, further indicating to some extent the similar patterns of the responses of these two oxidative stress response associated genes. However, the ycfR gene did not show an extremely high upregulation during the 2 mM H$_2$O$_2$ treatment, as the iroN gene showed (Figure 2). Another stress response gene, dps, had an unusual expression profile. During treatment with 1 mM H$_2$O$_2$ this gene was upregulated 44-fold, then during the 2 mM treatment the dps gene was downregulated 4-fold, and again upregulated 8.9-fold with 4 mM H$_2$O$_2$ treatment (Figure 2). This unusual expression profile of
the dps gene, which encodes a protein that protects DNA from reactive oxygen species (ROS), indicates a complex interaction of the oxidative stress response associated genes.

The genes associated with DNA replication and oxidoreductase processes, nrDM and trxC, had similar genes expressions profiles (Figure 2). Both genes exhibited 35- to 70-fold upregulation during the oxidative treatments (Figure 2). The ompF gene, which encodes for a major nonspecific OmpF porin, and the pocR gene, which is associated with anabolic processes, exhibited downregulation during the oxidative treatments (Figure 2). Interestingly, the downregulation gene expression patterns for both the ompF and pocR genes were the same. The greatest gene alterations (e.g., downregulation) were observed during 2 mM H$_2$O$_2$ treatment, whereas the 4 mM H$_2$O$_2$ treatment caused the lowest level of the gene expression alteration for both genes (Figure 2).

3.3. Global Transcriptome Response of S. Enteritidis to Oxidative Stress.

After the determination of the stress response patterns of a limited number of genes during treatment with different concentrations of H$_2$O$_2$, we carried out the global transcriptome analyses using a single concentration of H$_2$O$_2$. In total, 2051 genes were significantly differently expressed (SDE) in S. Enteritidis during the 3 mM H$_2$O$_2$ treatment compared to the reference transcriptome of the same S. Enteritidis strain with no H$_2$O$_2$ treatment. Of the 2051 SDE genes, 1111 were induced and 941 genes were repressed, showing at least a 2-fold alteration in gene expression and high reproducibility (false discovery rate < 0.5) across all three biological replications (Tables S1 and S2).

3.3.1. Induction of Transcriptome—Molecular Response of S. Enteritidis to Oxidative Stress

The most altered change in biological processes of oxidatively stressed S. Enteritidis was the induction of genes encoding Fe-S cluster biogenesis (Tables S1 and S2). Operons encoding sulfur utilization factor (SUF), sufABCSD and ynhAG, were upregulated from 173- to 903-fold and from 23- to 114-fold, respectively (Figure 3). The SUF operons were profoundly more induced compared to ones encoding iron-sulfur cluster (ISC), nifJUS, yfhFP and hscBA (Figure 3). Another group of highly induced genes were associated with the iron ion homeostasis. The operon sitABCD, iron/manganese ABC transporter, showed the greatest induction among operonsgenes associated with this biological function (Figure 3). Besides this Fe/Mn transporter, a group of operonsgenes encoding a wide range of iron transporters showed the greatest inductions, including iroN, ferric salmochelin siderophore receptor; fepBD, ferric enterobactin ABC transporter; fhuABCD, ferrichrome outer membrane transporter; ydiE, hemin transporter; cxbBD, iron siderophore bacteriocin transporter; fluE, ferric coprogen and ferric-rhodotorulic acid receptor as well as fluF, receptor for ferrooxamine B (Figure 3). Also, several operons and a gene responsible for the biosynthesis of salmochelin (iroBCE), enterobactin (ybdABZ, entABCDEF) and hem (hemH) were greatly induced during oxidative stress (Figure 3).

The significant induction showed a group of operonsgenes encoding proteins that govern the cell-redox homeostasis. Among the cell-redox encoding genes, the nrdH gene which encodes a classical antioxidant, glutaredoxin-like protein NrdH, showed the greatest induction, resulting in 144.3-fold upregulation compared to the reference strain (Figure 4). The trxC gene that encodes an effective cytoplasmic disulfide-reducing protein, thioredoxin 2, showed 76-fold induction (Figure 4). The grxAB operon, encoding the antioxidants glutaredoxin 1 and 2, showed a moderate induction compared to that of the nrdH gene. A major part of the cell-redox regulon was associated with the genes encoding proteins associated with the electron transport chain (Figure 4). The narIJHK operon, involved in nitrate assimilation and anaerobic electron transport, exhibited the greatest induction among this group of genes, followed by fdoHGI, dehydrogenases that transfer electrons from the periplasmic to the cytoplasmic heme; sdhABCD, cytochrome b556; cyoABCDE, cytochrome bo3 oxidase complex and s1hA, which converts NADPH to NADH (Figure 4).
**Figure 3.** Differential expression of genes involved in iron-cluster assembly and iron ion homeostasis. Transcriptional profiling of the wild type *S. Enteritidis* ATCC 13076. Isolation of RNA was carried out during the exponential growth phase. The transcriptomic profiles were determined using a HiSeq 4000 PE100 Illumina platform. The mean fold-increase of three biological replications in each gene expression after exposure to 3 mM of H$_2$O$_2$ compared with controls (no H$_2$O$_2$ treatment and the same growth phase) is indicated by the color scale bar. The biological functions of the induced gene were determined using the Database for Annotation, Visualization and Integrated Discovery (DAVID) as well as the National Center for Biotechnology Information (NCBI). Both sulfur utilization factor (SUF) and iron-sulfur cluster (ISC) belong to the same biological function “Iron-sulfur cluster assembly”.

| Gene   | Protein function/name                                      | Fold change | Biological function                                      |
|--------|------------------------------------------------------------|-------------|-----------------------------------------------------------|
| sufA   | Fe-S cluster assembly scaffold SufA                        |             | Iron-sulfur cluster assembly (SUF system)                 |
| sufB   | Fe-S cluster assembly protein SufB                         |             | Iron-sulfur cluster assembly (SUF system)                 |
| sufC   | Fe-S cluster assembly ATPase SufC                          |             | Iron-sulfur cluster assembly (SUF system)                 |
| sufS   | Bilfunctional cysteine desulphurase/selenocysteine lyase SufS |             | Iron-sulfur cluster assembly (SUF system)                 |
| sufD   | Fe-S cluster assembly protein SufD                         |             | Iron-sulfur cluster assembly (SUF system)                 |
| ynaA   | Cysteine desulfuration protein YnaA                         |             | Iron-sulfur cluster assembly (SUF system)                 |
| ynaG   | Putative LD-transpeptidase YnaG                            |             | Iron-sulfur cluster assembly (SUF system)                 |
| nfi    | Pyruvate-flavodoxin oxidoreductase IscJ                     |             | Iron-sulfur cluster assembly (SUF system)                 |
| nfu    | Fe-S cluster assembly scaffold IscU                         |             | Iron-sulfur cluster assembly (SUF system)                 |
| nsf    | Cysteine desulfurase IscS                                   |             | Iron-sulfur cluster assembly (SUF system)                 |
| yfhF   | Iron-sulfur cluster assembly protein IscA                   |             | Iron-sulfur cluster assembly (SUF system)                 |
| yfhP   | Fe-S cluster assembly transcriptional regulator IscR        |             | Iron-sulfur cluster assembly (SUF system)                 |
| hscA   | Fe-S protein assembly chaperone HscA                        |             | Iron-sulfur cluster assembly (SUF system)                 |
| hscB   | Fe-S protein assembly co-chaperone HscB                     |             | Iron-sulfur cluster assembly (SUF system)                 |
| sitA   | Iron/manganese transporter, periplasmic-binding protein SitA|             | Iron homeostasis                                           |
| sitB   | Iron/manganese transporter, inner membrane protein SitB     |             | Iron homeostasis                                           |
| sitC   | Iron/manganese transporter, inner membrane protein SitC     |             | Iron homeostasis                                           |
| sitD   | Iron/manganese transporter, inner membrane permease SitD    |             | Iron homeostasis                                           |
| iroN   | Salmochelin siderophore system receptor protein IroN        |             | Iron homeostasis                                           |
| fepB   | Fe$^{3+}$-enterobactin ABC transporter binding protein FepB  |             | Iron homeostasis                                           |
| fepD   | Ferric enterobactin transport protein FepD                   |             | Iron homeostasis                                           |
| fhuA   | Ferrichrome-iron receptor FhuA                              |             | Iron homeostasis                                           |
| fhuB   | Ferrichrome ABC transporter permease FhuB                   |             | Iron homeostasis                                           |
| fhuC   | Ferrichrome-iron transport ATP-binding protein FhuC         |             | Iron homeostasis                                           |
| fhuD   | Ferrichrome-iron binding periplasmic protein FhuD           |             | Iron homeostasis                                           |
| fhuE   | Ferric-rhodotorulic acid outer membrane transporter FhuE    |             | Iron homeostasis                                           |
| fhuF   | Siderophore-iron reductase FhuF                             |             | Iron homeostasis                                           |
| ydhE   | Hemin uptake protein Hdh                                      |             | Iron homeostasis                                           |
| exdB   | Biopolymer transport protein ExdB                            |             | Iron homeostasis                                           |
| exbD   | Biopolymer transport protein ExbD                            |             | Iron homeostasis                                           |
| iroB   | Salmochelin siderophore glycosyltransferase protein IroB    |             | Iron homeostasis                                           |
| iroC   | Multidrug ABC transporter ATP-binding protein IroC          |             | Iron homeostasis                                           |
| iroE   | Salmochelin siderophore system protein IroE                  |             | Iron homeostasis                                           |
| ybaD   | Enterobactin exporter EntS                                  |             | Iron homeostasis                                           |
| ybaB   | Thioesterase YbaB                                           |             | Iron homeostasis                                           |
| ybdZ   | Enterobactin biosynthesis protein YbdZ                       |             | Iron homeostasis                                           |
| entA   | 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase EntA        |             | Iron homeostasis                                           |
| entB   | Isochorismatase EntB                                        |             | Iron homeostasis                                           |
| entC   | Isochorismate synthase EntC                                  |             | Iron homeostasis                                           |
| entD   | Enterobactin synthase component D protein EntD              |             | Iron homeostasis                                           |
| entE   | Enterobactin synthase component E protein EntE              |             | Iron homeostasis                                           |
| entF   | Enterobactin synthase component F protein EntF              |             | Iron homeostasis                                           |
| hemH   | Ferrochelatase HemH                                         |             | Iron homeostasis                                           |
The significant induction showed a group of operons/genes encoding proteins that govern the cell-redox homeostasis. Among the cell-redox encoding genes, the \textit{nrdH} gene which encodes a classical antioxidant, glutaredoxin-like protein NrdH, showed the greatest induction, resulting in 144.3-fold upregulation compared to the reference strain (Figure 4). The \textit{trxC} gene that encodes an effective cytoplasmic disulfide-reducing protein, thioredoxin 2, showed 76-fold induction (Figure 4). The \textit{grxAB} operon, encoding the antioxidants glutaredoxin 1 and 2, showed a moderate induction compared to that of the \textit{nrdH} gene. A major part of the cell-redox regulon was associated with the genes encoding proteins associated with the electron transport chain (Figure 4). The \textit{narIJHKG} operon, involved in nitrate assimilation and anaerobic electron transport, exhibited the greatest induction among this group of genes, followed by \textit{fdoHGI}, dehydrogenases that transfer electrons from the periplasmic to the cytoplasmic heme; \textit{sdhABCD}, cytochrome b556; \textit{cyoABCDE}, cytochrome b oxidase complex and \textit{sthA}, which converts NADPH to NADH (Figure 4).

**Figure 4. Cont.**

| Gene   | Protein function/name                                      | Fold change | Biological function                       |
|--------|------------------------------------------------------------|-------------|------------------------------------------|
| nrdH   | Glutaredoxin-like protein NrdH                             |             |                                          |
| nrdI   | Ribonucleotide reductase assembly protein NrdI             |             |                                          |
| trxC   | Thioredoxin TrxC                                          |             |                                          |
| ahpC   | Peroxiredoxin AhpC                                        |             |                                          |
| ahpF   | Alkyl hydroperoxide reductase subunit F AhpF              |             |                                          |
| grxA   | Glutaredoxin GrxA                                         |             |                                          |
| grxB   | Glutaredoxin 2 GrxB                                       |             |                                          |
| narI   | Nitrate reductase 1, cytochrome b, gamma subunit NarI    |             |                                          |
| narJ   | Nitrate reductase assembly chaperone NarJ                |             |                                          |
| narK   | Nitrite extrusion protein NarK                            |             |                                          |
| narH   | Nitrate reductase subunit beta NarH                       |             |                                          |
| narG   | Nitrate reductase subunit alpha NarG                      |             |                                          |
| fdoH   | Formate dehydrogenase-O beta subunit FdoH                |             |                                          |
| fdoG   | Formate dehydrogenase alpha subunit FdoG                 |             |                                          |
| fdoI   | Formate dehydrogenase cytochrome b556 subunit FdoI       |             |                                          |
| sdhA   | Succinate dehydrogenase flavoprotein subunit SdhA         |             |                                          |
| sdhB   | Succinate dehydrogenase, Fe-S protein SdhB                |             |                                          |
| sdhC   | Succinate dehydrogenase cytochrome b556 subunit SdhC     |             |                                          |
| sdhD   | Succinate dehydrogenase cytochrome b556 subunit SdhO     |             |                                          |
| cyoA   | Cytochrome o ubiquinol oxidase subunit II CyoA            |             |                                          |
| cyoB   | Cytochrome o ubiquinol oxidase subunit I CyoB             |             |                                          |
| cyoC   | Cytochrome o ubiquinol oxidase subunit III CyoC           |             |                                          |
| cyoD   | Cytochrome o ubiquinol oxidase subunit IV CyoD            |             |                                          |
| cyoE   | Protohaeme IX farnesyltransferase CyoE                     |             |                                          |
| ycfR   | Multiple stress resistance protein BhsA                   |             |                                          |
| dps    | DNA starvation/stationary phase protection protein Dps    |             |                                          |
| yebG   | DNA damage-inducible protein YebG                         |             |                                          |
| recA   | Recombinase RecA                                           |             |                                          |
| recN   | DNA repair protein RecN                                    |             |                                          |
| polB   | DNA polymerase II PolB                                     |             |                                          |
| dinP   | DNA polymerase IV DinP                                      |             | DNA repair                                |
| dinl   | DNA damage-inducible protein I                            |             |                                          |
| dinF   | DNA damage-inducible protein F                            |             |                                          |
| dinG   | ATP-dependent DNA helicase DinG                            |             |                                          |
| deoB   | Phosphopentomutase DeoB                                   |             |                                          |
| deoC   | 2-deoxyribose-5-phosphate aldolase DeoC                   |             |                                          |
| deoD   | Purine-nucleoside phosphorylase                           |             |                                          |
| lbpA   | Heat shock protein LbpA                                    |             |                                          |
| lbpB   | Heat shock chaperone LbpB                                  |             |                                          |
| dnaK   | Molecular chaperone DnaK                                   |             | Protein protection, refolding and digestion |
| dnaJ   | Molecular chaperone DnaJ                                   |             |                                          |
| clpA   | ATP-dependent Clp protease ATP-binding subunit ClpA       |             |                                          |
| clpB   | ATP-dependent chaperone ClpB                               |             |                                          |
| pgtE   | Outer membrane protease PgtE                               |             |                                          |
| ythW   | Quercetin 2,3-dioxygenase YthW                             |             |                                          |
Figure 4. Differential expression of genes involved in cell redox homeostasis and various stress responses. The mean fold-increase in each gene expression after exposure to 3 mM of H$_2$O$_2$ compared with controls is indicated by the color scale bar. Note that a variety of biological functions including multiple stress response, DNA repair, protein protection, refolding and digestion, osmoprotectants, efflux, phosphate starvation, removal of reactive oxygen species, response to low Mg$^{2+}$ environment and programed cell death belong to a more general biological function called “Stress response”.

The most numerous group of altered operonsgenes in the transcriptome of oxidatively stressed S. Enteritidis cells was associated with various stress response processes as a distinct biological function indicated by the GO analysis. The greatest alteration in the entire transcriptome of S. Enteritidis under H$_2$O$_2$ stress was related to the ycfR gene. This gene, encoding the multiple stress resistance outer membrane protein YcfR, was induced 913-fold compared to that of the reference strain (Figure 4). The great induction exhibited the dps gene, which encodes the DNA-binding protein Dps that protects DNA. Besides dps, another group of genes encoding DNA repair proteins was significantly induced, including the DNA damage inducible yebG gene and the recAN, polB, dinPIG, and deoBCD genes (Figure 4). Similarly to genes encoding DNA repair proteins, a group of genes encoding molecular chaperones and proteases was significantly induced. Among this group of genes, the ibpBA gene,
encoding small heat shock proteins, showed the greatest induction, followed by molecular chaperones dnaKJ, yhhW, and clpAB, and protease pgtE (Figure 4).

The oxidative stress response regulon included upregulation of: Osmotically inducible genes envFR, osmYC, and yjbJ; efflux encoding genes yheR, yabL, yhcQP, dinF, and mdtAB; phosphate starvation inducible genes phoHE, yhBA, and phnTSUR; sensing and removal of ROS katG, ahpCF, yhaK, sodAC, soxSR, yphACDE, and yhcN; adaptation to low Mg\(^{2+}\) environment pagCDOK and mgtBC; and the programmed cell death inducible gene ybaJ (Figure 4).

The oxidative stress caused the induction of numerous operons/genes located on Salmonella pathogenicity islands (SPIs). The significant induction was observed for the ssrAB operon that positively controls the expression of the SPI-2 genes (Figure 5).

### Induced

| Gene   | Protein name                                      | Fold change | Biological function |
|--------|--------------------------------------------------|-------------|---------------------|
| ssaA   | Sensor kinase SsaA                                |             |                     |
| ssaB   | DNA-binding response regulator SsaB               |             |                     |
| ssaC   | Type III secretion system apparatus protein SsaC |             |                     |
| ssaD   | Type III secretion system outer membrane ring protein SsaD | |                     |
| ssaE   | Type III secretion system needle protein co-chaperone SsaE | |                     |
| ssaH   | Type III secretion system apparatus protein SsaH |             |                     |
| ssaI   | Type III secretion system inner rod protein SsaI |             |                     |
| ssaJ   | Type III secretion inner membrane ring protein SsaJ | |                     |
| ssaK   | Type III secretion system protein SsaK            |             |                     |
| ssaL   | Type III secretion system apparatus protein SsaL |             |                     |
| ssaM   | Type III secretion system protein SsaM            |             |                     |
| ssnA   | Type III secretion ATPase SsnA                    |             |                     |
| ssoQ   | Type III secretion system protein SsoQ            |             |                     |
| ssoV   | Type III secretion system export apparatus protein SsoV | |                     |
| sfaA   | Effector protein SfaA                             |             | Pathogenesis        |
| sfbB   | Effector protein SfbB                             |             |                     |
| sseAb  | Pathogenicity island II effector protein SseAb    |             |                     |
| sseBb  | Pathogenicity island II effector protein SseBb    |             |                     |
| sseJ   | Pathogenicity island II effector protein SseJ     |             |                     |
| sseF   | Pathogenicity island II effector protein SseF     |             |                     |
| sseL   | Secreted effector protein SseL                    |             |                     |
| sseE   | Pathogenicity island II effector protein SseE     |             |                     |
| spaQ   | Virulence associated secretory protein Spaq        |             |                     |
| spaS   | Surface presentation of antigens protein Spas      |             |                     |
| spaR   | Surface presentation of antigens protein Spar      |             |                     |
| spaO   | Surface presentation of antigens protein Spao      |             |                     |
| spaP   | Surface presentation of antigens protein Spap      |             |                     |
| iagB   | Invasion protein IagB                             |             |                     |
| sinH   | Inhibin protein SinH                              |             |                     |
| sinR   | Transcriptional regulator SinR                    |             |                     |
| scpE2  | Invasion-associated secreted effector protein ScpE2 | |                     |
| sicP   | Secretion chaperone SicP                          |             |                     |
| ymcC   | Colanic acid/biofilm transcriptional regulator YmcC | |                     |
| stbA   | Fimbrial major subunit StbA                       |             |                     |
| stbB   | Fimbrial chaperone protein StbB                   |             |                     |
| stbC   | Outer membrane fimbrial usher protein StbC        |             |                     |
| stbD   | Fimbrial protein StbD                             |             |                     |
| fimD   | Outer membrane usher protein FimD                 |             |                     |
| fimF   | Adhesin FimF                                     |             |                     |
| fimH   | Fimbrial adhesin FimH                            |             | Cell adhesion        |
| fimW   | Fimbriae W protein                               |             |                     |
| fimY   | Fimbriae Y protein                               |             |                     |
| sthA   | Fimbrial assembly chaperone SthA                  |             |                     |
| sthB   | Outer membrane fimbrial usher protein SthB        |             |                     |
| SEN4247| Fimbrial protein SEN4247                          |             |                     |
| sofA   | Fimbrial assembly chaperone SofA                  |             |                     |

Figure 5. Cont.
Indeed, a significant induction of the *ssaMCBDEVJKHILQN* operon encoding the type III secretion system (T3SS) apparatus of the SPI-2 as well as operons *sifAB* and *ssaABjBbFIE* encoding the SPI-2 effector proteins (Figure 5) was observed. Also, *spaQSROP* (translocon), *iagB*, *sinHR*, and *sopE2* (effectors) as well as *sicP* (effector chaperone) located on the SPI-1 were significantly induced (Figure 5). Not only genes encoding the T3SS apparatus and effector proteins responsible for the invasion of host cells and intracellular survival, but also genes encoding various cell adhesions were induced. This group of genes included *yjcC*, biofilm transcriptional regulator; *stbABCD*, *fimHYWDF*, *sfpAB* and SEN4247, fimbrial operons/genes; *safBCD* and SEN1978, pilin operon/gene as well as *csgABF*, curli operon (Figure 5).

Adaptation to oxidative stress involved induction of operons/genes that encode proteins associated with carbohydrate metabolism *yffR*, *ydfN*, *nagABCE* (glucosamine catabolic processes), *pduJKLMDQTNOPHUWSECVXG* (carboxysomes formation) and *yiaMOGN* (uptake of gluconate); amino acid metabolism *asnABC*, *yehEWX*, *dadAX*, *yefF*, *lysA*, *ynhHIG*, *ybaO*, and *lceCKGFMH* (uptake of branched-chain amino acids); nucleoside metabolism *safAB* (purine salvage pathway); outer membrane modification *pgp*, *nlpD*, *ybaN*, *wcaEFGHID* (colonic acid biosynthesis), *safA*, *ompX* (porin), and *ompA* (porin) and genes encoding proteins of unknown functions (Table S1).

### 3.3.2. Repression of Transcriptome—Molecular Response of *S. Enteritidis* to Oxidative Stress

There is a distinct segregation based on the biological functions between the induced and repressed transcriptome of oxidatively stressed *S. Enteritidis* cells. While the induced transcriptome showed a great association with the iron-sulfur cluster assembly, iron ion homeostasis, cell redox homeostasis, a variety of stress responses, pathogenesis and sessile lifestyle (cell adhesion), the repressed transcriptome was mainly associated with carbohydrate metabolic processes, ribosomal biogenesis, anaerobic respiration and cell motility (Figure 6).

**Figure 5.** Differential expression of genes involved in pathogenesis and cell adhesion. The mean fold-increase in each gene expression after exposure to 3 mM of H$_2$O$_2$ compared with controls is indicated by the color scale bar.

**Figure 6.** Gene ontology (GO) enrichment analysis. The figure portrays the most altered biological functions of *S. Enteritidis* during the H$_2$O$_2$ treatment. In the GO analysis were included operons/genes.
that showed the greatest alterations compared to their counterparts in the control sample. Each gene showed false discovery rate (FDR) $p < 0.05$ and high reproducibility across the biological replications.

The \textit{tdcA} gene, encoding a transcriptional activator for the \textit{tdcABCD} operon, showed the greatest repression, resulting in 131-fold downregulation compared to that of the reference strain (Figure 7). Indeed, other genes of the \textit{tdcABCD} operon, which are involved in L-threonine and L-serine catabolic processes, showed significant repression (Figure 7). Also, additional operons/genes associated with amino acid metabolism showed significant repression including \textit{yhdG} (amino acid permease), \textit{gcvTHR} (glycine, serine and threonine metabolism), \textit{pepETQD} (hydrolases of peptide bonds), \textit{selAB} (selenocysteine synthesis) and \textit{sdaBC} (serine uptake and degradation) (Figure 7).

| Gene     | Protein function/name                                      | Fold change | Biological function                  |
|----------|-----------------------------------------------------------|-------------|-------------------------------------|
| tdcA     | Transcriptional regulator TdcA                            | 131         | Amino acid metabolism               |
| tdcB     | Catalytic threonine dehydratase TdcB                      | 131         |                                     |
| tdcC     | Threonine/serine transporter TdcC                         | 131         |                                     |
| tdcD     | Propionate/acetate kinase TdcD                            | 131         |                                     |
| yhdG     | tRNA dihydrouridine synthase DusB                         | 131         |                                     |
| gcvT     | Glycine cleavage system protein T                          | 131         |                                     |
| gcvR     | Glycine cleavage system protein H                          | 131         |                                     |
| pepE     | Dipeptidase E                                            | 131         |                                     |
| pepT     | Aminopeptidase PepT                                       | 131         |                                     |
| pepQ     | Proline dipeptidase                                       | 131         |                                     |
| pepD     | Cysteine nonspecific dipeptidase                          | 131         |                                     |
| selA     | L-seryl-tRNA(Sec) selenium transferase SeLA               | 131         |                                     |
| selB     | Selenocystein-tRNA-specific translation elongation factor SeLB | 131         |                                     |
| sdaB     | L-serine ammonia-lyase                                    | 131         |                                     |
| sdaC     | HAAAP family serine/threonine permease SdaC              | 131         |                                     |
| gusD     | D-galacturonic deaminase                                  | 131         |                                     |
| gusP     | Galacturonic/glucarate/glycerate transporter GudP         | 131         |                                     |
| garD     | Galactarate dehydratase GarD                              | 131         |                                     |
| garL     | 2-dehydro-3-deoxyglucarate aldolase GarL                 | 131         |                                     |
| garK     | Glycerate 2-kinase GarK                                   | 131         |                                     |
| garR     | 2-hydroxy-3-exopropionate reductase GarR                  | 131         |                                     |
| evtS     | Ethanolamine utilization protein EutS                     | 131         |                                     |
| evtP     | Ethanolamine utilization protein EutP                     | 131         |                                     |
| evtQ     | Ethanolamine utilization protein EutQ                     | 131         |                                     |
| evtT     | Ethanolamine utilization protein EutT                     | 131         |                                     |
| nanA     | N-acetylasparaginase lyase                                | 131         |                                     |
| nanE     | N-acetylasparagine-6-phosphate 2-aminase NanE             | 131         |                                     |
| nanK     | N-acetylasparaginase kinase NanK                          | 131         |                                     |
| nanT     | Sialic acid transporter NanT                              | 131         |                                     |
| meA      | Alpha-galactosidase MeA                                   | 131         |                                     |
| meB      | Melibiose-sodium symporter MeB                            | 131         |                                     |
| meR      | Transcriptional regulator MeR                             | 131         |                                     |
| sghA     | PTS glucitol/sorbitol transporter subunit IIIC SghA       | 131         |                                     |
| sghR     | Glucitol operon transcriptional repressor SghR            | 131         |                                     |
| kgdT     | 2-keto-3-deoxyglucuronate permease KgdT                   | 131         |                                     |
| kgdK     | Ketonodeoxyglucokinas KgdK                                | 131         |                                     |
| idnD     | L-idonate 5-dehydrogenase IdnD                            | 131         |                                     |
| idnT     | Glucosate permease IdnT                                  | 131         | Carbohydrate metabolism             |
| gntR     | Transcriptional regulator GntR                            | 131         |                                     |
| gntT     | Glucosate transporter GntT                                | 131         |                                     |
| molB     | Malto ABC transporter permease MolB                       | 131         |                                     |
| molR     | Transcriptional regulator MolR                            | 131         |                                     |
| molK     | Malto/maltodextrin transport ATP-binding protein MolK     | 131         |                                     |
| molM     | Maltoase regulation protein MolM                         | 131         |                                     |
| molS     | Maltoase transporter permease MolS                        | 131         |                                     |
| ydeW     | Putative transcriptional repressor YdeW                  | 131         |                                     |
| ydeZ     | Sugar ABC transporter YdeZ                                | 131         |                                     |
| ydeY     | Sugar ABC transporter YdeY                                | 131         |                                     |

Figure 7. Cont.
Carbohydrate metabolism was the biological function most commonly affected within the repressed transcriptome. This group of operons genes, encoding a wider range of proteins, was involved in catabolic pathways, including guaDP (D-glucarate), garDLKR (galactarate), eutSPQT (ethanolamine), nanAEKT (sialic acid), melABR (melibiose), srlAR (sorbitol), kdgTK (D-glucuronate), idnDT, and gntRT (D-gluconate) (Figure 7). Besides this group of operons genes associated with catabolism, there was repression of operons genes encoding sugar uptake and anabolism such as malFTKMG (maltose), ydeWZYV (carbohydrate permease), yadBEI (a major carbohydrate active transport system), mglABC (galactose/methyl galactoside) and citCDEFD2E2 (citric operon involved in anabolic processes of the tricarboxylic acid cycle) (Figure 7).

Other central metabolic processes repressed during oxidative stress of S. enterica included anaerobic respiration (napABCDFGH, dmsA2A3A1AB1B, glpABCFTXK, frdABC, nrfABCD, hybABCFG, deuABC), ribosomal biogenesis and rRNA processing (yjjRO, rpsUTPLGOSCFH4JQBR, rplUKSNNXVBCDAEPLWYM, rimJMK, rpmAGBHC), fatty acid metabolic processes (accBD, fadHRL, fabGIBAH), vitamin biosynthesis (pocR, ynfKA, cbmADEF, menDAB, yfbBSI), ATP synthesis (atpBIEHFGA), cellular protein modification and transport processes (hypAODBCE and oppCBD), aerobic respiration (yhhUTCSQPQV), the ethanolamine catabolic process (eutSPQT), the carnitine metabolic process (caiFEA) and the pyrimidine nucleoside biosynthetic process (pyrHDLE) (Figure 6).

3.4. Validation of RNA-seq Data by Real-Time PCR

To validate the RNA-seq data, the expressions of nine randomly chosen genes were tested by quantitative real-time PCR (qRT-PCR) under the same experimental conditions. The qRT-PCR showed the same pattern of expressions, including seven upregulated genes (dinP, dnal, eutC, malA, osmY, pagO, and spaR) and two downregulated genes (malK and nrdD) (Figure 8). It can be observed that generally, the expression values measured by qRT-PCR were greater compared to those measured by RNA-seq analysis. The difference in expression values between these two techniques is consistent with the fact that qRT-PCR generally gives higher expression values compared to RNA-seq [17,22].
was proportional with \( \text{H}_2 \) physiological characteristics of this pathogen. In this study, it was shown that incrementally increased antioxidative stress or Fe-limited inducible system [35]. Indeed, the transcriptomic analyses showed that the ISC system is a housekeeping system [34], while the SUF system was shown to be an inducible system. In this study, the SUF system showed a significantly greater induction than the ISC system. This finding correlates with previous studies that pointed out that the ISC is a housekeeping system [34], while the SUF was shown to be an inducible system. In general, there are two Fe-S cluster formation systems, the ISC system and the SUF system [33]. In this study, the SUF system showed the greatest change. The Fe-S clusters are cofactors of proteins and they are involved in various critical metabolic processes including DNA replication, regulation, and repair, substrate uptake, respiration, and RNA modification [33]. In general, there are two Fe-S cluster formation systems, the ISC system and the SUF system [33]. In this study, the SUF system showed the greatest gene expression alteration, either induction (iroN) or repression (pocR, ompF and dps). Recently, Mitosch et al. [30], using a dual-reported model, demonstrated the importance of timing of the gene expression during exposure of the microbial cell to an oxidative agent. Besides the importance of temporal gene expression response, this study points to a multifaceted effect of oxidative agent concentration on gene expression during oxidative stress.

The comparative transcriptomic analyses revealed that the expression of 45% of the S. Enteritidis genome was altered at least two-fold upon exposure to \( \text{H}_2\text{O}_2 \). This finding showed that oxidative stress has a much greater impact on the physiology of this pathogen than previously anticipated [18,31,32]. Out of numerous biological functions altered by oxidative stress, the SUF mechanism of Fe-S cluster formation exhibited the greatest change. The Fe-S clusters are cofactors of proteins and they are involved in various critical metabolic processes including DNA replication, regulation, and repair, substrate uptake, respiration, and RNA modification [33]. In general, there are two Fe-S cluster formation systems, the ISC system and the SUF system [33]. In this study, the SUF system showed a significantly greater induction than the ISC system. This finding correlates with previous studies that pointed out that the ISC is a housekeeping system [34], while the SUF was shown to be an oxidative stress or Fe-limited inducible system [35]. Indeed, the transcriptomic analyses showed the

4. Discussion

Salmonella Enteritidis, one of the most important non-typhoidal Salmonella serovars from the public health and veterinary medicine perspectives [23–26], has developed a robust anti-oxidative response not only to survive endogenous oxidative stress [27] but also to survive exogenous stress imposed by a host [28] or environment [29]. In other words, for successful host invasion, intracellular proliferation and/or environmental survival, possession of an efficient oxidative stress response is one of the key physiological characteristics of this pathogen. In this study, it was shown that incrementally increased concentrations of oxidative agents caused various patterns of gene expressions. While inductions of genes encoding antioxidant (thioredoxin 2) and DNA replication regulator (NrdM) were constant regardless of \( \text{H}_2\text{O}_2 \) concentrations, induction of a gene encoding iron/manganese transporter, SitA, was proportional with \( \text{H}_2\text{O}_2 \) concentrations. In contrast to the pattern of sitA induction, the ycfR gene, encoding multiple stress-response protein, exhibited disproportionate induction regarding \( \text{H}_2\text{O}_2 \) concentration. The greatest concentration of \( \text{H}_2\text{O}_2 \) resulted in the lowest ycfR induction. Besides these three patterns of gene expressions, the most common gene expression alteration during this assay was associated with an unpredictable pattern, where the middle concentration of \( \text{H}_2\text{O}_2 \) caused the greatest gene expression alteration, either induction (iroN) or repression (pocR, ompF and dps). Recently, Mitosch et al. [30], using a dual-reported model, demonstrated the importance of timing of the gene expression during exposure of the microbial cell to an oxidative agent. Besides the importance of temporal gene expression response, this study points to a multifaceted effect of oxidative agent concentration on gene expression during oxidative stress, further indicating another dimension of cellular adaptation complexity to oxidative stress.
significant induction of numerous Fe acquisition mechanisms, including an iron/manganese transporter, ferrichrome, hemin, ferroxamine, ferric coprogen and ferric-rhodotorulic acid uptake systems, then biosynthesis and uptake of salmochelin, enterobactin, as well as hydroxamate siderophores, clearly indicating a state of Fe-limitation in oxidatively stressed cells. These data showed the activation of the various Fe-acquisition systems in S. Enteritidis cells under oxidative stress, further suggesting their critical importance in cellular adaptation to oxidative stress.

Hydroxyl free radical, a product of the Fenton reaction, represents the most damaging compound during H$_2$O$_2$ treatment [36]. This highly reactive oxygen species interacts with numerous biomolecules further causing multiple effects on the treated procaryotic cells [37]. The comparative transcriptomics analyses showed multifaceted responses of oxidatively treated cells including induction of not only the OxyR (katG, ahpCF) and the SoxRS (soxR, soxS, sodA, sodC), two oxidative stress regulons, but also stress responses involved in DNA and protein repairs, osmoprotection, multidrug efflux, phosphate and magnesium starvation. During the oxidative stress response, this human pathogen induced a robust DNA repair system, which included base repair (deoB), single base mismatch repair (dinIG), early double-strand break repair (yebG), multiple double-strand breaks repair (recAN) and DNA replication (polB, dinP). Also, the dps gene which encodes a multifunctional Dps protein was greatly induced. Dps non-specifically binds DNA, forming a highly stable Dps-DNA nucleoprotein complex which further protects DNA from the deleterious effects of ROS [38]. Similarly to DNA, protein repair systems were induced. The ibpAB genes, encoding small heat-shock proteins IbpA and IbpB, showed the greatest induction among various protein repair systems. Induction of genes encoding molecular chaperones (dnaK), clpB and proteases (clpA, pgtE) indicates a tendency of proteins to aggregate or to undergo a process of denaturation during oxidative stress. Multifaceted responses of oxidatively treated cells was also reflected by induction of key genes associated with osmotic stress (osmYC, envFR), manganese starvation (sitABCD, pagCDOK, mgtBC), phosphate starvation (phoEH, yjbA, phnTSUR), multidrug efflux (mdlAB, yhcQP) and outer membrane modification (wcaEFH, yncCJ, yjbAEH). Adaptation of S. Enteritidis to oxidative stress was not only confined to the induced transcriptome but it also included the repressed transcriptome. The significant repression of the major small (rpsUTPLGSOCHJQR) and large (rplUKJSNXVBCDAEPLWYW) units of ribosomal encoding genes indicates that oxidatively stressed cells activated a stringent response. The main purpose of a stringent response is saving cellular energy and precursors required for ribosomal synthesis. If cells continue to synthesize ribosomes, the majority of these organelles would end up being inactive due to the disruption of central metabolic pathways (i.e., lack of amino acids) and this would be an unnecessary and costly expenditure of resources [37].

Besides the alterations of a large pool of genes encoding various central metabolic and stress response pathways, the comparative transcriptome analyses revealed a clear division, during the H$_2$O$_2$ treatment, in the gene expressions associated with the genes encoding for motility (planktonic) and the adhesion genes (sessile) forms of life. The exposure of S. Enteritidis to oxidative stress had an extensive and significant positive effect on the expression of adhesion encoding genes, including fimbrial stbABCD, fimHYWDF, sthB, SEN4247; curli csgABF, and pilin safBCD, SEN1978 (type IV pilin), yibEHG and yncC operons and genes. At the same time, it negatively affected expressions of the major flagellar operons flgHECFGJDBMN and fljMRLNKFOPOHEBG clearly indicating a switch from a planktonic to a sessile (i.e., biofilm) life forms of oxidatively treated cells. Recently, it was shown that a transition from planktonic to sessile life form in S. Enteritidis is followed by repression of the major flagellar operons and induction of numerous fimbrial, curli, and pili operons [22]. This transcriptional change is in agreement with the “swim-or-stick” theory, which postulates that motility and biofilm development are mutually-exclusive processes [39]. Our transcriptomic data indicate that oxidatively stressed cells transition from a planktonic to a biofilm form of life. Another important feature of oxidatively stressed cells was an extensive induction of salmonellae-essential virulence genes located on SPI 1, SPI 2, and SPI 5. Induction of core virulence genes involved genes encoding not only T3SS apparatus of the SPI 2 and translocan of SPI 1 but also numerous effectors (sifAB, sseA,bBFIE, iagB,
sinHR, sopE2, sicP) essential for intracellular survival of this pathogen [40]. Fu and colleagues [18] studying the oxidative stress response of S. Typhimurium found that this organism repressed the synthesis of core virulence proteins encoded on SPI 1. Discrepancy between their and our results can be most likely explained by different experimental setups. While Fu and colleagues [18] exposed the cultures of S. Typhimurium to H$_2$O$_2$ at their mid-exponential growth phases (0.5 at OD$_{600}$) and harvested them at their early stationary growth phase (0.9 at OD$_{600}$), we started H$_2$O$_2$ treatment at the mid-exponential growth phase (0.4 at OD$_{600}$) and harvested the cultures at their still exponential growth phase (~0.5 at OD$_{600}$). Taken together, our experimental approach ensured that all transcriptional changes will be consequences of oxidative stress, while the approach employed by Fu et al., [18] reflects not only oxidative stress response but additional stress responses associated with the stationary growth phase of this organism [41,42].

5. Conclusions

This study employing a combination of incremental oxidative stress assays and the next generation sequencing approach revealed additional levels of the oxidative stress response complexity in non-typhoidal Salmonella. It was shown that the oxidative stress response is intricately linked with not only Fe-S cluster formation, iron homeostasis, DNA repair, and cell redox homeostasis but also with heat, osmotic, stringent, manganese and phosphate starvation stress responses as well as multidrug efflux and decreased passive influx of small hydrophilic molecules. In addition to these multifaceted cellular adaptation processes, it was shown that S. Enteritidis during oxidative stress simultaneously repressed the key motility encoding genes and massively induced the essential adhesion and salmonellae core-virulence encoding genes, crucial for the biofilm formation and host cell invasion. Based on these data, we hypothesize that the oxidative stress response of non-typhoidal Salmonella is closely associated with intracellular survival and that oxidative stress may act as a stimulus for eukaryotic cell invasion and formation of Salmonella-containing vacuoles. It has been already found that intracellular biofilm formation plays an important step in virulence of uropathogenic Escherichia coli, another facultative intracellular pathogen [43], suggesting that a similar approach can be explored by non-typhoidal Salmonella.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3921/9/9/849/s1, Figure S1. The effect of different H$_2$O$_2$ concentrations on the survivability of S. Enteritidis over 90 min. Error bars correspond to the standard deviations. Table S1. List of induced genes during the 3 mM H$_2$O$_2$ treatment of the S. Enteritidis ATCC 13076 strain compared to the control. Table S2. List of repressed genes during the 3 mM H$_2$O$_2$ treatment of the S. Enteritidis ATCC 13076 strain compared to the same H$_2$O$_2$ untreated strain.

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