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Transcriptome sequencing reveals genes and adaptation pathways in *Salmonella* Typhimurium inoculated in four low water activity foods

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ABSTRACT

Salmonella enterica serotypes have been reported as the agent of various outbreaks occurred after the consumption of low water activity (a_w) foods. When the pathogen encounters harsh conditions, several regulatory networks are activated through dynamic differential gene expression that lead to cell survival for prolonged periods. In this work, the transcriptome of *S. enterica* serovar Typhimurium using RNA-Seq, after cells' inoculation in four distinct types of low a_w foods (milk chocolate, powdered milk, black pepper, and dried pet food), following storage at 25 °C per 24 and 72 h was studied. The findings of this study suggest that gene regulation is influenced by the food composition mainly in the first 24 h post-inoculum, proceeded by the induction of similar genes shared among all samples. It was possible to evaluate the differences on each type of food matrix regarding the bacteria adaptation, as well as the similarities provoked by low a_w. The results reveal genes that may play key roles in response to desiccation in *Salmonella*, as well as the pathways in which they are involved.

1. Introduction

Salmonella enterica serotypes are amongst the most common causes of food-related diseases in the world. It is estimated that *S. enterica* is responsible for causing 94 million of diseases per year globally, with more than 80 million cases attributed to the consumption of contaminated food (Majowicz et al., 2010). Foodborne salmonellosis is also the leading cause of hospitalizations and an estimated number of 155,000 deaths per year (Majowicz et al., 2010; Scallan et al., 2011). Foodborne salmonellosis outbreaks are associated mainly with the consumption of raw or undercooked foods of animal origin, but recently there has been an increased link of salmonellosis with low a_w products (Podolak et al., 2010). Several reports in the last decades have confirmed *Salmonella* outbreaks involving the consumption of such products, including almonds, chocolate, peanut butter, infant formula, dried pet foods, red and black peppers, among others (CDC, 2016; Finn et al., 2013a).

The presence of *Salmonella* in foods is a cause of significant concern for public health. Besides, the presence of this bacterium can be even more concerning depending on the food composition and the host susceptibility. In some instances, even the consumption of foods contaminated with low numbers of *Salmonella* can be sufficient to cause an infection (D'Aoust et al., 1975; De Jong and Ekdahl, 2006). For example, in a salmonellosis outbreak associated with chocolate consumption in Canada, it was reported that the product samples presented *Salmonella* concentrations as low as 0.005 CFU/g (Komitopoulou and Peñaloza, 2009). Also, most low a_w foods do not require cooking steps for their consumption and have a long shelf life. These two characteristics, combined with low levels of *Salmonella* commonly found in low a_w foods, may lead to the occurrence of extended outbreaks as the detection of low levels of pathogens and sampling plans become a major challenge (Santillana Farakos et al., 2013). Thus, for microorganisms whose infective dose can be low, even small populations may result in considerable risks for consumers (Finn et al., 2013a).

Salmonella is resistant to several stress conditions, including those employed during food processing such as heating and dehydration. On the one hand the thermal inactivation kinetics of *Salmonella* has been widely studied and understood (Doyle and Mazzotta, 2000; Ng et al., 1969; Van Asselt and Zwietering, 2006), on the other hand, the survival and persistence of this bacterium under desiccation conditions, mainly from molecular point of view, is still not well established. The exposure of *Salmonella* cells to sublethal conditions, such as found in dried foods, may lead to adaptations that influence subsequent survival, persistence and enhanced pathogenicity (Wesche et al., 2009). Studies have shown that, for instance, the reduction of a_w may increase the resistance of *Salmonella* to heat and that the cells remain in a state of low metabolic activity that is resumed upon return to favorable environmental growth conditions (Podolak et al., 2010). In addition, the composition of the

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food matrix and the presence of solutes may aid in the resistance of the microorganism, such as in fat-rich foods, in which a synergistic effect is observed culminating in the survival of *Salmonella* at the extreme acid conditions of the host's stomach (Krapf and Gantenbein-Demarchi, 2010). A better understanding of *Salmonella* response to desiccation is critical for food processing as the knowledge can be used for the development of effective control strategies.

As of the overall gene expression under desiccation may help to elucidate the adaptations that lead to the physiological changes necessary for *Salmonella* cells survival. A few transcriptome studies of microorganisms have been conducted in food matrices, most of them using microarrays, to identify differentially expressed genes (Bassi et al., 2016; Bergholz et al., 2009; Cretenet et al., 2011; Fratamico et al., 2011; Liu and Ream, 2008; Makhzami et al., 2008). Excitingly, the advent of RNA-Seq and bioinformatics approaches have recently allowed many genes to be identified in *Salmonella*, many of which are induced after exposure of the cells to various stress conditions (Amin et al., 2016; Deng et al., 2012; Kröger et al., 2013). Previous work investigated the transcriptome of *S.* Enteritidis in peanut oil (a_w 0.3), using RNA-Seq (Deng et al., 2012). Other studies analyzed *Salmonella* desiccation responses in plastic or stainless steel surfaces, and different results regarding the induced genes were reported (Finn et al., 2013b; Gruzdev et al., 2012).

Given the above and considering the significant differences in the gene expression profile depending on the conditions the cells are subjected, this study was conducted aiming to expand the knowledge of *Salmonella* response to desiccation using different low a_w food products. The study was designed to analyze the similarities and differences in the patterns of gene expression by *Salmonella* according to the combination of the food composition and low a_w. Therefore, four types of low a_w foods, with distinct levels of fat, sugar, proteins and other nutrients were selected objecting to analyze *Salmonella enterica* serovar Typhimurium strain SL1344 responses through RNA-Seq. The food products included milk chocolate, powdered skim milk, dried black pepper and dried dog food, all of them previously involved in *Salmonella* outbreaks. Sequencing was performed in transcripts obtained from *S.* Typhimurium SL1344 cells after inoculation in food products and storage for 24 h and 72 h. To our knowledge, bacterial transcriptome investigation of food matrices through the use of RNA-Seq was employed in only two studies. Furthermore, one of the studies did not use the final product but a raw material in liquid form to inoculate the bacteria (peanut oil), and the other used a liquid matrix (beer) (Bergsveinson et al., 2016; Deng et al., 2012). Thus, our study is unprecedented and aims to clarify the central adaptation pathways in *S.* Typhimurium SL1344 cells within complex, solid food matrices, through a global analysis of gene expression.

2. Materials and methods

2.1. Strain and growth conditions

S. enterica serovar Typhimurium strain SL1344 was grown and maintained on Luria-Bertani (LB) agar (Tryptone 1%, NaCl 1%, Yeast Extract 0.5%, Agar 1.5%). SL1344 was chosen for this study for three main reasons: i) Several strains of *S. enterica* were submitted to desiccation, and SL1344 showed a high survival rate, being recovered in selective medium [Xylose Lysine Deoxycholate (XLD) agar] after more than 90 days of desiccation in polystyrene plates (data not shown); ii) this strain has already been studied regarding other stress responses such as acid stress, desiccation, heat, exposure to sanitizers (Greenacre et al., 2006; Gruzdev et al., 2011; Pin et al., 2012), some of which may be related to the responses also encountered in low water activity foods; iii) the serovar Typhimurium is a relevant model to be studied in food matrices, as it represents one of the leading causes of human gastroenteritis and invasive non-typhoidal salmonellosis in the world (Amin et al., 2016; Ferrer-Navarro et al., 2016; Spector and Kenyon, 2012).

In order to prepare the cell inoculum, a fresh overnight-grown culture was streaked onto LB Agar plates (2% agar, to facilitate cell scraping) and grown overnight at 37 °C. On the next day, cells were harvested using sterile saline solution (NaCl 0.9%) with a sterile cell scraper and washed three times in sterile saline solution (4000 × *g* for 5 min) at room temperature. Washings were discarded, and the tubes were inverted and left to drain briefly on sterile, absorbent paper, before inoculum on the respective food matrices. These drained cells were used for inoculum as well as control sample. A final average concentration of 10^6 CFU/g of sample was obtained.

2.2. Food preparation and inoculation

Four low a_w food products were inoculated with *S.* Typhimurium SL1344: milk chocolate, black pepper, powdered skim milk, and dog food. All foods were purchased from a local market in Campinas, São Paulo (Brazil). The products were previously analyzed to ensure the absence of *Salmonella* spp. employing the ISO 6579 method (ISO 6579:2002(E), 2002). The inoculation procedure of the foods was generally similar. A total of 20 g of each food sample was inoculated with *Salmonella* cells obtained from two scraped Petri dishes, in triplicate. The samples were then incubated at 25 °C for 24 h, and 72 h and the relative humidity was kept at 40% during the storage period. Dehydration conditions were determined based on a previous *S.* Typhimurium study, with an extension of incubation time up to 72 h to increase results reliability (Gruzdev et al., 2012). The temperature used in this study was determined to be similar to those from grocery stores and consumer's home storage, *i.e.*, 23-25 °C.

For the milk chocolate, the inoculum was performed according to Kotzekidou et al. (2008) with modifications (Kotzekidou et al., 2008). Briefly, milk chocolate pieces (composition: sugar, whole milk powder, cocoa butter, cocoa liquor, vegetable fat, lactose, cocoa powder, dehydrated butterfat, emulsifying soy lecithin and polyglycerol polyricinoleate and flavoring – a_w = 0.6) were melted in a water bath at 80 °C, chilled to 35 °C and inoculated with *Salmonella*. The powdered skim milk (composition: skim milk, minerals, and vitamins) inoculated with *Salmonella* were placed on the bottom of sterile Petri dishes using sterile filter paper as lids and dried at 37 °C until the a_w reached the original value (final a_w = 0.4). Whole *Piper nigrum* fruits were washed for 1 min on 70% ethanol, 30 s on 200 ppm sodium hypochlorite followed by three washing in distilled water. After 15 min drying at 37 °C, whole pepper grains were inoculated with *Salmonella*. The mixtures were placed in sterile Petri dishes covered with sterile filter paper aiming to allow water evaporation. The pepper grains were dried at 50 °C for 18 h (final a_w = 0.5). Sterilized dried dog food (composition: crude protein, ethereal extract, fiber, minerals, amino acids, fructans) was macerated before inoculation with *Salmonella*. The mixtures were placed on the bottom of sterile Petri dishes using sterile filter paper as lids and dried at 37 °C until reached the original a_w (a_w = 0.6). After inoculation with *Salmonella*, the food samples were placed in sterile Petri dishes, following storage in a Biochemical Oxygen Demand (BOD) incubator at conditions described above. All the samples were stored in the same chamber to avoid temperature and RH variations. Colony count was *performed using* Xylose Lysine Deoxycholate agar (XLD) (Thermo Fisher Scientific, MA, USA) after each condition and control samples.

2.3. RNA isolation

Total RNA from samples (control and food samples) was isolated using the PowerMicrobiome™ RNA Isolation Kit (MO BIO Laboratories, CA, USA), following manufacturer's instructions. This kit was chosen due to the good performance on samples containing high PCR inhibitors (such as food matrices that may present inhibitors like polysaccharides, xylan, proteases, calcium ions, among others), along with efficient extraction of tough bacteria during the bead beating step.

RNA concentration and quality were evaluated using the spectrophotometer Biospec-Nano (Shimadzu Corp., Kyoto, Japan) and stored at −80 °C. For shipment to the sequencing company, RNAs were previously ethanol–precipitated. For precipitation, 0.1 volume of 3 M Sodium Acetate (NaOAc, pH5.5) was added to the RNA solutions and mixed gently, followed by the addition of two volumes of 100% ethanol per sample. NaOAc solution was prepared using DEPC-treated water. Samples were shipped with blue ice packs.

2.4. Sequencing and bioinformatics analyses

RNA-Seq was performed to analyze *Salmonella* differential responses after inoculum in different, low a_w food matrices for 24 h and 72 h. Before mRNA sequencing, RNA integrity was accessed using a 2100 Bioanalyzer (Agilent Technologies, CA, USA) and rRNA were depleted with the Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina, CA, USA). Transcripts were sequenced at Macrogen Inc (South Korea) using TruSeq stranded total RNA library preparation with bacterial rRNA depletion, and Illumina NovaSeq 100bp paired-end lane. Sequencing provided ~40 million reads per sample. FastQC was used to check quality on the raw sequences before analysis to confirm data integrity. Trimming was performed using the program Trimmomatic. Paired-end sequences were mapped to *Salmonella* Typhimurium SL1344 available genome (RefSeq assembly accession GCF_000210855.2) using Bowtie (Langmead et al., 2009). HTSeq was used in the RNA-Seq data for differential expression analysis by counting the overlap of reads with genes (Anders et al., 2014). Reads were normalized using the RPKM approach. Gene expression was analyzed by comparison between treatment and the control (overnight grown scraped cells). Genes were defined as up- or down-regulated when they presented at least 2-fold difference ($p < 0.05$) to the control.

For all the samples, if more than one RPKM value was 0, it was not included in the analysis. Therefore, from a total of 5092 genes, 149 were excluded, and 4943 genes were used for statistical analysis. To facilitate log2 transformation, +1 was added to the raw signal (RPKM). This process was performed because raw signals are scattered along the wide range, and most signals are concentrated on the low signal value, so log transformation reduces the range of the signals and produces more even data distribution. After log transformation, to reduce systematic bias, quantile normalization was used with “preprocessCore” library (https://www.bioconductor.org/packages/release/bioc/html/preprocessCore.html).

Gene ontology analysis was performed using the UniProt mapping tool (http://www.uniprot.org/mapping/), and gene-set enrichment analysis was performed based on the KEGG database (http://www.genome.jp/kegg/). The data were submitted in the BioProject database under BioProject ID: PRJNA490179 (http://www.ncbi.nlm.nih.gov/bioproject/490179).

2.5. Quantitative real-time PCR (RT-qPCR)

RT-qPCR was performed to confirm RNA-Seq data. The reactions were prepared using DNAse-treated RNA and the iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad, CA, USA), according to the manufacturer's instructions. Six genes were selected to validate the RNA-Seq results: *cspA, cspJ, arfA, trbB, pilP* and *cspH*. Glucose-6-phosphate isomerase (*pgi*) was used as a control to normalize the values. The analysis was performed for three biological replicates from each sample in a 384-well plate containing 5 μL of iTaq universal SYBR® Green reaction mix, 0.125 μL of the iScript reverse transcriptase, 300 nM of each forward and reverse primers, 45 ng of RNA and nuclease-free water to a total reaction mix volume of 10 μL. Primers for qRT-PCR were designed using Primer3Plus (Untergasser et al., 2007). The sequences are presented in the Supplementary table. Reactions were run in technical triplicates with the CFX384 Touch Real-Time PCR Detection System (Bio-Rad, CA, USA), using the following program: initial reverse transcription reaction at 50 °C for 10 min, polymerase activation, and DNA denaturation at 95 °C for 1 min, followed by 40 cycles of amplification at 95 °C for 10 s and 60 °C for 15 s. A control without reverse transcriptase was included. The specificity of the amplification was verified using melting curves. Gene expression was calculated via the Delta-Delta cycle threshold method (Schmittgen and Livak, 2008). All statistical comparisons were performed using Student's t-test ($P < 0.05$).

3. Results and discussion

3.1. Determination of viable S. Typhimurium counts and RNA isolation

Following the incubation times of 24 h and 72 h, colony-forming units (CFU) were counted for each sample. Accordingly, the initial CFU/g (time = 0) were: i) black pepper 8.0×10^6, ii) chocolate 2.4×10^6 CFU/g, iii) dried pet food 6.8×10^6 and iv) powdered milk 2.3×10^6. After 24 h and 72 h incubation, the CFU/g were (respectively): i) black pepper 9.1×10^2 and 9.7×10^2, ii) chocolate 9.2×10^3 and 6.0×10^3, iii) dried pet food 4.5×10^5 and 3.2×10^5, iv) powdered milk 2.8×10^4 and 5.5×10^3.

After RNA isolation, absorbance ratios at 260 and 280 nm (A260/280) and 260 and 230 nm (A260/230) were measured and the sample values were in the range of 1.9–2.1 and 2.0–2.3, respectively. The RNA Integrity numbers (RIN) were measured prior to RNA-Seq library preparation and were all in the range of 5.6–6.1. These RIN might seem low but are inherent to the strain used in this work, due to intervening sequences in the rRNA genes (Bhagwat et al., 2013).

3.2. Sequencing summary

To map cDNA fragments obtained from RNA sequencing, GCF_000210855.2 was used as a reference genome. *S.* Typhimurium SL1344 behavior was analyzed when cells were inoculated in different food samples and stored for 24 h and 72 h. An average of 30 million processed reads was obtained per sample. Trimmed data presenting quality score (Q-score) of Q30 were mapped to the reference genome, with an average mapping ratio varying from 20% to 78%. Some low mapping data are believed to be due to the nature of the samples, including contaminant RNA from the food matrix itself and technical difficulties in isolating high-quality RNA from these complex environments. Also, rRNA depletion might not be entirely well succeeded, remaining contaminant rRNA, especially in the 24 h samples. This may have occurred because the first 24 h represented an adaptation period in which the cells were actively translating essential molecules for survival in the unfamiliar environment, still demanding significant levels of rRNA. As the rRNA genes are distributed in repeats over the genome, many reads were suppressed by multiple mapping, as suggested by the results. Despite the low mapping rates, the sequencing data were confirmed by qPCR, and therefore the Seq-RNA results are reliable. Table 1 shows the statistics obtained from Bowtie aligner. Read count per gene was extracted from known gene annotations with HTSeq program.

3.3. Overall differential gene expression (DGE) analysis

The present work focused on comparisons of *S.* Typhimurium gene expression based on the food type where they were inoculated, where all the products share one common attribute - the low water activity. In addition, only the first 72 h after inoculation were analyzed, an interval that is not representative of the long-term survival of *Salmonella.* Nonetheless, the 72- h period was sufficient to show important differential gene expression related to adaptations that might lead to long-term survival and/or resistance. As previous RNA-Seq works with *Salmonella* related to desiccation were only performed in abiotic surfaces and the only published research using a low water activity food

Table 1
Mapped data statistics. CTRL = control; BP24 = black pepper 24 h-storage; BP72 = black pepper 72 h-storage; CH24 = chocolate 24 h-storage; CH72 = chocolate 72 h-storage; DF24 = dog food 24 h-storage; DF72 = dog food 72 h-storage; PM24 = powdered milk 24 h-storage; PM72 = powdered milk 72 h-storage.

Sample ID	# of processed reads	# of mapped reads	# of failed to align reads	# of suppressed reads by multiple mapping
CTRL	30,682,098	23,961,598 (78.10%)	5,478,505 (17.86%)	1,241,995 (4.05%)
BP24	27,167,441	6,873,060 (25.30%)	11,403,840 (41.98%)	8,890,541 (32.72%)
BP72	28,785,724	7,489,809 (26.02%)	7,172,195 (24.92%)	14,123,720 (49.07%)
CH24	38,536,577	7,585,312 (19.68%)	9,016,534 (23.40%)	21,934,731 (56.92%)
CH72	27,661,414	17,638,512 (63.77%)	9,450,367 (34.16%)	572,535 (2.07%)
DF24	34,258,058	7,678,453 (22.41%)	8,760,300 (25.57%)	17,819,305 (52.01%)
DF72	27,521,949	17,353,449 (63.05%)	9,236,372 (33.56%)	932,128 (3.39%)
PM24	31,369,082	22,244,712 (70.91%)	8,328,777 (26.55%)	795,593 (2.54%)
PM72	27,276,757	20,238,175 (74.20%)	6,681,723 (24.50%)	356,859 (1.31%)

matrix was a liquid oil (peanut oil), we decided to extend this knowledge to inoculation in solid, complex food matrices, represented by final products that have already been related to *Salmonella* outbreaks. It is important to note, however, that our experimental design considered a simulation of contamination after processing and in very high levels (10^6 CFU/g), which may not reflect the real-world scenario.

3.3.1. Hierarchical clustering analysis

Using each sample's Log2 (RPKM + 1) value, the high expression similarities were grouped (Fig. 1A). Accordingly, the heatmap shows the results of hierarchical clustering analysis which clusters the similarity of genes and samples by expression level (normalized value) (Fig. 1B).

The analysis shows a grouping pattern most related to the time of sample storage. When comparing all the samples and control, there were two main groups, one of which represents the control and samples stored for 24 h and the other group containing samples stored for 72 h. Interestingly, the only exception was the cells present in powdered milk for 24 h, which remained clustered with the 72 h samples. Therefore, it can be deduced that the powdered, nonfat milk represents the most distinct environment compared to milk chocolate, black pepper, and dog food and may have induced stress response pathways more quickly than the other samples. Bearing in mind that all the food samples in this study present low a_w activity, the fact that the skim powdered milk is the only sample that does not contain any fat (0%, according to the manufacturer information) may be the reason for such contrast. Accordingly, previous studies have shown that fat content can be pivotal to the survival and adaptation of *Salmonella* to harsh conditions, acting as a protective factor against stresses like heat, desiccation and gastric acid (Aviles et al., 2013; Finn et al., 2013a).

3.3.2. Significant gene results

Considering the inoculated cells compared against the control and an $|FC| > = 2$, there was an average of 344 upregulated genes and 393 downregulated genes for 24 h stored food samples. As for 72 h stored samples, there was an average of 685 upregulated genes and 800 downregulated genes. In this section, an $|FC| > 2$ ($p < 0.05$) will be considered for overall significant differential gene expression, and $|FC| > 5$ ($p < 0.05$) will be used to discuss the most up- and downregulated genes. A complete list of up- and downregulated genes is shown in the *Supplementary table*.

Considering DGE after 24 h of storage, it was found that only eight were upregulated in all four different food samples (Fig. 2). The eight genes are represented by four hypothetical proteins, a xenobiotic-response family transcriptional regulator, a chaperone encoded in the pathogenic island 2, a plasmidial conjugal transfer protein and a cytochrome c protein related to oxidative response. Such a result reinforces the adaptation process to the food matrices, as most DGE's are specific for each type of sample, which were stored at the same conditions after inoculation and desiccation. In the case of the black pepper, the high temperature (50 °C) used previously during the desiccation might also have influenced the pattern of gene expression in the first 24 h. In addition, there were no groups of upregulated genes with $|FC| > 5$ shared among all food samples at 24 h storage. On the other hand, for the 24 h storage downregulated genes there were two genes shared among all samples and with an $|FC| > 5$. Interestingly, these two genes code cold-shock proteins and were also identified as being downregulated in all the 72 h samples (Table 2).

Cold-shock proteins are expressed under normal conditions and usually are upregulated when a temperature drop occurs. Studies suggest they are chaperones that act unfolding mRNA molecules thus promoting read-through transcription and may be required for optimal cell growth (Zhang et al., 2018). In the present study, the incubation temperatures were set at 25 °C, and the continuous expression of such genes significantly decreased when compared to control (cells were grown at 37 °C). The adaptation of the cells to the new environments (food matrices) might have activated response pathways that lead to the downregulation of these genes. The results reveal that the first 24 h after contamination comprise a period of adaptation, in which the gene responses appear to be more related to the food matrices than to the diminution of water availability (Fig. 2A and B).

As shown in Fig. 2, there was a significant increase in the number of upregulated genes that were shared among the four food samples, totaling 309 genes ($|FC| > 2$) after 72 h of storage. Unlike the 24 h-storage, among the 72 h-storage samples, the differential gene expression appears to be more related to the desiccation response, given the high number of upregulated sequences shared among all samples. Strikingly, some transfer RNAs (tRNAs) were upregulated in all the samples. This might be related to a reduction in the efficiency of the post-transcriptional process due to desiccation. Up-regulation of tRNAs were also observed and discussed in a previous study on *S.* Typhimurium desiccation responses (Maserati et al., 2017).

For 72-h samples, GO analysis on the 309 identified sequences shows the following results (for molecular function): catalytic activity > binding > transporter activity > DNA binding transcription factor activity > structural molecule activity > toxin activity (data not shown). The upregulated genes that were shared among all samples after 72 h storage are located mainly in *S.* Typhimurium plasmids. Considering the plasmids sizes of strain SL1344 (pSLT = 93.8 kb; pCol1B9 = 86.9 kb; pRSF1010 = 8.7 kb) compared to the chromosome size (~4.9 Mb), the level of upregulated genes from plasmids shared among all samples was extremely high. In this case, the top upregulated genes (72 h samples, $|FC| > 5$) were 100% located in plasmids (Table 2). It should be highlighted that only 146 (47%) genes (among the 309 upregulated genes ($|FC| > 2$)) found in the 72 h samples are chromosomal, and the others are all plasmidial genes. Especially concerning plasmid RSF1010, the smaller one, whose size is only 8.7 kb and contains 12 identified coding genes, 6 of them (50%) were highly expressed ($|FC| > = 5$) in all four kinds of food samples after 72 h of storage. If $|FC| > 2$ is considered, a total of 10 out of 12 genes were upregulated (Table 2). pRSF1010 is part of the incompatibility group Q (IncQ), represented by small, promiscuous plasmids that can be

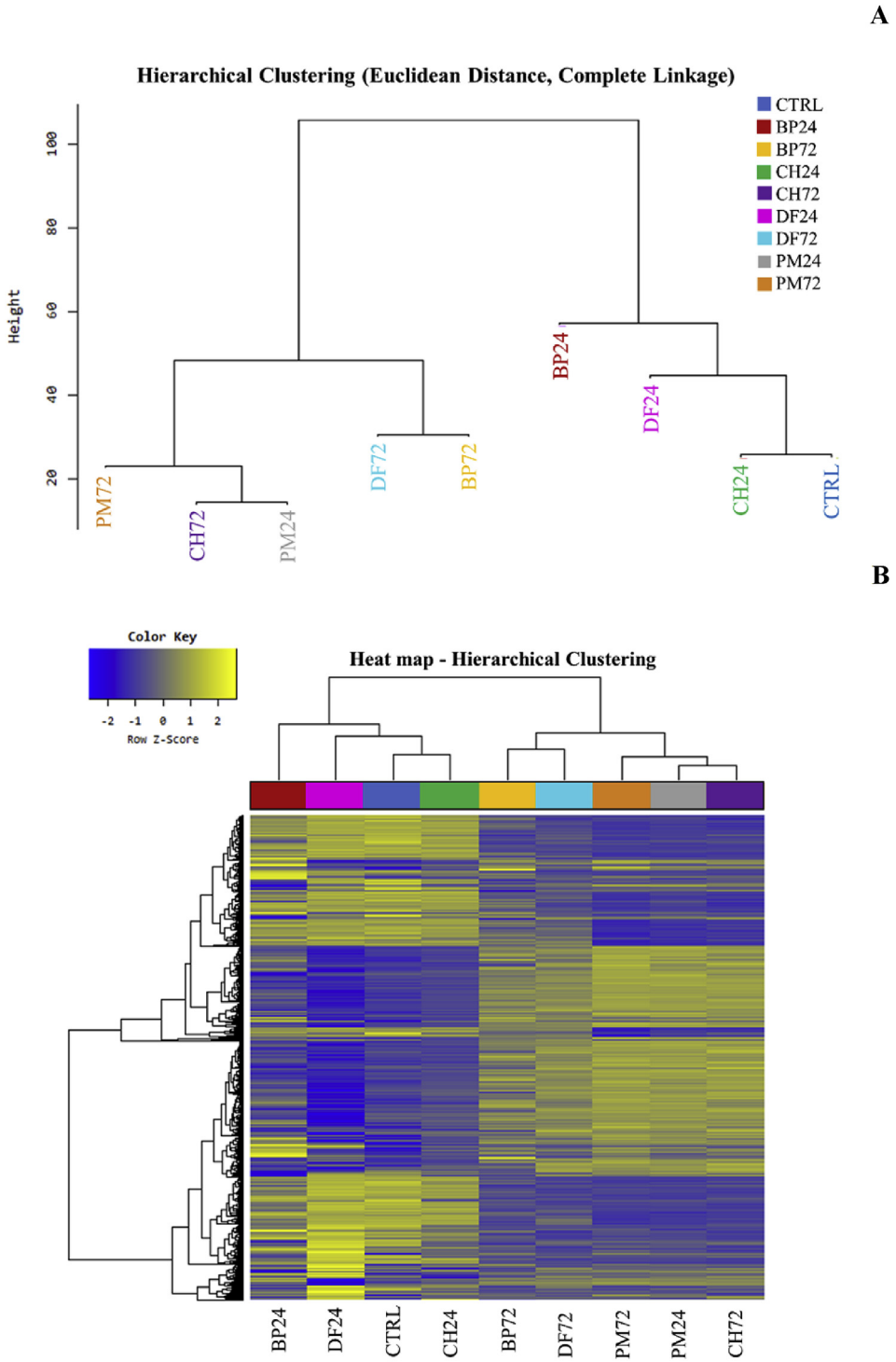

Fig. 1. Hierarchical clustering. **A.** Using each sample's Log2(RPKM + 1) value. High expression similarities were grouped together. Distance metric = Euclidean distance, linkage method = complete linkage **B.** Heat map of the two-way hierarchical clustering (2547 genes satisfying with FC2) using Z-score for normalized value (log2 based).

replicated in several bacterial hosts and exhibit mobilization by various self-transmissible plasmids (Rawlings and Tietze, 2001; Scholz et al., 1989). This plasmid is encountered in high-copy number in bacterial cells (~10–16 copies) and contains streptomycin and sulfonamide resistance genes in addition to genes responsible for its own, host-independent replication (Meyer, 2009; Rawlings and Tietze, 2001; Scholz et al., 1989). The upregulation in all samples indicates that the conditions applied to *S.* Typhimurium in this study lead to pRSF1010 replication in the cells, causing fluctuations in their plasmid copy numbers.

It is well-known that several stress responses lead to the diminution of ribosomal proteins and reduction of cell growth (Garbeva et al., 2011). As expected, among the 35 most downregulated genes that were identified in all 72 h samples ($|FC| > 5$), nearly ¼ code ribosomal proteins (Table 2). GO analysis for the 35 most downregulated genes regarding their molecular functions are: binding > structural molecule activity > catalytic activity > transmembrane transporter activity > transcription antitermination factor activity (RNA binding).

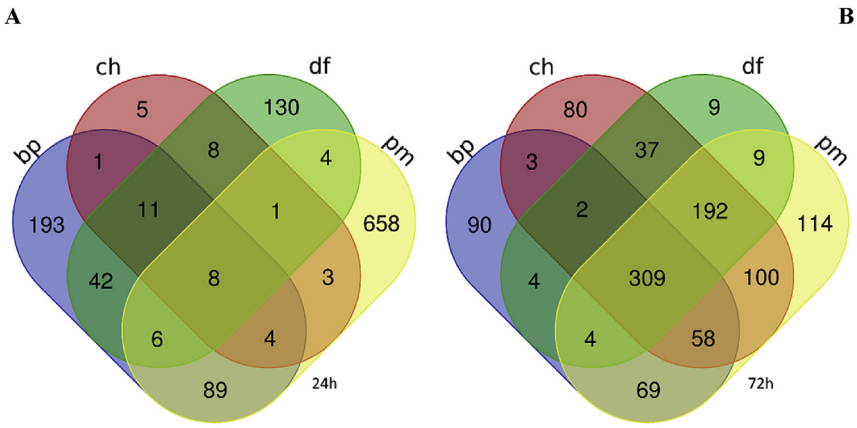

Fig. 2. Venn diagram showing number of induced genes (FC ≥ 2) for 24 h (A) and 72 h (B) samples as well as their intersections. bp = black pepper; ch = chocolate; df = dog food; pm = powdered skim milk.

Table 2
Top differentially expressed genes shared among all four food samples. ^{P} = plasmidial gene; ^{P1} = pSLT/^{P2} = pCol1B9/^{P3} = pRSF1010.

Top up-regulated genes identified in all samples			
Storage	**Locus tag**	**Protein ID**	**Product**
72h	SL1344_RS24720^{P3}	WP_000238497.1	mobilization protein C
|FC| > 5	SL1344_RS26350 ^{P2}	WP_001330800.1	Replication protein RepA4
	SL1344_RS24515 ^{P2}	WP_000086957.1	Conjugal transfer protein TraR
	SL1344_RS24700 ^{P3}	WP_000190708.1	regulatory protein RepA
	SL1344_RS24710 ^{P3}	WP_000455501.1	hypothetical protein
	SL1344_RS24715 ^{P3}	WP_001395566.1	mobilization protein A
	SL1344_RS24725 ^{P3}	WP_001120891.1	IS91 family transposase
	SL1344_RS24730 ^{P3}	WP_000480968.1	aminoglycoside O-phosphotransferase APH(6)-Id
	SL1344_RS23690 ^{P1}	WP_000055840.1	type-F conjugative transfer system pilin assembly thiol-disulfide isomerase TrbB
Top down-regulated genes identified in all samples			
24h	SL1344_RS18810	WP_000014594.1	cold-shock protein CspA
|FC| > 5	SL1344_RS09980	WP_000208509.1	cold-shock protein CspJ
72h	SL1344_RS09980	WP_000208509.1	cold-shock protein CspJ
|FC| > 5	SL1344_RS17670	WP_001140434.1	50S ribosomal protein L30
	SL1344_RS17720	WP_000644742.1	50S ribosomal protein L29
	SL1344_RS17740	WP_001138115.1	30S ribosomal protein S19
	SL1344_RS17705	WP_000729185.1	50S ribosomal protein L24
	SL1344_RS17680	WP_000358956.1	50S ribosomal protein L18
	SL1344_RS25980	WP_024131133.1	hypothetical protein
	SL1344_RS21280	.	tRNA-Thr
	SL1344_RS22545	WP_001519453.1	primosomal replication protein N
	SL1344_RS09065	WP_001185666.1	cell division topological specificity factor
	SL1344_RS17070	WP_000271396.1	50S ribosomal protein L21
	SL1344_RS03705	WP_000254356.1	succinate dehydrogenase, hydrophobic membrane anchor protein
	SL1344_RS25540	WP_014344104.1	hypothetical protein
	SL1344_RS19270	WP_000135058.1	DNA-directed RNA polymerase subunit omega
	SL1344_RS22540	WP_001216673.1	30S ribosomal protein S6
	SL1344_RS24925	WP_014343956.1	hypothetical protein
	SL1344_RS18810	WP_000014594.1	cold-shock protein CspA
	SL1344_RS15760	WP_000351186.1	hypothetical protein
	SL1344_RS25350	WP_071525150.1	hypothetical protein
	SL1344_RS17785	WP_000031748.1	elongation factor Tu
	SL1344_RS08430	WP_001274953.1	envelope stress response membrane protein PspB
	SL1344_RS17615	WP_001682446.1	ribosome alternative rescue factor ArfA
	SL1344_RS02255	WP_000179833.1	cytochrome o ubiquinol oxidase subunit III
	SL1344_RS25140	WP_001752570.1	hypothetical protein
	SL1344_RS17780	WP_000289082.1	bacterioferritin-associated ferredoxin
	SL1344_RS01200	WP_000062330.1	Rho-binding antiterminator
	SL1344_RS20530	WP_000649858.1	DUF1040 domain-containing protein
	SL1344_RS11110	WP_001261696.1	membrane protein
	SL1344_RS03270	WP_001269947.1	hypothetical protein
	SL1344_RS00915	WP_000621526.1	aspartate 1-decarboxylase
	SL1344_RS09105	WP_000457328.1	YoaH family protein
	SL1344_RS22550	WP_000135199.1	30S ribosomal protein S18
	SL1344_RS10540	WP_001012956.1	microcompartment protein PduM
	SL1344_RS26215	WP_001520310.1	hypothetical protein
	SL1344_RS11370	WP_000241015.1	bifunctional murein DD-endopeptidase/murein LD-carboxypeptidase

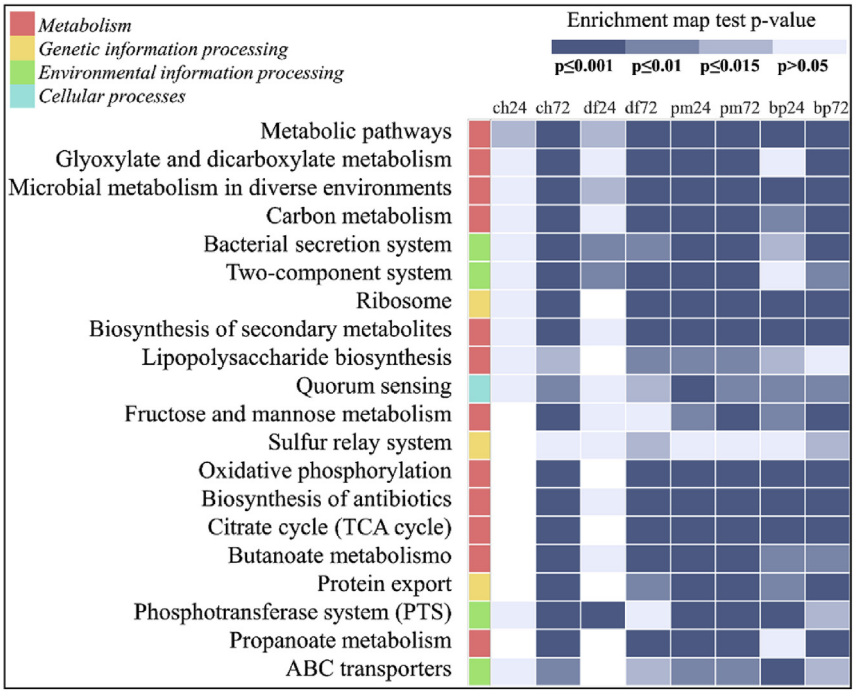

Fig. 3. KEGG analysis. Result of gene-set enrichment analysis (p-value top 20).

3.4. KEGG enrichment analysis

To access the main cell pathways induced in each treatment, KEGG database was used to achieve the pathway map, colored by fold change for significantly expressed genes by each comparison pair (treatment and control). The gradient legend shows the level of enrichment raw p-value from the modified Fisher's exact test to determine the enrichment of each gene from the gene set. A raw p-value < 0.05 means that the pathway has been significantly enriched. According to KEGG, the cellular responses of samples ch24 and df24 have more proximity to the control, while the remaining treatments activated pathways essential for survival under stress conditions. For example, glyoxylate metabolism (highly enriched in five treatments, $p \leq 0.001$) may be related to the glyoxylate shunt pathway, already shown to be involved in the adaptation of *Salmonella* to starvation, anaerobic growth in the presence of hydrogen, persistence during infection in mice and desiccation (Gruzdev et al., 2012). The resultant heat map shows the enrichment analysis for each of the top 20 pathway terms (Fig. 3).

3.4.1. Individual DGE analysis

To evaluate the particularities of each food composition, DGE analysis was conducted separately for the samples studied. A complete list of up- and downregulated genes for each sample is available in the *Supplementary table*.

3.4.2. Milk chocolate and powdered milk

The fact that the chocolate used in this study has milk as the second main ingredient in its composition grouped these two samples as presenting similar expression profiles. Thus, in this session, the two food matrices will be discussed at once.

Chocolate (a_w = 0.4–0.5) has been linked to several *Salmonella* outbreaks in the last decades (Krapf and Gantenbein-Demarchi, 2010). The highly induced genes of *Salmonella* in chocolate after 24 h of storage ($|FC| > 5$) were only ribosomal (16S and 23S). This result has also been found in other studies during *Salmonella* dehydration process (Gruzdev et al., 2012; Maserati et al., 2018). The authors suggest that the dying cells during this process release nutrients that will function as nutrient sources to the remaining ones; thus transcription and translation activities might be actively occurring (Finn et al., 2013b). It is interesting to note that it only happened in the sample of 24 h-stored chocolate samples, as the high sugar and fat contents may have retarded the desiccation stress response. As for 72 h-stored chocolate samples, the profile of induced genes was analogous to the milk samples.

Powered skim milk comprised the food matrix presenting more induced genes ($|FC| > 5$) shared between 24 h and 72 h samples. As stated before, this was the only sample that did not present fat in its composition, which could have led to quicker desiccation stress responses. GO analysis of 72 h-stored chocolate, and both milk samples showed catalytic, binding and transporter activities regarding their main molecular functions induced.

Mobilization protein C was the most induced gene; followed by a transposase and an aminoglycoside O-phosphotransferase, all belonging to the RSF1010 plasmid. The most induced chromosomal gene is a DNA polymerase V subunit UmuC, which participate in DNA damage control and translesion DNA synthesis (Reuven et al., 1999). This gene might play an important role since the harsh conditions can lead to cell death by DNA mutagenesis and damage from specific metabolites. DNA repair genes were also up-regulated in a previous study using *S.* Typhimurium global transcriptional analysis, after desiccation in abiotic surface (Maserati et al., 2017). Elevated levels of some tRNA expressions (tRNA-Sec, tRNA-Pro, tRNA-Met, and tRNA-Leu), pili-encoding genes, and a sulfonamide-resistance gene were also observed. The results indicate metabolically active cells with induced genes that contribute to their survival and adaptation to the chocolate and milk compositions, with characteristics that may lead to the expression of virulence factors important to colonize host cells.

The downregulated genes for all the samples were identified as two cold-shock proteins and a membrane protein (WP_001261696.1; WP_000014594.1; WP_001261696.1). The top downregulated genes shared among 72 h-stored chocolate, and milk samples were mainly ribosomal proteins, a ribosome modulation factor, some stress-response molecules, and transcriptional regulators, indicating a change in the cell metabolism. A complete list of up- and downregulated genes is shown in the *Supplementary table*.

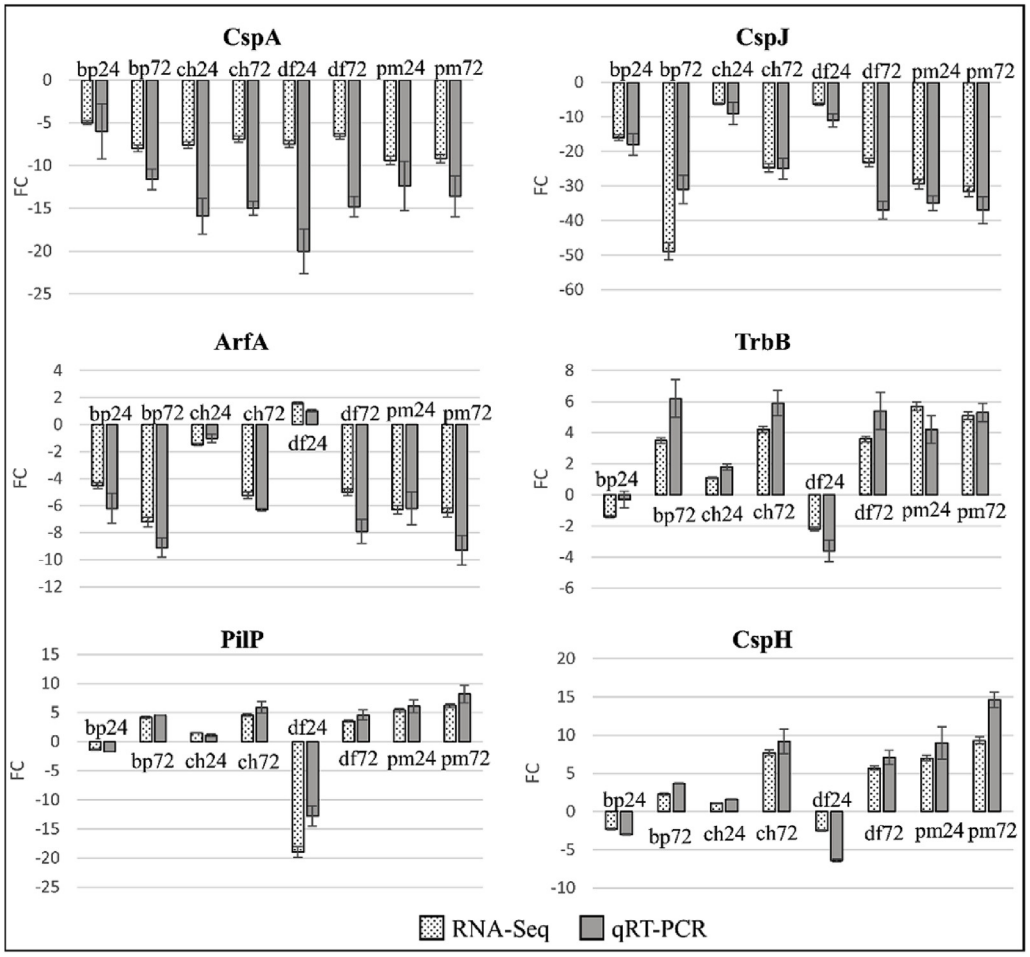

Fig. 4. Validation of RNA-Seq using qRT-PCR. Six genes (*cspA, cspJ, arfA, trbB, pilP* and *cspH*) were evaluated for each sample/condition. The gene *gpi* was used to normalize the data, and the fold change was calculated using the $2-\Delta\Delta CT$ method.

3.4.3. Black pepper

The top induced genes ($|FC| > 5$) shared between 24 h- and 72 h-stored black pepper were mainly 23S and 16S ribosomal RNA. These results are in accordance to the results of a previous study on desiccation response of *Salmonella*, although some genes coding for proteins of the 50S ribosomal subunit was downregulated in the black pepper samples (Finn et al., 2013b). The noncoding 6S RNA was also highly induced in both treatments but especially in the 24 h-stored samples. This RNA regulates RNA polymerase activity, binding to the σ70 holoenzyme reducing its activity (Wassarman and Storz, 2000). Some transfer RNAs were upregulated (tRNA-Gln, tRNA-Leu, tRNA-Met), while others were downregulated (tRNA-Lys, tRNA-Val, tRNA-Asp) in both treatments. Sequences of plasmid pCol1B9, responsible for horizontal gene transfer, were highly induced in the 72 h-black pepper sample, several of which were more than 2-fold overexpressed compared to the 24 h sample.

In the case of black pepper (and other spices), the presence of *Salmonella* is very common, as the production is executed many times by rudimentary process, e.g., sun drying of the pepper in open spaces. The contamination during dehydration leads to adaptations that will make the cells more resistant to the subsequent stresses present during the production steps. In fact, it should be noted that in this work, *Salmonella* was inoculated in pepper grains that were subsequently dehydrated at 50 °C. Although we haven't sequenced the transcriptome in the first hours of dehydration process, the 24-h sample (incubated at 25 °C after dehydration) showed only 8 of the upregulated genes as the same encountered in the other food samples (in which desiccation occurred in lower temperatures), but this number increased to 309 in 72 h. It suggests that, once the dried black pepper is contaminated, even the use of relative high temperatures in the process is not sufficient to eliminate the pathogen. Furthermore, if a favorable condition occurs (like a humidity increase in the environment), the cells may resume their regular metabolism as the pepper composition itself is sufficient for *Salmonella* growth (Keller et al., 2013). Therefore, ideal conditions throughout the whole production chain of spices must be strictly followed to avoid any possible contaminations.

3.4.4. Dried pet food

Dried pet food (dog food) was the more complex sample, which presented the more discrepant expression profile between 24 h- and 72 h-stored samples. Only one gene was overexpressed in the two samples, the 6S RNA, which was also overexpressed in both 24- and 72 h-stored black pepper samples. Most of the genes presented different patterns of expression, as several were downregulated in the 24 h sample while the same sequences were upregulated in the 72 h sample. Among the top induced genes for the 24 h-sample ($|FC| > 5$) were 23S and 16S ribosomal RNA; transfer RNAs (tRNA-Arg, tRNA-His, tRNA-Leu); transcriptional regulators and cellulose biosynthesis protein (which may be related to biofilm formation). None of the top induced genes ($|FC| > 5$) was plasmidial. As for 72 h-stored sample, the top induced genes included various plasmidial genes, being most of them downregulated at 24 h. For the chromosomal top overexpressed genes after 72 h, transfer RNAs were also identified (tRNA-Sec, tRNA-Leu, tRNA-Pro, tRNA-Gln, tRNA-Met), as well as a bacteriophage protein, and the cold-shock protein CspH. Unfortunately, some proteins identified in this condition, highly induced, still don't have any known

functions.

Interestingly, for this sample, the pilus assembly protein PilP was downregulated in the first 24 h, but at 72 h, the expression levels significantly increased, indicating a modified level on the virulence pattern between the two samples. The same occurred to a glycosyltransferase, a chaperone, and a LysR transcriptional regulator. The expression profile suggested that the *Salmonella* cells were metabolically active in the first 24 h of storage of dog food, with great transcription and translation activities while plasmidial genes were not induced as in the 72 h sample. The dog food was the most complex sample analyzed in this study, and it is expected to be a balanced nutritional supply (containing the calories, macronutrients and micronutrients to a balanced animal diet), which may explain the findings. Several genes were downregulated in the first 24 h, shifting to upregulated with more time of exposure.

3.5. Quantitative real-time PCR

The expression of six selected genes using qRT-PCR was experimentally verified to endorse the results obtained from RNA-Seq. Gene *pgi* (glucose-6-phosphate isomerase) was used as endogenous control, as its expression remained unchanged in all the conditions applied in this study. The primers were first checked by conventional PCR followed by an efficiency test through serial dilutions. Only primers producing single amplicons and with efficiency in the range of 90–110% were used for RNA-Seq validation. Fig. 4 presents the fold change (FC) results for selected genes for the two analyzes for each sample. The results ($p < 0.05$) presented a good correlation with those obtained from the sequencing, confirming that the RNA-Seq data reproduced real changes in the gene expression for the different treatments. The difference in FC values observed for some genes between the two techniques may be explained by the better sensitivity of qRT-PCR in the quantification of gene expression compared with RNA-Seq (Git et al., 2010).

4. Conclusions

In this work, the transcriptome of *S.* Typhimurium strain SL1344 after inoculation in four different low a_w food products (milk chocolate, powdered skim milk, black pepper and dried dog food) was analyzed. The data acquired by RNA-Seq enabled the verification of the differential expression of genes in each sample after 24 h and 72 h, compared to the control (*i.e.*, fresh cells before the inoculum). According to the results, it was possible to verify that the initial responses are more related to the food composition itself, followed by an adaptation to the low a_w activity that remained considerably similar among different samples. Besides, it was observed that nutrient-rich foods with high fat and protein contents form protection that keeps *Salmonella* metabolism closer to the control group at least in the first day post-contamination. Plasmidial genes of *S.* Typhimurium SL1344 were highly induced after 72 h of inoculum, suggesting a relevant role in the desiccation stress response. Several genes coding proteins without known functions were induced in all samples, and their connection with desiccation (and maybe other stresses) responses in *Salmonella* should be investigated.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.03.016.

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