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1 **Quantification of *Campylobacter jejuni* gene expression after successive**  
2 **stresses mimicking poultry slaughtering steps**

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7

8 **Highlights**

- 9 • *C. jejuni* molecular response studied after consecutive thermal stresses by RT-qPCR  
10 • Similar gene expression patterns shared between two of the three strains tested  
11 • Up-regulated genes mainly involved in the heat shock response  
12 • Down-regulated genes belonged to metabolic pathways (lipid, amino-acid metabolisms)  
13 • A strain-specific gene expression profile consistent with atypical stress response

14

15 **Abstract**

16 Broiler meat is considered as the most important source of the foodborne pathogen *Campylobacter*  
17 *jejuni*. Exposure to stress conditions encountered during the slaughtering process may induce  
18 bacterial adaptation mechanisms, and enhance or decrease pathogen resistance to subsequent stress.  
19 This adaptation may result from changes in bacterial gene expression. This study aims to accurately  
20 quantify the expression of selected *C. jejuni* genes after stresses inspired from the poultry  
21 slaughtering process. RT-qPCR was used to quantify gene expression of 44 genes in three strains  
22 after successive heat and cold stresses. Main results indicated that 26 genes out of 44 were  
23 differentially expressed following the successive thermal stresses. Three clusters of genes were  
24 differentially expressed according to the strain and the stress condition. Up-regulated genes mainly  
25 included genes involved in the heat shock response, whereas down-regulated genes belonged to  
26 metabolic pathways (such as lipid, amino-acid metabolisms). However, four genes were similarly  
27 overexpressed in the three strains; they might represent indicators of the thermal stress response at  
28 the species scale. Advances in the molecular understanding of the stress response of pathogenic  
29 bacteria, such as *Campylobacter*, in real-life processing conditions will make it possible to identify  
30 technological levers and better mitigate the microbial risk.

31

32 **Keywords:** RT-qPCR, strain variability, food safety, foodborne pathogen, cell history

33

34 **1 Introduction**

35 *Campylobacter* has been the main cause of bacterial enteritis worldwide in humans for many years  
36 (EFSA and ECDC, 2019). The number of reported confirmed cases of campylobacteriosis is often  
37 under-estimated: it was 246,571 in 2018 with an EU notification rate of 64.1 *per* 100,000 population  
38 (EFSA and ECDC, 2019). *C. jejuni* infection is usually benign, but may also lead to chronic sequelae  
39 such as Guillain-Barré syndrome (GBS) and Miller-Fisher syndrome (MFS) (Altekruse et al., 1999;  
40 Silva et al., 2011; WHO, 2013). Consumption of animal products, mainly poultry, is a major cause of  
41 infection in humans (Zhong et al., 2016). Although chicken meat is the main source of this pathogen,  
42 other foods have been involved in campylobacteriosis outbreaks such as milk (EFSA and ECDC,  
43 2019).

44 *Campylobacter* are apparently fragile organisms that cannot grow under aerobiosis conditions; they  
45 cannot multiply outside the animal host and are highly susceptible to a number of environmental  
46 conditions (Park, 2002). Nevertheless, *Campylobacter* are paradoxically considered as one of the  
47 main foodborne pathogens (Solomon and Hoover, 1999). During poultry processing, bacteria are  
48 submitted to numerous stressful steps assimilated to heat stress, cold stress, acid stress or oxidative  
49 stress. To mitigate stressful effects, the pathogen sets up various defense strategies like production of  
50 heat shock proteins (*e.g.* DnaK, GroEL, ClpB) in response to heat or acid stress (Konkel et al., 1998;  
51 Reid et al., 2008a; Reid et al., 2008b; Stintzi, 2003; Thies et al., 1998; Thies et al., 1999a; Thies et  
52 al., 1999b), but also enzymes (*e.g.* AhpC, KatA, SodB) involved in cell detoxification (Baillon et al.,  
53 1999; Grant and Park, 1995; Pesci et al., 1994; van Vliet et al., 1999). Such mechanisms enable  
54 *Campylobacter* to adapt to environmental conditions.

55 Unlike other foodborne pathogens, *Campylobacter* does not grow effectively in the food environment  
56 or on food products. However, it can survive under stressful conditions (Duqué et al., 2019; Klančnik  
57 et al., 2009; Murphy et al., 2003a, b; Oh et al., 2015; Reid et al., 2008b). Adaptation to stress has  
58 been investigated at the transcriptomic level and has determined which genes could be involved.  
59 Most studies addressed the acid, oxidative or heat stress responses (Reid et al., 2008a; Reid et al.,  
60 2008b; Stintzi, 2003; Varsaki et al., 2015), in which single stresses were applied independently.  
61 Many genes play a role in the stress response, such as genes implied in the heat shock response or in  
62 the amino acid or lipid metabolisms (Cameron et al., 2012; Guccione et al., 2017; Palyada et al.,  
63 2009; Stintzi, 2003). In order to decipher how this pathogen can survive along the food chain, it  
64 seemed relevant to evaluate the impact of various food production and preservation conditions on the  
65 stress adaptation potential of this foodborne pathogen. Consequently, we transposed consecutive heat  
66 and cold stresses inspired from the poultry slaughtering process to the laboratory. The aim was to  
67 assess the impact of successive hot and cold temperature stresses on *C. jejuni* gene expression and  
68 better understand the adaptation capacity of this pathogen.

69 To our knowledge, the molecular mechanism underlying the adaptive response of *C. jejuni* resulting  
70 from exposure to successive stresses, especially stresses similar to those encountered during the  
71 poultry processing steps, has not been studied yet. Exposure to a stress may indeed induce a general  
72 stress response enabling bacterial cross-protection from other stress conditions (Kim et al., 2015). In  
73 a previous study, we evaluated the response of *C. jejuni* to the application of successive stresses  
74 inspired from the poultry slaughtering process at the phenotypic level (Duqué et al., 2019): the  
75 inactivation of *C. jejuni* induced by cold storage significantly depended on the previously applied  
76 heat stress and differed according to the strain. Our results pointed out the complexity of bacterial  
77 behavior and strain variability, and highlighted the need to decipher the molecular mechanisms  
78 underlying adaptive responses through gene expression profiling.

79 The investigation of bacterial adaptive responses requires the use of molecular approaches. In this  
80 respect, reverse transcription quantitative PCR (RT-qPCR) is still considered as a standard method  
81 for accurate and sensitive measurement of gene expression (Nolan et al., 2006). In addition to the  
82 recommendations about the Minimum Information for Publication of Quantitative Real-Time PCR  
83 Experiments (MIQE) (Bustin et al., 2010; Bustin et al., 2009), stringent quality controls at each  
84 critical point throughout the entire RT-qPCR workflow are necessary (Desriac et al., 2017). Besides,  
85 to disentangle experimental variation from true biological variation, an internal control to compensate  
86 for experimental errors has to be used (Huggett et al., 2005). Housekeeping genes are generally used  
87 as internal controls. However, finding appropriate internal control genes whose expression remains  
88 stable whatever the experimental conditions is still quite a challenge (Ritz et al., 2009).

89 In the current study, three *C. jejuni* strains were submitted to successive stresses inspired from those  
90 encountered during the poultry slaughtering process. First, an accurate method of RT-qPCR was  
91 developed (Rez e et al., 2019). Second, this method was used to study the influence of successive hot  
92 and cold temperatures on the expression of selected genes to investigate how bacteria modulate gene  
93 expression after two successive stresses.

94

## 95 **2 Materials and methods**

### 96 **2.1 Bacterial strains and culture conditions**

97 The culture conditions described in Duqu e et al. (2019) were adopted. To sum up, three strains  
98 originally isolated from poultry were used: *C. jejuni* C09MJLT205, *C. jejuni* RM1221, and *C. jejuni*  
99 C97anses 640 (Fouts et al., 2005; Guyard-Nicodeme et al., 2015; Guyard-Nicodeme et al., 2013).  
100 The cultures were stored at -80 C in brain heart infusion (BHI, Biom erieux, Marcy l'Etoile, France)  
101 supplemented with 20% (v/v) glycerol. Prior to the experiments, they were cultured on Karmali  
102 (Oxoid, Dardilly, France) agar plates at 42 C for 48-72 h under microaerobic conditions. Then, two  
103 successive cultures were incubated in Mueller-Hinton broth (MH, Oxoid, Dardilly, France) for 20 h  
104 and 18 h, respectively, under microaerobic atmosphere and shaking. Finally, 10-fold dilutions were  
105 prepared to obtain the final cultures, which were enumerated.

### 106 **2.2 Induction of stress conditions**

107 The experimental procedure was as described previously, with slight modifications (Duqu e et al.,  
108 2019). Briefly, *C. jejuni* strains were submitted to stresses inspired from conditions encountered  
109 during the poultry slaughtering process and considered as stressful. These steps are scalding and  
110 chilling, and are associated with heat and cold stresses.

111 The experimental design used "strain" and "hot bath temperature" as variables. For each strain, ten  
112 mL of the culture at an initial concentration of 8 log<sub>10</sub> CFU.mL<sup>-1</sup> were transferred into a glass tube  
113 and successively submitted to heat and cold. The glass tubes had the following dimensions: 150 mm  
114 in length, 18 cm in diameter and 1 mm in thickness. Heat stress consisted in immersing *C. jejuni*  
115 cultures in hot water baths at 46, 51 or 54 C for 4 min. After exposure to heat stress, the tubes were  
116 immediately cooled for 5 min in a water bath at 22 C. Cold stress was applied immediately after  
117 cooling by immersing the cultures into freezing-cold water containing ethylene glycol (-4 C, for 2 h).

118 In parallel, 10 mL from the same initial culture were kept as a control unstressed culture.

119 After each step, viable counts of *C. jejuni* were enumerated on Columbia sheep blood agar plates  
120 (Biomérieux, Marcy l’Etoile, France) and incubated for 48 h at 42°C under microaerobic conditions  
121 using a SPIRAL plater (EasySpiral Interscience, Saint Nom, France) to determine the viability loss,  
122 expressed in  $\Delta\log_{10}$ , resulting from each step. Besides, a volume of culture was kept after each step  
123 for RNA extraction. Each experiment was performed in three independent replicates.

## 124 **2.3 Transcriptomic analysis by RT-qPCR**

125 The molecular response of *C. jejuni* was investigated using RT-qPCR instead of a global approach  
126 like RNA-seq because the final objective of this work will be to correlate the phenotypic response of  
127 *C. jejuni* (inactivation) with its transcriptomic response, using mathematical modeling to identify  
128 biomarkers of stress. The search for biomarkers represents a challenge, and this proof of concept was  
129 investigated using RT-qPCR as already performed by Desriac et al (Desriac et al., 2015; Desriac et  
130 al., 2012; Desriac et al., 2013). To this end, quantitative data had to be provided using a quantitative  
131 method. This was why we chose RT-qPCR rather than RNA-seq, which only provides the relative  
132 abundance of reads. Moreover, RT-qPCR is appropriate to study well-known phenotypes and  
133 associated genes well described in the literature.

### 134 **2.3.1 Gene selection**

135 Using RT-qPCR to evaluate the modulation of gene expression requires selecting a limited number of  
136 genes to be studied. To compare the transcriptomic response of *C. jejuni* following one stress *versus*  
137 two consecutive stresses, we chose forty genes already known to be involved in the *C. jejuni* stress  
138 response according to the literature. The selection was based on a decision tree as described in Figure  
139 1. A literature search was first performed on the Web of Science platform by seeking articles with the  
140 following keywords in their title and/or their abstract: adaptive AND response AND *Campylobacter*,  
141 oxidative AND stress AND *Campylobacter*, transcriptomic AND *Campylobacter*, RT-PCR AND  
142 *Campylobacter*, heat AND stress AND *Campylobacter*, cold AND stress AND *Campylobacter*, acid  
143 AND stress AND *Campylobacter*, osmotic AND stress AND *Campylobacter*, alkaline AND stress  
144 AND *Campylobacter*, gene expression AND stress AND *Campylobacter*, proteomic AND stress  
145 AND *Campylobacter*, microarray AND stress AND *Campylobacter*, RNA-seq AND stress AND  
146 *Campylobacter*. Based on these criteria, 1, 096 publications were retrieved.

147 One hundred and twenty studies were selected out of the initial batch of 1,096 publications because  
148 they showed differential gene expression following stresses or the involvement of a given gene in the  
149 stress response. Around 700 genes were considered in these studies, *i.e.* approximately 43% of the *C.*  
150 *jejuni* whole genome. To avoid selecting marginally studied genes, only genes studied in at least two  
151 different laboratories were kept. This represented 430 genes. All the regulators involved in the *C.*  
152 *jejuni* stress response (13 genes) were retained because we assumed that their expression would  
153 probably change under stress conditions. Then, we stringently selected 27 genes among the 430  
154 genes studied in at least two different laboratories by trying to capture the variability of the metabolic  
155 pathways in which they were involved. To do so, the gene classification according to metabolic  
156 pathways was retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.  
157 Finally, at least one gene from each metabolic pathway was picked up, but genes involved in several  
158 metabolic pathways and involved in different stress responses were preferentially selected. Out of  
159 1,600 initially selected genes, 40 were eventually kept for analysis (Table 1).

160 Only the genes amplified by PCR and therefore present in the three strains were kept. Consequently,  
161 two genes – *putA* and *rprB* – were excluded from the analysis because they were not amplified in the

162 *C. jejuni* C09MJLT205 strain and in the RM1221 and C94anses640 strains, respectively. The  
163 expression levels of the 38 remaining genes were then analyzed.

### 164 **2.3.2 Selection of reference genes**

165 Reference genes are needed for normalized expression. They are usually housekeeping genes, and  
166 their expression should remain constant whatever the experimental conditions. Reference genes were  
167 selected among a pool of 44 genes composed of i) six genes previously described as potential  
168 reference genes for RT-qPCR in *C. jejuni* (Ritz et al., 2009), and ii) the 38 genes selected as  
169 potentially involved in the stress response.

170 Stability of gene expression was assessed as demonstrated by Vandesompele et al. (2002). The gene  
171 expression stability measure (M) was calculated using the geNorm module in qBase+ software  
172 (version 3.2, Biogazelle). To determine the optimal number of reference genes, the pairwise variation  
173  $V_{n/n+1}$  was calculated between the two sequential normalization factors ( $N_{Fn}$  and  $N_{Fn+1}$ ) for all  
174 samples. The software program recommended that V should be less than 0.15.

### 175 **2.3.3 Primer design**

176 For each gene, primers were designed using Primer3Plus, a web interface developed by Untergasser  
177 et al. (2007) based on the genome of strain RM1221 – the only sequenced strain in this study. Pairs  
178 with the smallest penalty score were retained, and sequences were tested for specificity using NCBI  
179 BLAST software, and compared to all available genomes of *C. jejuni* species. Oligonucleotide  
180 sequences of the targeted genes with BLAST score values greater than 30 were retained, as suggested  
181 by Desriac et al. (2017).

### 182 **2.3.4 Determination of primer efficiency**

183 DNA was isolated from unstressed *C. jejuni* RM1221 cultures. Briefly, 1 mL was centrifuged at  
184 10,000 x g for 6 min. Then, the supernatant was removed, and DNA was extracted using a Dneasy®  
185 Blood and Tissue Kit (Qiagen, Courtaboeuf, France). Combined lysozyme (15 mg.mL<sup>-1</sup>) and  
186 proteinase K (20 mg.mL<sup>-1</sup>) digestion was used for lysis. DNA quantity and purity were measured  
187 using an Implen NanoPhotometer and stored at -20°C.

188 After *in silico* design, the PCR efficiency (E) of each primer pair was assessed (Table 2). Briefly,  
189 after thawing, each DNA suspension was serially diluted tenfold before amplification and  
190 quantification. Then, for each primer pair, Ct values were plotted on a logarithmic scale along with  
191 corresponding DNA concentrations. Efficiency was calculated from a linear regression curve through  
192 the data points, using the following equation:  $E = -1 + 10^{(-1/\text{slope})}$ . All primer pairs showed satisfactory  
193 efficiency values ranging between 97 and 109% with an R<sup>2</sup> higher than 0.97.

### 194 **2.3.5 RNA isolation**

195 RNA was isolated from cultures (i) after the heat stress, and (ii) after the cold stress applied  
196 following the heat stress. RNA was also extracted from unstressed cultures that remained under  
197 optimal conditions (42°C in microaerobiosis) and considered as the control.

198 Briefly, 30 mL of stressed or unstressed culture were centrifuged at 10,000 x g at 4°C for 4 min.  
199 Thirty mL of culture were necessary to have enough RNA to extract (mostly after stress application).  
200 Consequently, the centrifugation step was performed before adding RNA protect to resuspend the  
201 pellet in a smaller volume of RNA-protect, as performed by Desriac *et al.* (Desriac et al., 2015;  
202 Desriac et al., 2012; Desriac et al., 2013). After removal of the supernatant, 1 mL of RNA protect

203 (Qiagen, Courtaboeuf, France) was added to suspend the pellet. The suspension was incubated for 5  
204 min at ambient temperature and then centrifuged at 5,000 x g at 4°C for 10 min as recommended by  
205 the manufacturer. The supernatant was removed, and the pellet was snap-frozen in liquid nitrogen  
206 and stored at -80°C for up to 1 month.

207 After cell pellets were thawed, RNA extraction was performed using an RNeasy® Mini Kit (Qiagen,  
208 Courtaboeuf, France) according to the manufacturer's recommendations. Combined lysozyme (15  
209 mg.ml<sup>-1</sup>) and proteinase K (20 mg.ml<sup>-1</sup>) digestion was used for cell lysis. Contaminant genomic DNA  
210 was removed from each sample using an Invitrogen Turbo DNA-free™ Kit (Ambion, Cambridge,  
211 USA). A first incubation of 30 min at 37°C was performed, and the supernatant was extracted. A  
212 second incubation of 30 min at 37°C was performed to efficiently remove contaminant genomic  
213 DNA.

214 Quality controls of the extracted RNAs were carried out to validate RNA isolation. First, RNA  
215 quantity and purity were measured using an Implen NanoPhotometer. Then, after RNA denaturation  
216 at 70°C for 9 min, RNA integrity was assessed by electrophoresis on agarose gel (1.2%). Profiles  
217 presenting RNA degradation were automatically discarded. The absence of genomic DNA in the  
218 RNA samples was checked by PCR. Amplification was performed using primers 341F and 758R  
219 (Rodrigues et al., 2016). The thermo-cycling program consisted of 95°C for 5 min, followed by 30  
220 cycles of 30 s at 95°C, 30 s at 59°C and 30 s at 68°C. The absence of DNA was checked using  
221 agarose gel (1.2%) electrophoresis.

222 Each extraction was performed on three independent replicates.

### 223 **2.3.6 Reverse Transcription (cDNA synthesis)**

224 As recommended by Stahlberg et al. (2004), reverse transcription was performed on 200 ng of the  
225 total RNA extract using an iScript cDNA synthesis kit (Bio-Rad, Marnes la Coquette, France). The  
226 cDNA synthesis program consisted in initial priming at 25°C for 5 min, followed by reverse  
227 transcription at 46°C for 20 min, and inactivation at 95°C for 1 min. cDNA was then stored at -20°C.

### 228 **2.3.7 Quantitative PCR**

229 Amplification and quantification were performed using a CFX connect device (Bio-Rad, Mitry-  
230 Mory, France). For each sample, the reaction mixture contained 10 µL of SoAdvanced SYBR Green  
231 Supermix (Bio-Rad, Marnes la Coquette, France), 1 µL of each forward and reverse primer, 4 µL of  
232 water and 4 µL of cDNA. The program consisted in initial denaturation at 95°C for 3 min, followed  
233 by 40 cycles of 15 s at 95°C and 30 s at 60°C.

234 A sample maximization method was used, as advised by Hellemans et al. (2007). All samples were  
235 assayed in the same run, but genes were analyzed separately. This method avoided the need for an  
236 inter-run calibrator (IRC).

### 237 **2.3.8 Data analysis**

238 Gene expression quantification was performed after each step of the experimental setup (*i.e.* after  
239 heat stress and after successive heat and cold stresses). RT-qPCR results were expressed in relative  
240 quantity scaled to the expression of the same gene in the control sample (*i.e.* unstressed culture) using  
241 the delta Ct transformation with efficiency correction. Then, expression levels were normalized  
242 relatively to the expression levels of the reference genes. Reference genes were selected among the  
243 44 genes.

244 The normalization factor (NF) was calculated as the geometric mean of reference gene expression  
245 (Vandesompele et al., 2002). After normalization, relative gene expression was  $\log_2$  transformed for  
246 further ANOVA analysis (Bengtsson et al., 2005).

247 For each strain, ANOVA analysis ( $\alpha=5\%$ ) using XLSTAT software (version 2018.5) was performed  
248 to assess if the application of successive heat and cold stress had a significant impact on the gene  
249 expression compared to the gene expression after the heat stress only. Besides, ANOVA analysis was  
250 also performed on genes whose absolute relative expression level (in  $\log_2$ ) was altered more than 2-  
251 fold, to assess the impact of the different temperatures of heat stress (Reid et al., 2008a). Fisher's  
252 least significant difference test ( $P<0.05$ ) was applied to determine significant differences in mean  
253  $\log_2$  fold changes between stresses.

254 Hierarchical cluster analysis of significantly differentially expressed transcripts was performed using  
255 the function heatmap.2 of the gplots package in R. Only genes with an expression level above a 2  
256  $\log_2$  fold change or below a -2  $\log_2$  fold change were considered (Reid et al., 2008a).

## 257 **3 Results**

### 258 **3.1 Selection of reference genes**

259 As depicted by Desriac et al. (2017), appropriate quality procedures and quality controls should be  
260 set up and assessed carefully to reliably interpret RT-qPCR results. The selection of good reference  
261 genes is one of the numerous critical points in the procedure. A good reference gene should display  
262 constant expression whatever the condition, and the number of reference genes has to be adapted  
263 according to their stability.

264 To select genes with the most stable expression, all 44 genes were considered as candidate reference  
265 genes, including those selected as targets. Two criteria were adopted to select the reference genes: (i)  
266 the M value, calculated for each gene and for each strain (Supplementary Figure S1), and (ii)  
267 pairwise variation –  $V_{n/n+1}$  –, calculated for each strain. The  $V_{2/3}$  pairwise variation values were <  
268 0.15, meaning that the optimal number of reference genes in this experimental situation was 2  
269 (Supplementary Figure S2). Finally, the two most stable genes were retained for each strain (Table  
270 3). Unfortunately, the reference genes were not the same for all three strains.

### 271 **3.2 *C. jejuni* inactivation following application of successive stresses**

272 *C. jejuni* encounters several stresses during the poultry slaughtering process. For example, the  
273 scalding and chilling steps can be assimilated to heat and cold stresses, respectively. The inactivation  
274 generated by these stresses varied from  $-0.28 \pm 0.10$  to  $0.54 \pm 0.04$  according to the strain and the  
275 stress applied (Table 4). Negative values mean no inactivation due to the uncertainty from  
276 enumeration method.

277 Since bacteria may respond differently and use different mechanisms of defense according to the  
278 stress encountered, their gene expression may also differ from one stress to another. Thus, the gene  
279 expression was expected to be modified following the application of the second cold stress in  
280 comparison with the application of the only first heat stress. This was evaluated in the next section.

### 281 **3.3 Differential gene expression of *C. jejuni* between heat and cold stress**

282 Gene expression was evaluated both after heat stress alone and after successive application of heat  
283 and cold stresses. Analysis of variance was performed *per* strain to determine if gene expression was



284 altered in a stress-dependent manner. For C97anses640 strain, gene expression was shown to  
285 significantly depend on gene, temperature of the heat stress and the application or not of the second  
286 stress, i.e. the cold stress ( $P < 0.001$ ). For the other two strains, the application of the cold stress was  
287 shown to affect expression of specific genes only when heat stress had been performed at some  
288 temperatures (temperature\*coldstress and gene\*coldstress significant interactions;  $P < 0.001$ ). It  
289 appeared that the application of the second stress altered more expression of some genes when strains  
290 had been previously submitted to heat stress at 46°C, rather than at 51°C or at 54°C. Genes whose  
291 expression was significantly altered at the different temperatures of the heat stress are listed in  
292 supplementary materials (Table S1). However, the difference in expression between both stresses  
293 remained very low, generally below 2 log<sub>2</sub> fold changes. Among these genes, it is noteworthy that  
294 four genes were differentially expressed by all strains after application of 46°C. These are: *kata*,  
295 *lysR*, *pebC* and *racR*. Expression of these genes after the heat stress applied at 46°C with or without  
296 subsequent application of the cold stress is described in Figure 2. It appears that heat stress followed  
297 by cold stress indeed induced a slight change in expression, but in the same direction (up or  
298 downregulation) whatever the strain. Three genes, i.e. *kata*, *lysR*, *pebC*, were downregulated  
299 following the heat plus cold stresses whereas *racR* was upregulated. The highest change in  
300 expression between the heat stress at 46°C and the heat plus cold cold stresses was observed for  
301 *pebC*, downregulated from -2 to -3.9 log<sub>2</sub> fold changes depending on the strain.

### 302 **3.4 Differential *C. jejuni* gene expression following the consecutive stresses**

303 Gene expression following heat + cold stresses slightly differed from gene expression following heat  
304 stress.

305 Only genes differentially expressed by more than 2 log<sub>2</sub> fold change (in absolute value), were  
306 considered (Reid et al., 2008a). Overall, among the 44 selected genes, 26 were differentially  
307 expressed in at least one of the three heat stress conditions and in one or more strains, with a  
308 maximum log<sub>2</sub> fold change of 6.21 and a minimal fold change of -4.17 (Supplementary Table S2).  
309 Consequently, these 26 differentially expressed genes were subjected to hierarchical clustering  
310 analysis and were grouped into 3 main clusters designated as A, B, and C (Figure 3).

311 The four genes belonging to cluster A were globally up-regulated in the three strains, and their  
312 overexpression varied between 2 and 6 log<sub>2</sub>-fold change according to the gene and the strain.  
313 However, the expression of all four genes decreased at 54°C in strain C09MJLT205, compared with  
314 46 and 51°C. This cluster mainly included genes associated with bacterial heat shock responses,  
315 namely the transcriptional regulator *hrcA* and several chaperones and co-chaperones considered as  
316 heat shock proteins (HSPs) (*grpE*, *dnaK* and *clpB*), in agreement with heat stress (Figure 4A).

317 Clusters B and C were composed of genes differentially expressed according to the strains (Figure 3).  
318 Strain C97anses640 had a rather different pattern of gene expression from that of the other two  
319 strains.

320 Cluster B harbored two categories of genes. A first pool was mainly overexpressed in strain  
321 C97anses640 when temperature increased, with a maximum of 3.1 log<sub>2</sub> fold change, while they were  
322 little or not differentially expressed in the other two strains. This group included genes encoding  
323 three regulators – *cbrR*, *hspR* and CJE1780 –, and the chaperone *groEL*, and *nuoL* (Figure 4B). The  
324 *hspR* gene encodes a repressor of the previously described operon *clpB-grpE-dnaK*, which encodes  
325 HSPs. The second group of genes was mainly under-expressed in strains C09MJLT205 and RM1221,  
326 down to -2.88 log<sub>2</sub> fold change, while it remained relatively stable or slightly increased in strain  
327 C97anses640. This group was included *pebC*, *rpoB*, *gyrA*, *thiC*, *trpD*, *ilvC* (Figure 4C).

328 Lastly, cluster C contained eleven genes significantly down-regulated in the three strains from 51°C.  
329 Down-regulation varied between -2 and -4 log<sub>2</sub> fold change. It seemed to increase as temperature  
330 increased beyond 46°C for strain C09MJLT205, and 54°C for the other two strains, suggesting that  
331 the regulation threshold could be strain dependent. This cluster included a two-component sensor  
332 histidine kinase CJE1361, a *fliP* gene, and genes belonging to the amino acid metabolism (*argF*,  
333 *slyD*), the lipid metabolism (*kdtA*, *yciA*) and the energy metabolism (*atpA*). The regulators *cosR*  
334 (Figure 4D) and *csrA*, and the *katA* gene – which are involved in the bacterial oxidative stress  
335 response – also belonged to this cluster.

336

#### 337 4 Discussion

338 The objective of the study was to assess the impact of successive hot and cold temperatures on *C.*  
339 *jejuni* gene expression to better understand the adaptation capacity of this pathogen. In addition, we  
340 investigated the strain variability response at the molecular level.

341 Studying gene expression requires an accurate and validated RT-qPCR method to quantify  
342 transcripts. Following the recommendations of Desriac et al. (2017), each step was validated (Rezé et  
343 al., 2019). Besides, the use of reference genes is mandatory to normalize the expression of target  
344 genes. A suitable reference gene should display stable expression under the experimental conditions  
345 of the study. Otherwise, the selection of inappropriate reference genes may bias gene expression  
346 quantification (Dhedda et al., 2005; Hyytiainen et al., 2012; Udvardi et al., 2008). In most cases,  
347 reference genes are *a priori* selected among genes commonly found in the literature, such as  
348 housekeeping genes. We did not limit our selection to genes previously identified in the literature,  
349 but considered a pool of 44 genes to evaluate the stability of their expression following heat and cold  
350 stresses. The  $\Delta\Delta C_t$  method is largely used to quantify gene expression. However, according to this  
351 method, only one reference gene is used to normalize gene expression (Livak and Schmittgen  
352 (2001)). The use of two reference genes increased quantification accuracy. The pairs of reference  
353 genes we chose varied according to the strains (the development of universal reference genes is not  
354 always feasible (Bustin et al., 2009). Five different reference genes were selected for the three *C.*  
355 *jejuni* strains: *rrs* – common to all three strains –, and *asd*, *proS*, *thiC* and *dsbI*. *rrs* has been  
356 commonly used as a reference gene in several previous works (Bronnec et al., 2016; Hyytiainen et  
357 al., 2012; Turonova et al., 2017). *asd*, *proS*, and *thiC* are involved in the amino-acid biosynthesis  
358 pathway, and *dsbI* is part of the *C. jejuni* redox system required for disulfide bond formation in some  
359 proteins. Surprisingly, *rpoA* and *slyD* were considered as reference genes for *C. jejuni* (Phongsisay et  
360 al., 2007; Ritz et al., 2009; Stintzi, 2003), but turned out to be pretty bad reference genes in the  
361 current study because their expression changed following stress. This highlights the importance of a  
362 robust strategy to select reference genes.

363 The expression of 44 genes was quantified by RT-qPCR in three *C. jejuni* strains (C09MJLT205,  
364 RM1221, and C97anses640) after exposure to stresses inspired by the poultry slaughtering process.  
365 In order to mimic conditions that may be encountered by *Campylobacter*, three steps were  
366 successively applied, namely a hot stress assimilated to the scalding step, followed by a short resting  
367 time at 22°C assimilated to the time poultry is left at ambient temperature between scalding and  
368 chilling, and finally a cold step, assimilated to chilling. These conditions were determined after  
369 visiting several slaughterhouses in France (Duqué et al., 2019).

370 In addition to transcriptional analyses, the three strains were enumerated following these stresses.  
371 The combined heat and cold stresses barely inactivated all three strains. Despite this low inactivation,  
372 these stresses influenced *C. jejuni* gene expression, since 24 genes were differentially expressed  
373 under at least one of the temperature conditions and in one or more strains. By selecting genes *a*  
374 *priori*, we found that 59% of them were differentially expressed in at least one of these stressful  
375 conditions, showing that such a selection procedure may be suitable when a limited number of genes  
376 is targeted. Global transcriptomic approaches highlighted that around 20% of the genes were  
377 differentially expressed after a thermal stress (Riedel et al., 2020a; Stintzi, 2003). This represents a  
378 higher number of coding sequences than in the present study.

379 Different profiles of strain-dependent gene expression emerged after thermal stress. Genes  
380 expressed in the same manner after stress whatever the strain were mainly involved in the heat shock  
381 response. Whereas genes differentially expressed according to the strain were involved in the  
382 oxidative stress response or the central metabolism. Most of these genes (14/24) were under-  
383 expressed in *C. jejuni* C09MJLT205 and RM1221 strains from 51°C or 54°C, respectively. Besides,  
384 strain variability was higher than biological variability (reproducibility of biologically independent  
385 replicates) and was consistent with the literature (Aryani et al., 2015; den Besten et al., 2017). This  
386 result highlights the reproducibility of the experimental method.

387 The comparison of the *C. jejuni* transcriptional response following application of one stress  
388 *versus* two consecutive stresses revealed that only few genes were differentially expressed following  
389 heat stress alone *versus* heat plus cold stresses. Although the transcriptomic response to cold stress  
390 was not investigated alone (without pre-adaptation to heat-shock), the comparison between gene  
391 expression after heat stress alone (H) and successive heat stress + ambient temperature + cold stress  
392 (H+C), seemed to show that the heat stress had a limited pre-adaptation impact on the *C. jejuni*  
393 transcriptional response to cold stress (H ~ H+C). The molecular response to the cold stress is poorly  
394 documented in *C. jejuni*. No gene encoding cold shock proteins has been identified in genomes  
395 available (Parkhill et al., 2000), which suggests that this pathogen may have other tolerance  
396 mechanisms to respond to cold stress. Studies so far have shown oxidative stress defense, particularly  
397 SodB, played an important role in the cold stress tolerance of *Campylobacter* (Garenaux et al., 2009;  
398 Stead and Park, 2000; Stintzi, 2003). In comparison with heat shock, cold stress applied in the current  
399 study induced repression of *pebC* gene and overexpression of *racR* in the three *C. jejuni* strains.  
400 Some studies have already investigated the implication of RacR in *C. jejuni* thermoregulation under  
401 elevated temperatures (Apel et al., 2012; Brás et al., 1999). Our work suggests a potential role of this  
402 regulator in a wider range of temperatures. Regarding *pebC* gene, the transition from heat to cold  
403 stress led to a dramatically expression decrease by 4 log<sub>2</sub> fold changes, which is different from the  
404 results obtained by Shi (2014) (Shi, 2014). The *pebC* gene encodes a component of the amino acid  
405 ABC transporter involved in aspartate and glutamate metabolism and required for the microaerobic  
406 growth (Stahl et al., 2012). This drop may be linked to a decrease in glutamate utilization during cold  
407 stress, because of its rapid metabolization during stationary phase before stress induction (Stahl et al.,  
408 2012).

409 Following the successive heat and cold stresses, the gene expression profiles of each strain were  
410 compared by hierarchical clustering analysis. Two strains – C09MJLT205 and RM1221 – had a  
411 rather similar profile compared with the third one (C97anses640). The 24 genes differentially  
412 expressed following the two stresses were mainly categorized into three different groups: i) genes  
413 involved in the general stress response, ii) regulators, and iii) genes involved in the central  
414 metabolism.

415 Among the 24 differentially expressed genes, four genes – the transcriptional regulator *hrcA* and  
416 the chaperone-encoding genes *clpB*, *dnaK*, *grpE* – were up-regulated by thermal stress whatever the  
417 strain. This suggests that these genes can be considered as biomarkers of sublethal thermal stress in  
418 *C. jejuni*. The up-regulation of these genes was consistent with several results demonstrating the  
419 involvement of these HSPs in response to heat, acid or oxidative stresses in *C. jejuni* but also in *C.*  
420 *coli* and *C. lari* (Andersen et al., 2005; Cameron et al., 2012; Flint et al., 2014; Holmes et al., 2010;  
421 Konkel et al., 1998; Palyada et al., 2009; Reid et al., 2008a; Reid et al., 2008b; Riedel et al., 2020a;  
422 Riedel et al., 2020b; Stintzi, 2003; Sulaeman et al., 2012). They act by repairing and preventing  
423 damage caused by the accumulation of unfolded proteins (Stintzi, 2003). Several heat shock proteins  
424 (e.g., DnaK) also play a crucial role under optimal physiological conditions by assisting in the proper  
425 folding of newly synthesized proteins (Stintzi, 2003). However, other genes were expected to be  
426 differentially expressed following thermal stress. For example, *hspR* – encoding a transcriptional  
427 regulator – and the chaperone-encoding gene *groEL*, both involved in the *C. jejuni* heat stress  
428 response, were not significantly over-expressed in any of the three strains.

429 The heatmap analysis also highlighted that three transcripts were significantly less abundant in  
430 the three strains at the highest temperature. These genes included the two regulators *cosR* and *csrA*,  
431 which are involved in the *C. jejuni* oxidative stress response (Fields and Thompson, 2008; Garénaux  
432 et al., 2008; Hwang et al., 2011b). The differential expression of genes involved in the oxidative  
433 stress response was no surprise because *C. jejuni* were exposed to atmospheric oxygen in addition to  
434 heat stress. Furthermore, oxidative stress is related to the *C. jejuni* response to temperature stress.  
435 This pathogen is indeed more susceptible to oxidative stress at high temperatures (42°C) than at low  
436 temperatures (4°C), suggesting that temperature affects oxidative stress resistance in *C. jejuni*  
437 (Garenaux et al., 2008). In *Escherichia coli*, increasing heat increases the generation of reactive  
438 oxygen species (ROS), which leads to an oxidative stress response (Marcén et al., 2017).  
439 Interestingly, the expression levels of genes involved in the oxidative stress response, namely *cosR*,  
440 *csrA* and *katA*, were lower with increased temperature. This result can be linked to oxygen solubility  
441 in water, which decreases as temperature increases. This may have resulted in a lower oxygen  
442 concentration in contact with *C. jejuni* cells, and in turn lower expression of these genes at 54°C than  
443 at 46°C.

444 CosR (*Campylobacter* oxidative stress regulator) is an OmpR-type response regulator involved in the  
445 control of oxidative stress resistance in *C. jejuni* (Hwang et al., 2011a; Hwang et al., 2011b; Hwang  
446 et al., 2012). Autoregulation of CosR could also be influenced by the post-transcriptional regulator  
447 CsrA (Fields et al., 2016). CosR may respond selectively to superoxide stress (compared to peroxide  
448 stress), as superoxide is the first toxic by-product of the oxygen reduction cycle (Hwang et al.,  
449 2011b). Resistance of *C. jejuni* to superoxide stress (and to aerobiosis conditions) may ensue from  
450 reduced CosR protein levels, which would derepress *sodB*. SodB is the only enzyme able to detoxify  
451 superoxide ions in *C. jejuni*. In addition, decreased *cosR* expression may lead to reduced *katA* and  
452 *ahpC* gene expression, which cannot contribute to superoxide detoxification (Hwang et al., 2012). In  
453 the present study, *cosR* and *katA* gene expression decreased at the highest temperature in the three  
454 strains, in line with the literature (Hwang et al., 2011a; Hwang et al., 2011b; Hwang et al., 2012).  
455 However, *sodB* and *ahpC* expression was not significantly affected. It should be recalled that CosR is  
456 not the only regulator of ROS detoxification genes. Regulators PerR and Fur also play a role  
457 (Butcher et al., 2015; Kim et al., 2011; Palyada et al., 2009; van Vliet et al., 1999). Therefore, these  
458 findings underline the complexity of the regulation of gene expression and an interaction between co-  
459 regulators and the environment.

460 Eleven genes implied in the lipid, amino acid and energy metabolisms were down-regulated in the  
461 two C09MJLT205 and RM1221 strains from 51°C and at 54°C, respectively. Among them, four  
462 genes were involved in amino-acid transport and metabolism (*slyD*, *argF*, *trpD*, *ilvC*), in line with  
463 previous studies dealing with the effect of cold or heat stresses on *Campylobacter* (Guccione et al.,  
464 2017; Shi, 2014). Furthermore, the down-regulation of *csrA* in the three strains at the highest  
465 temperature could explain the low level of transcripts involved in the amino acid metabolism (*ilvC*,  
466 *argF*), the energy metabolism (*atpA*) and the stress response (*kataA*), as shown by Fields et al. (2016)  
467 in *csrA* mutants. CsrA appears to regulate a number of cellular processes, including transcription and  
468 translation, the nucleotide / amino acid metabolism, DNA repair, energy homeostasis, etc. (Fields et  
469 al., 2016).

470 Genes encoding cell surface components were also down-regulated, such as *yciA* involved in the lipid  
471 metabolism, *fliP* in flagellum biosynthesis, and *kdtA* encoding for lipid A of the lipooligosaccharide  
472 (LOS). *C. jejuni* LOS consists of a lipid A moiety, an inner core composed of a conserved  
473 trisaccharide, and a strain-variable outer core consisting of various sugars (Karlyshev et al., 2005).  
474 This structure is important for many pathogens to bypass the host immune defenses. The effect of  
475 temperature on LOS length is strain-dependent (Semchenko et al., 2010), suggesting a role in the  
476 pathogen's adaptive mechanisms or stress response. *kdtA* can also be down-regulated under acid or  
477 oxidative stress conditions (Guccione et al., 2017; Kaakoush et al., 2009; Palyada et al., 2009; Reid et  
478 al., 2008a), but not under heat stress conditions (Stintzi, 2003). In addition, cell envelope proteins can  
479 be down-regulated following cold shock (Shi, 2014; Stintzi and Whitworth, 2003). These findings  
480 could suggest a remodeling of the membrane and cell wall structures in response to stress. The cell  
481 membrane composition is generally modified by bacteria under unfavorable environments such as  
482 heat or cold stress in order to maintain membrane integrity against stressful conditions (Hughes et al.,  
483 2009; Siliakus et al., 2017). The rapid chilling of *C. jejuni* cultures, analogous to poultry carcass  
484 chilling, would result in a rapid and large-scale shutdown of cellular processes (Hughes et al., 2009).

485 Interestingly, two strains, C09MJLT205 and RM1221, had rather similar transcriptomic  
486 profiles compared with the third one, C97anses640, in agreement with the results of a previous study.  
487 Indeed, *C. jejuni* C97anses640 previously submitted to successive heat and cold stresses was less  
488 resistant than strains C09MJLT205 and RM1221 to a subsequent stressing step of refrigerated  
489 storage under a modified atmosphere (Duqué et al., 2019). These results suggest a possible  
490 correlation between stress-induced gene expression and future bacterial behavior.

## 491 **Conclusion**

492 Consecutive thermal stresses inspired from the broiler slaughtering process induced a limited  
493 inactivation of *C. jejuni* populations, but altered the molecular response of the pathogen at the  
494 transcriptomic level. Among the 44 genes under study, 24 were differentially expressed following the  
495 two stresses and belonged to three different groups: i) genes involved in the general stress response,  
496 ii) regulators, and iii) genes involved in the central metabolism. Our results pointed out  
497 transcriptomic variability of *C. jejuni* strains exposed to stresses based on the poultry slaughtering  
498 process. Indeed, thermal stress application induced strain-dependent gene expression patterns, with  
499 one strain exhibiting a profile significantly different from the two other strains. Genes involved in the  
500 heat shock response were mainly overexpressed after stress application in the same manner no matter  
501 the strain. In contrast, genes implicated in the oxidative stress response or the central metabolism  
502 were differentially expressed according to the strain. For example, it appeared that among the  
503 differentially expressed genes, some genes involved in oxidative response like *CbrR*, *CJE1780* and  
504 *nuoL*, were mainly more expressed in the C97anses640 than in the two other strains whereas genes

505 involved in amino acid metabolism, lipid metabolism and energy metabolism were less down  
506 regulated in the atypical strain. These results suggest that the adaptative stress response in bacteria is  
507 strain-dependent. Since the atypical C97anses640 strain was also shown to behave differently  
508 according to stress, these results pave the way for further investigations on bacterial adaptation.

509 Further work is indeed needed to consider the possible correlation between the altered gene  
510 expression patterns highlighted in the current work and enhanced *C. jejuni* resistance to a subsequent  
511 stress. If this is confirmed, these genes could be used as biomarkers to improve the prediction of  
512 bacterial fitness and adaptation because biomarkers are a promising avenue for next-generation  
513 predictive models.

514

## 515 **5 Conflict of Interest**

516 The authors declare that the research was conducted in the absence of any commercial or financial  
517 relationships that could be construed as a potential conflict of interest.

## 518 **6 Author Contributions**

519 J-M.M., S.G. and N.H. conceived the original idea. B.D., J-M.M., S.G. and N.H. conceived and  
520 planned the experiments. B.D., S.R., and A.R. carried out the experiments. B.D., S.R., S.G. and N.H.  
521 contributed to the interpretation of the results. B.J., S.G. and N.H. took the lead in writing the  
522 manuscript. All authors provided critical feedback and helped shape the research, analysis and  
523 manuscript.

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820

821 **Table 1.** Function of the forty target genes of *C. jejuni* used in this study.

Categories according to the tree decision	Gene	Accession number of <i>C. jejuni</i> RM1221	Annotations	Involvement in stress response						References
				oxidative	heat	cold	acid	basic	osmotic	
<b>Regulation/regulator</b>	<i>hrcA</i>	CJE0848	heat-inducible transcriptional repressor	X	X		X		X	(Cameron et al., 2012; Flint et al., 2014; Holmes et al., 2010; Palyada et al., 2009; Reid et al., 2008b; Stintzi, 2003)
	<i>perR</i>	CJE0367	peroxide stress response regulator	X			X			(Burgess et al., 2016; Palyada et al., 2009; Reid et al., 2008b)
	<i>cbrR</i>	CJE0746	two-component response regulator	X					X	(Cameron et al., 2012; Kaakoush et al., 2009)
	<i>Fur</i>	CJE0449	fur ferric uptake regulator	X						(Askoura et al., 2016; Burgess et al., 2016; Butcher et al., 2015; Palyada et al., 2009; van Vliet et al., 1999)
	<i>hspR</i>	CJE1365	heat shock transcriptional regulator		X		X		X	(Askoura et al., 2020; Cameron et al., 2012; Holmes et al., 2010; Stintzi, 2003)
	<i>lysR</i>	CJE1080	transcriptional regulator	X						(Dufour et al., 2013)
	<i>CJE1780</i>	CJE1780	two-component regulator	X			X			(Guccione et al., 2017; Reid et al., 2008b)
	<i>cmeR (tetR)</i>	CJE0417	transcriptional regulator	X			X			(Butcher et al., 2015; Guccione et al., 2017; Reid et al., 2008b)
	<i>cosR</i>	CJE0404	two-component regulator	X			X			(Garenaux et al., 2009; Hwang et al., 2011b; Varsaki et al., 2015)
	<i>racR</i>	CJE1397	two-component regulator	X	X				X	(Apel et al., 2012; Brás et al., 1999)
	<i>rprA</i>	Cj1546	transcriptional regulator	X						(Gundogdu et al., 2015)
	<i>rprB</i>	Cj1556	transcriptional regulator	X						(Gundogdu et al., 2015)
	<i>csrA</i>	CJE1246	carbon storage regulator	X						(Fields and Thompson, 2008)
<b>Involvement in one stress response</b>	<i>clpX</i>	CJE0324	ATP-dependent protease ATP-binding subunit	X						(Cohn et al., 2007)
<b>Involvement in two stress responses</b>	<i>atpA</i>	CJE0100	Synthase subunit alpha	X					X	(Cameron et al., 2012; Kaakoush et al., 2009)
	<i>fliP</i>	CJE0907	flagellar biosynthesis protein					X	X	(Butcher et al., 2015; Palyada et al., 2009)
	<i>CJE1361</i>	CJE1361	two-component sensor histidine kinase				X		X	(Butcher et al., 2015; Kaakoush et al., 2009; Palyada et al., 2009; Varsaki et al., 2015)
	<i>putA</i>	CJE1676	proline dehydrogenase /delta-1-pyrroline-5-carboxylate dehydrogenase	X			X			(Guccione et al., 2017; Reid et al., 2008b)
	<i>dsbl</i>	CJE0017	disulfite bond formation protein	X			X			(Butcher et al., 2015; Guccione et al., 2017; Reid et al., 2008b)
	<i>pebC</i>	CJE1000	amino acid ABC transporter ATP-binding	X			X			(Butcher et al., 2015; Guccione et al., 2017; Reid et al., 2008b)

*Campylobacter* molecular response to stress

Categories according to the tree decision	Gene	Accession number of <i>C. jejuni</i> RM1221	Annotations	Involvement in stress response						References
				oxidative	heat	cold	acid	basic	osmotic	
			protein							
	<i>ahpC</i>	CJE0379	alkyl hydroperoxide reductase	X			X			(Baillon et al., 1999; Birk, 2012; Kaakoush et al., 2009; Oh et al., 2015; Palyada et al., 2009; Reid et al., 2008b; Varsaki et al., 2015)
	<i>trpD</i>	CJE0395	anthranilate synthase subunit II	X			X			(Guccione et al., 2017; Reid et al., 2008b)
	<i>yidC</i>	CJE1038	membrane protein insertase	X			X			(Guccione et al., 2017; Reid et al., 2008b)
	<i>hupB</i>	CJE0991	NA-binding protein	X		X				(Guccione et al., 2017; Shi, 2014)
	<i>proS</i>	CJE0647	proline--tRNA ligase				X	X		(Shi, 2014; Varsaki et al., 2015)
	<i>asd</i>	CJE1167	aspartate-semialdehyde dehydrogenase	X		X				(Butcher et al., 2015; Guccione et al., 2017; Shi, 2014)
<b>Involvement in ≥ 3 stress responses</b>	<i>grpE</i>	CJE0849	heat shock protein	X	X		X		X	(Askoura et al., 2020; Cameron et al., 2012; Flint et al., 2014; Palyada et al., 2009; Reid et al., 2008b)
	<i>groEL</i>	CJE1356	Chaperone protein	X	X		X			(Askoura et al., 2020; Cameron et al., 2012; Reid et al., 2008b; Stintzi, 2003; Sulaeman et al., 2012)
	<i>dnaK</i>	CJE0850	Chaperone protein	X	X		X		X	(Cameron et al., 2012; Flint et al., 2014; Reid et al., 2008b; Stintzi, 2003; Sulaeman et al., 2012)
	<i>katA</i>	CJE1576	catalase A	X			X			(Cameron et al., 2012; Oh et al., 2015; Palyada et al., 2009; Reid et al., 2008b; Varsaki et al., 2015)
	<i>argF</i>	CJE1074	delta-aminolevulinic acid dehydratase	X		X	X			(Guccione et al., 2017; Shi, 2014; Varsaki et al., 2015)
	<i>rpoB</i>	CJE0528	DNA-directed RNA polymerase subunit beta	X		X	X			(Guccione et al., 2017; Reid et al., 2008b; Shi, 2014)
	<i>yciA</i>	CJE0993	acyl-CoA thioesterase	X			X	X		(Butcher et al., 2015; Guccione et al., 2017; Reid et al., 2008b)
	<i>kdtA</i>	CJE0807	3-deoxy-D-manno-octulosonic acid transferase	X	X		X			(Kaakoush et al., 2009; Palyada et al., 2009; Reid et al., 2008b; Stintzi, 2003)
	<i>clpB</i>	CJE0616	Chaperone protein		X		X		X	(Cameron et al., 2012; Reid et al., 2008b; Stintzi, 2003)
	<i>sodB</i>	CJE0164	superoxide dismutase							(Cameron et al., 2012; Kaakoush et al., 2009; Oh et al., 2015; Stintzi, 2003)
	<i>htrA</i>	CJE1363	serine protease	X	X		X		X	(Baek et al., 2011; Boehm et al., 2015; Brondsted et al., 2005; Cameron et al., 2012; Reid et al., 2008b; Sulaeman et al., 2012)
	<i>sdhA</i>	CJE0488	succinate dehydrogenase	X	X		X			(Palyada et al., 2009; Reid et al., 2008b; Stintzi, 2003)

Categories according to the tree decision	Gene	Accession number of <i>C. jejuni</i> RM1221	Annotations	Involvement in stress response						References
				oxidative	heat	cold	acid	basic	osmotic	
			flavoprotein subunit							
	<i>htrB</i>	CJE1276	lipid A biosynthesis lauroyl acyltransferase	X	X		X		X	(Phongsisay et al., 2007; Trigui et al., 2017)
	<i>nuoL</i>	CJE1739	NADH-quinone oxidoreductase subunit L	X			X		X	(Cameron et al., 2012; Kaakoush et al., 2009; Varsaki et al., 2015)

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824 **Table 2.** Primer pairs sequences for *C. jejuni* genes used in this study

Genes	Forward	Reverse	Amplicon size (pb)	Efficiency (%)	R <sup>2</sup>
<b>Control of RNA samples</b>					
<i>341F/758R</i>	CCTACGGGAGGCAGCAG	CTACCAGGGTATCTAATCC	439	/	/
<b>Potential reference genes</b>					
<i>rpoA</i>	CGAGCTTGCTTTGATGAGTG	AGTTCCCACAGGAAAACCTA	80	100	1.00
<i>rrs</i>	AAGGGCCATGATGACTTGAC	AGCGCAACCCACGTATTTAG	107	100	1.00
<i>ilvC</i>	GCATGCAGAACGCAAAAATA	TGATCCAAGGCATCATAGCA	109	92	1.00
<i>gyrA</i>	GTTATTATAGGTCGTGCTTT	CTATGAGGTGGGATGTTTGT	92	89	1.00
<i>slyD</i>	TACGATGAAAATGCCGTTCA	TTCGCCAAAAGCTCCATAC	84	95	1.00
<i>thiC</i>	TTATCTTTGGGCGATGCTTT	CATCCCAAGCCCTTTGAGTA	109	93	1.00
<b>Target genes</b>					
<i>hrcA</i>	GGGCTTAAGGTAGATGCGCA	TCATGCCGCCTCCTTTATGT	111	93	0.97
<i>groEL</i>	AGTAATCGGTGGTGGTGCAG	CTTTCAACGATAGCTGCGCC	96	109	1.00
<i>atpA</i>	CGTGAAGCTTATCCAGGCGA	GCAATGCCGTCAAAGAACCA	103	98	1.00
<i>clpX</i>	AGCTGCAGATGGAGATGTGC	AAGCTTGTTGCACACCCTCT	131	94	1.00
<i>dnaK</i>	CACGCGGTATGCCACAAATC	CCTCGCTAAGTCCGCTTGAA	132	97	1.00
<i>sodB</i>	CAAACTTCAAATGGGGGCGT	ACAGCCACAGCCTGTACTTG	97	94	1.00
<i>katA</i>	CAGGCGCAAAGGACCTTTG	TTCCCTTGGCATGAACGGTT	105	93	1.00
<i>perR</i>	CCTTCAATCTTTAGCGACGG	CCACATTTGGTGCAAACAACA	146	90	1.00
<i>cbpR</i>	CTTGCCAGATGCCCAAATG	GCCTTATCACCCTAGCCGT	90	98	1.00
<i>CJE1780</i>	TCCAACAAGCAAAGCAGTGC	AGAGATTGTTTTCTGTGGGAAGT	136	94	1.00
<i>cmeR (tetR)</i>	TCCAATTGGCAAGATGTCTATCT	TCAATCAACCAGAAGCTGTAGC	81	93	1.00
<i>cosR</i>	GGGAAGCAGAAACGGTATCCT	TGCCATCTGGGAAGAACCTG	141	99	1.00
<i>fur</i>	CCATTTCTTTTGGTTCAGCAGGT	GCAATCAAGGCTTGCTGTCT	136	92	1.00
<i>hspR</i>	AGAGCCAAGTAGAACCGATGG	ACTCCAGCAAGATTGATTCCCA	111	93	1.00
<i>lysR</i>	CGCTCTCAAACGCAGAATTCA	AGCTGGATCAAGTTCTGCTTCA	93	93	1.00
<i>putA</i>	GCTAGTGGTGCAGCTATGGT	TTCGCACCTAAGGGCATAGG	83	106	1.00
<i>racR</i>	TTGTGGGGCTTCAAATCGGT	TCAACTCTTTTTGTGCGACGA	106	93	1.00
<i>rprA</i>	CGCTCAAATTCCACCCAAGG	TGCCCCATTTGCTCATAGCT	89	99	0.98
<i>rprB</i>	CGAGATTTGTTGCAAGGGACTAA	CTTTGCGTTTGATCAGTTTTGC	139	93	1.00
<i>fliP</i>	TTTGCCTCCCATAGCTTGT	TGCGCTCGCTCCAAGTATAG	92	92	1.00
<i>dsbl</i>	AAGCCCTACTGCTGTCATGA	GCTGGGGACAAAGAGAAGCA	116	90	1.00
<i>pebC</i>	GGTTTCTGGATCAAGGGCTGA	AGCAACCCTTTCAGGTGGAC	112	91	1.00
<i>htrA</i>	TGCCGTTATTTCCACCACCA	CAGGTGGAGCTTTGGTGGAT	84	103	1.00
<i>ahpC</i>	GAAACAGTTCGCCATGCTGTG	TCGCCCTTATTCCATCTGC	133	97	0.99
<i>grpE</i>	TGAAAAACATGGGGTGGCTCT	AGCACTTGAACCCTTCACCA	117	98	1.00
<i>hupB</i>	TCTTTTGGCGCTTGCTTTTG	GCTAGAGTACCAAGCACAGGA	112	97	1.00
<i>proS</i>	GGCTATTGGTGGAAGTGGCT	GCAGCTTCAACATTAGCCGC	108	94	1.00
<i>asd</i>	GCATTCAGGAACCACCAAAGG	GCAGGTGGAAGTGTGAGTGA	120	91	1.00
<i>sdhA</i>	GGTAGATCAAGACGCGGCTT	AAACGCTGAGCTACTCCACC	117	92	1.00

Genes	Forward	Reverse	Amplicon size (pb)	Efficiency (%)	R <sup>2</sup>
<i>Control of RNA samples</i>					
<i>htrB</i>	TGCGGCTAAATATGGTGC GA	AAATTGGGTGCGACTTTGGC	91	95	1.00
<i>yciA</i>	TCCTGAACGCGTTGTAACCA	CGCTGATTGAAGTGTGCCC	119	93	1.00
<i>argF</i>	ATCCTTTACAGGCTCGCCAC	CCATAACAGAGCTTGGCGGA	80	100	1.00
<i>rpoB</i>	GGCTAATGGCGTTGATGCAG	TCACCTGGGCGCATAACTTT	141	90	1.00
<i>CJE1361</i>	TCGCATTGCAATTTCTTTGGC	GCAAATTACTTCAGGGGCTGC	88	94	1.00
<i>YidC</i>	TCACAACGATAGCCCAACCC	ATCCACGCCTTGAAGCAGTT	120	92	1.00
<i>trpD</i>	AAGCGGTAGAATGCAAGGGG	GCCACTAAAGGCGAACTTGC	96	95	0.99
<i>kdtA</i>	TGCCAAACCTAAGGAAAAGCT	TAAAACGCTCAGGGTGTCTGT	131	97	0.99
<i>csrA</i>	GAAAGTATAATTATCGGAGAAGG	CAACAGAATGCAAATTTTCATC	151	87	1.00
<i>nuoL</i>	ACCAAATAAACCCCGCTGT	CACACTTGGCTTGCTGATGC	92	87	1.00
<i>clpB</i>	GGCGCACCTAAGAGTCTTGA	GGCCAACAGGGGTAGGAAAA	136	87	1.00

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827 **Table 3.** Reference genes selected and associated M value for each *C. jejuni* strain. In bold, the two  
 828 genes selected as internal reference for each strain, according to their M-value (< 0.5).

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<i>C. jejuni</i> strain	Gene	M value
C09MJLT205	<i>asd</i>	<b>0.16</b>
	<i>dsbI</i>	0.475
	<i>proS</i>	<b>0.16</b>
	<i>thiC</i>	0.255
	<i>rrs</i>	0,485
RM1221	<i>asd</i>	<b>0.41</b>
	<i>dsbI</i>	0.735
	<i>proS</i>	0.790
	<i>thiC</i>	0.865
	<i>rrs</i>	<b>0,410</b>
C97anses640	<i>asd</i>	0,525
	<i>dsbI</i>	<b>0.30</b>
	<i>proS</i>	0.375
	<i>thiC</i>	<b>0.30</b>
	<i>rrs</i>	0,565

830

831 **Table 4.** Mean inactivation (expressed in  $\Delta\log$  CFU.mL<sup>-1</sup>) induced by heat stress only, cold stress  
 832 only and cumulated stresses, with respective standard deviation, of the three strains of *C. jejuni*  
 833 (C97anses40, C09MJLT205 and RM121) as a function of the heat stress temperature (54, 51 and  
 834 46°C).

835

Heat stress temperature (°C)	Strain	$\Delta\log$ (CFU.mL <sup>-1</sup> )		
		Heat stress	Cold stress	Cumulated stress
54	C97anses640	0.37 ± 0.11	-0.03 ± 0.13	0.34 ± 0.01
	C09MJLT205	0.35 ± 0.22	0.15 ± 0.19	0.51 ± 0.04
	RM1221	0.16 ± 0.03	0.26 ± 0.10	0.44 ± 0.07
51	C97anses640	0.03 ± 0.05	0.19 ± 0.19	0.23 ± 0.11
	C09MJLT205	-0.06 ± 0.14	0.01 ± 0.22	-0.05 ± 0.25
	RM1221	-0.02 ± 0.10	0.33 ± 0.04	0.31 ± 0.08
46	C97anses640	-0.06 ± 0.07	-0.04 ± 0.06	-0.10 ± 0.06
	C09MJLT205	-0.13 ± 0.11	-0.15 ± 0.20	-0.28 ± 0.10
	RM1221	0.01 ± 0.02	0.16 ± 0.04	0.17 ± 0.03

836 Negative values mean no inactivation due to the uncertainty from enumeration method.

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840 **List of figures**

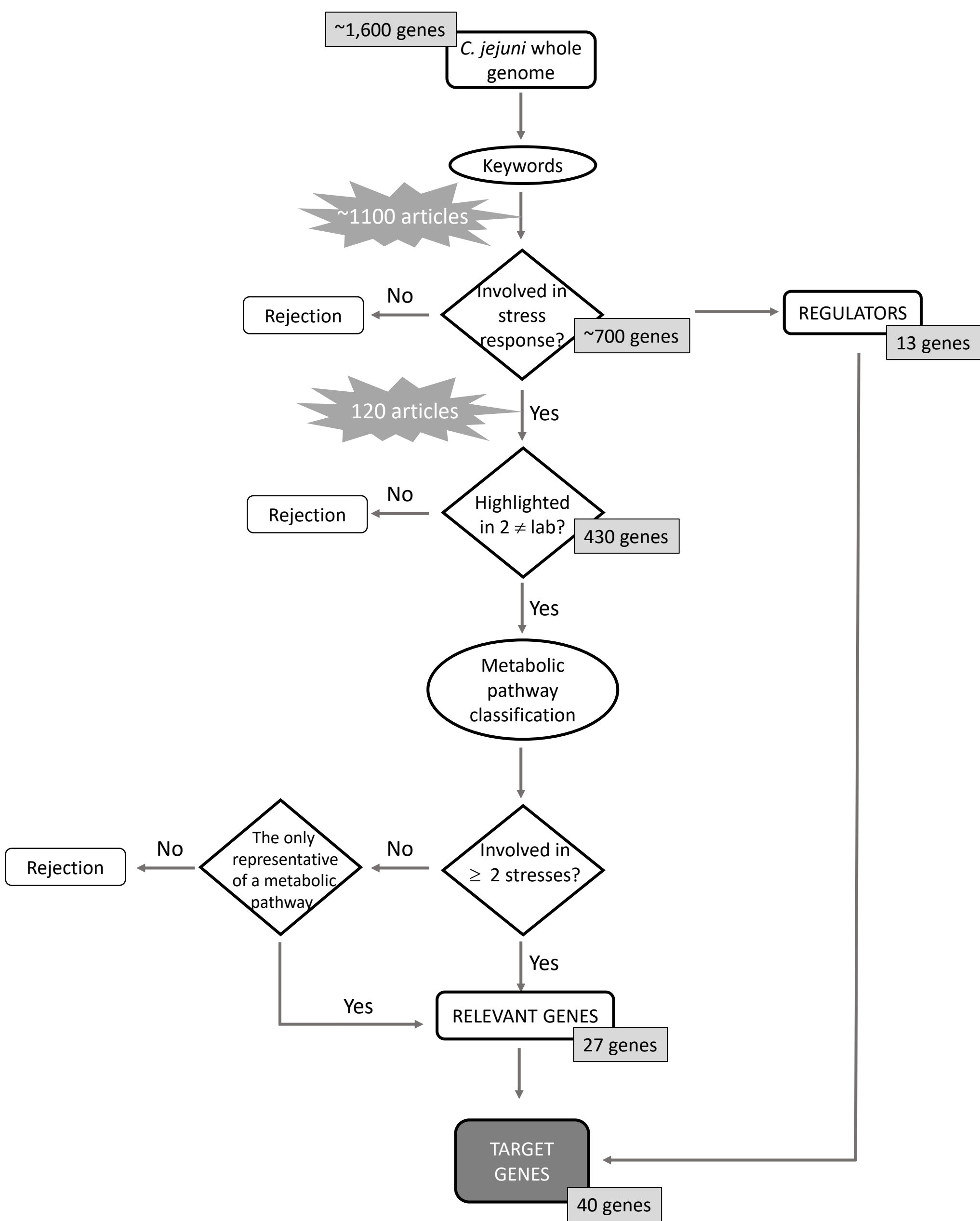
841 **Figure 1.** Decision tree for the selection of target genes according to the literature analysis.

842 **Figure 2.** Expression of *katA*, *lysR*, *pebC* and *racR* following heat stress at 46°C with or without  
843 subsequent application of the cold stress. Symbols correspond to mean log<sub>2</sub> fold change (●: RM1221  
844 ; ▲: C09MJLT205 ; ■: C97anses640) and bars to 95% confidence intervals.

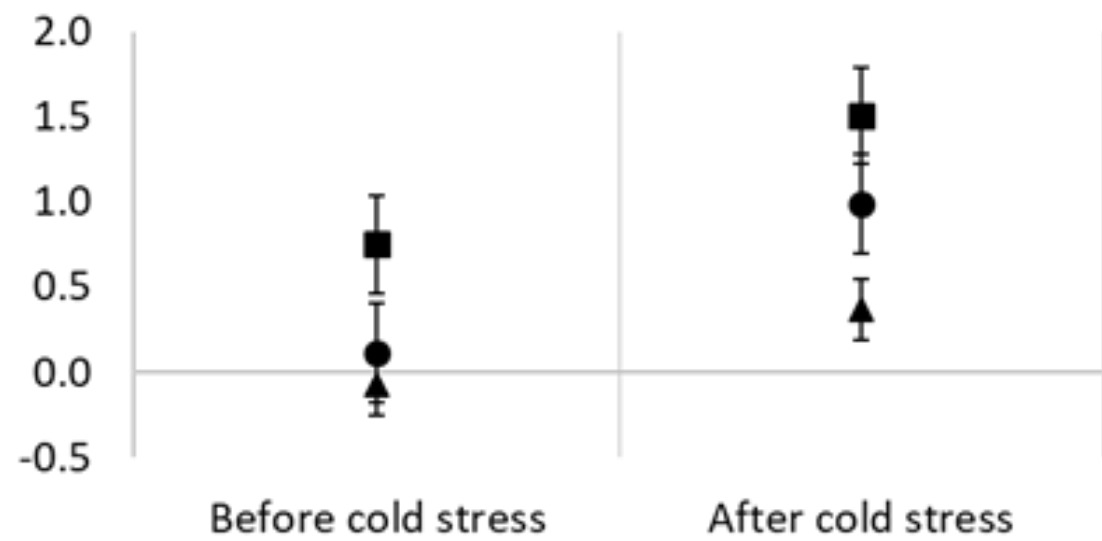
845 **Figure 3.** Hierarchical clustering analysis of gene expression for three *C. jejuni* strains  
846 (C09MJLT205, C97anses640, RM121) after application of successive stresses: heat (46°C, 51°C and  
847 54°C during 3 min) and cold temperature (-4°C during 2 h). A threshold log<sub>2</sub> value of 2 was used in  
848 this figure. The intensity of the color is proportional to expression change. Data were normalized  
849 with the control condition, which was the unstressed culture. The color variation indicates level of  
850 gene expression as compared to the normalizing condition, *i.e.* green, downregulation; red,  
851 upregulation. Each capital letter corresponds to a cluster after the hierarchical clustering was  
852 performed.

853 **Figure 4.** Representative genes from each cluster which were differentially expressed after  
854 application of the consecutive heat and cold stress (open bars, grey bars and black bars correspond to  
855 46, 51 and 54°C, respectively) for the three strains of *C. jejuni* C09MJLT205, RM121 and  
856 C97anses640). For each strain and each gene, different letters (a-c) show significant differences  
857 between the different temperatures ( $p \leq 0.05$ ).

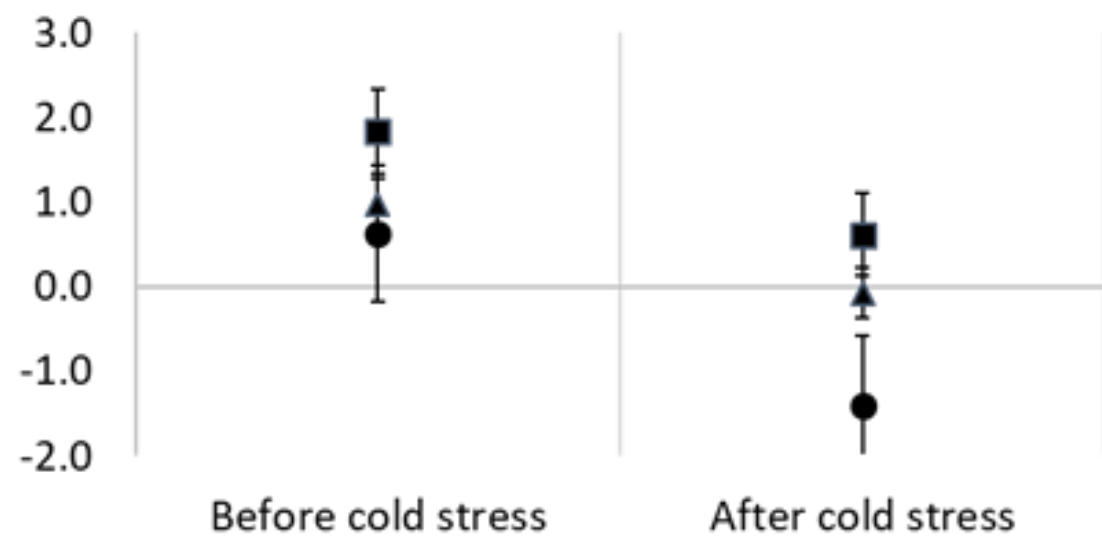
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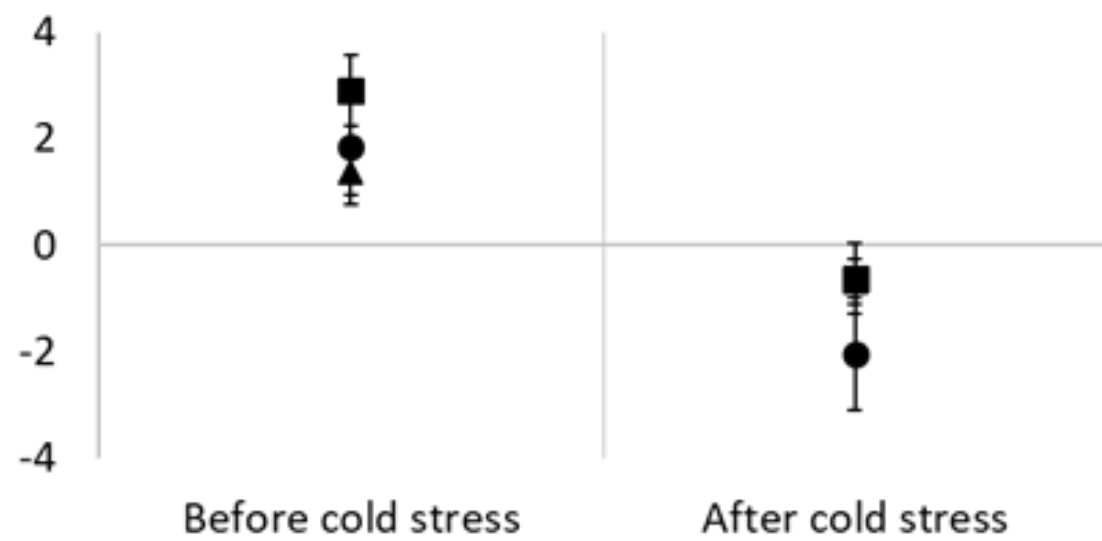
*racR*



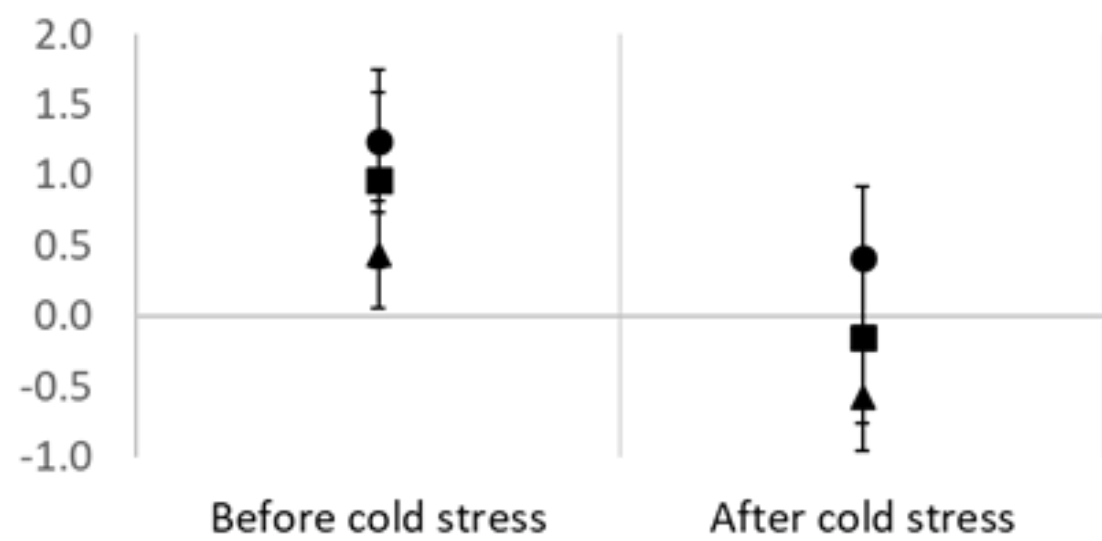
*lysR*



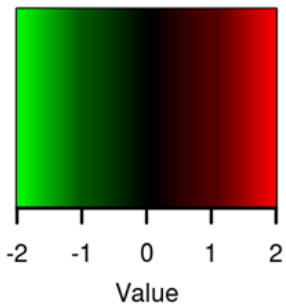
*pebC*



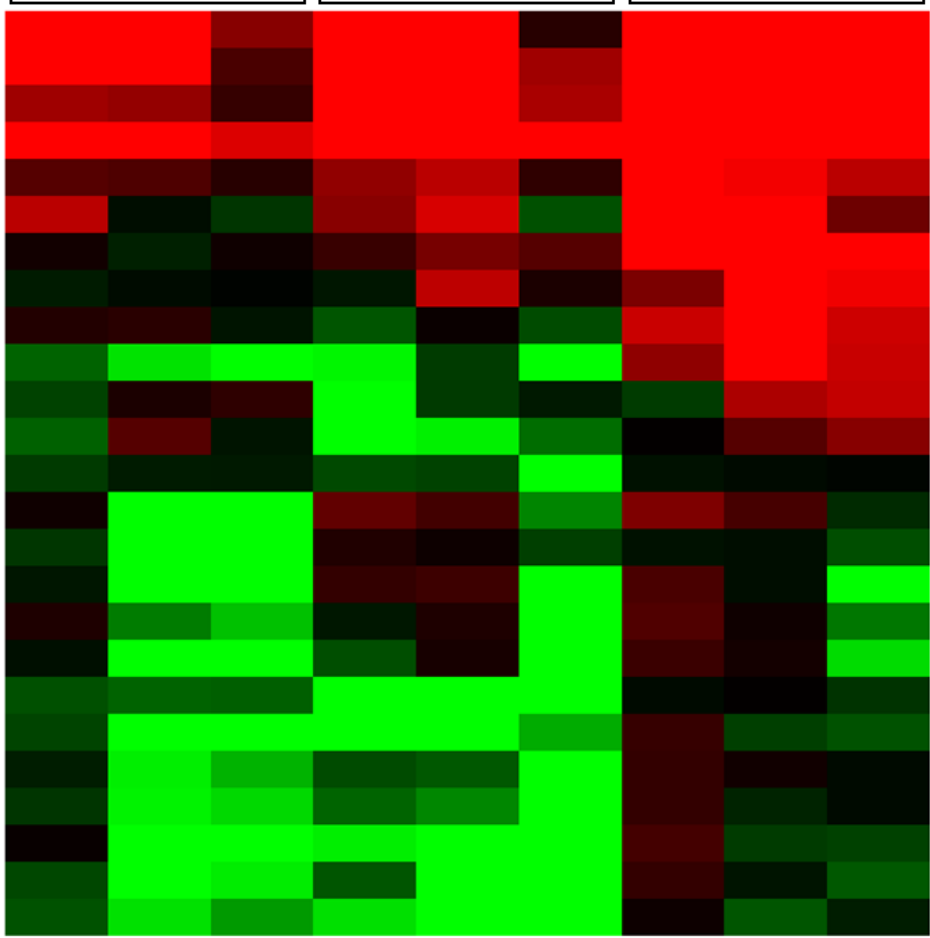
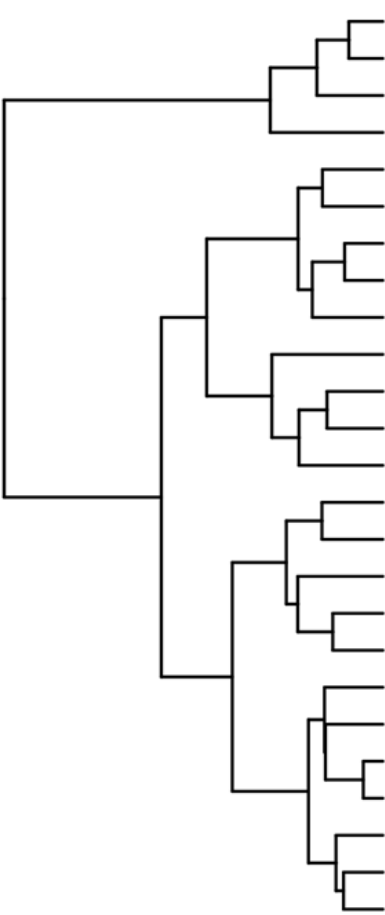
*katA*



### Color Key



CJ09MJLT205 RM1221 C97anses640



hrcA  
grpE  
dnaK  
clpB  
groEL  
hspR  
CJE1780  
cbrR  
nuoL  
gyrA  
pebC  
rpoB  
thiC  
flpP  
katA  
yciA  
csrA  
cosR  
ilvC  
kdtA  
rpoA  
argF  
CJE1361  
atpA  
slyD

46°C 51°C 54°C 46°C 51°C 54°C 46°C 51°C 54°C



