Caractérisation de la réponse au stress digestif *in vitro* « batch » d'une bactérie à Gram-négatif et d'une bactérie à Gram-positif par des outils moléculaires

1. Présentation de l'article 4 en construction

L'objectif de cette dernière partie de la thèse était de caractériser plus finement la réponse au stress digestif de deux micro-organismes, leur choix ayant été effectué en fonction des résultats obtenus dans les chapitres précédents. Des outils moléculaires ont été utilisés avec (i) de la transcriptomique, (ii) de la protéomique, et (iii) la validation de certaines hypothèses par la mise en évidence de métabolismes particuliers. Les stress majeurs auxquels sont soumis les micro-organismes lors de la digestion sont la chute de pH, la présence de sels biliaires et la raréfaction puis la quasi absence d'oxygène (anoxie). Le stress éventuel de la rencontre avec le microbiote intestinal et ses métabolites n'a pas été abordé dans ces travaux.

Les travaux disponibles actuellement sur la réponse au stress digestif de bactéries, concernent surtout les micro-organismes probiotiques ou pathogènes. La résistance au stress acide des bactéries à Gram-positif a été revue de manière assez exhaustive par Cotter and Hill (2003) et, bien que cette revue ait plus de 10 ans, elle reste encore très pertinente. Une revue plus récente de Krulwich et al. (2011) complète les observations de la première revue, en y apportant un aspect mécanistique plus poussé traitant des bactéries à Gram-négatif. Citons aussi les travaux importants et de l'équipe de J. Slonczewski (2009) portant sur le maintien du pH intracellulaire lors de stress acide intense et, notamment, la mise en place de méthode permettant de mesurer ce pH. Les mécanismes de résistance aux sels biliaires ont été décrits dans la revue de Begley et al. (2005) et, surtout, bien caractérisés chez les entérobactéries ainsi que chez certains probiotiques comme les lactobacilles et le genre *Propionibacterium*.

Dans ce contexte de littérature ne traitant pas de micro-organismes « alimentaires » au sens technologique, notre premier choix s'est porté sur la bactérie à Gram-positif *Staphyloccocus equorum* Mu2, cette souche ayant montré un potentiel anti-inflammatoire (Chapitre 1) ainsi qu'une certaine sensibilité au stress gastrique batch, tout en résistant au passage à travers le DIDGI (Chapitre 2). Le second choix a été la bactérie à Gram-negatif *Hafnia alvei* GB001, cette bactérie s'étant montrée la plus résistante à toutes les conditions de digestion (Chapitre 1) et possédant un profil anti-inflammatoire (Chapitre 1).

Nous avons construit un plan d'expérience reprenant la démarche du stress batch utilisée dans le Chapitre 1, tout en modifiant les conditions de stress gastrique de manière à ce que *S.equorum* Mu2, qui ne survivait pas à ces conditions trop drastiques, conserve une viabilité suffisante pour permettre l'extraction de son ARN pour l'analyse RNAseq. Le stress duodénal a été conservé à l'identique. Le stress combiné a consisté en l'application du stress gastrique modifié, suivi du stress duodénal.

En parallèle, une extraction des protéines a été réalisée concernant les deux micro-organismes après application du stress acide seulement dans l'objectif d'une analyse protéomique ultérieure.

2. Faits marquants

- ✓ Nous avons développé une méthode de digestion *in vitro* statique en deux étapes
- ✓ Les bactéries à Gram-positif et à Gram-négatif ont différé par leurs réponses.
- ✓ Nous avons caractérisé la réponse transcriptomique au stress digestif in vitro d'une bactérie à Gram-positif et d'une bactérie à Gram-négatif
- ✓ La réponse au stress combiné de la bactérie à Gram-positif ressemble à la réponse au stress gastrique
- ✓ La réponse au stress combiné de la bactérie à Gram-négatif est intermédiaire entre la réponse au stress gastrique et a réponse au stress duodénal

Insights of the transcriptomic *in vitro* digestive stress response of a Gram-positive and a Gram-negative bacterium from cheese origins.

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Abstract

Although large numbers of viable microorganisms are ingested through ripened cheese consumption, little is known about the microorganisms' ability to withstand digestion. We investigated the resistance to digestive stress of a Gram-positive – *Staphylococcus equorum* Mu2 – and a Gram-negative bacterium – *Hafnia alvei* – constitutive of that ripened cheese microflora. The approach mimicked gastric and/or duodenal digestion. Transcriptional changes were measured using a global RNA-Seq transcriptomic approach and viability of both strains was assessed. *S.equorum* Mu2 was far more sensitive to gastric stress (viability decrease of 3 log CFU/mL) than *H.alvei* GB001 (no significant decrease of viability) and, therefore, more sensitive to a combined stress. Both strains were equally resistant to duodenal challenge. Overall, 1730, 1761 and 2308 genes in *H. alvei* GB001 and 573, 796 and 510 genes in *S.equorum* Mu2, were differentially modified following, respectively, gastric-like, duodenal-like and combined *in vitro* batch stress (*p*-value <0.05) among a total of 4692 CDS in *H. alvei* GB001 and a total of 2932 CDS in *S. equorum* Mu2. Following gastric-like stress, *H. alvei* GB001 exhibited up-regulated genes of the cysteine metabolism (*cysND, cysC* and *cysIJ*) along with glutamate decaboxylation metabolism. Hydrogenases encoding genes from the Hyd complexes were both up- (*hyfFG*) and down-regulated (*hybABCO*, *hypBCD*). *S.equorum* Mu2 up-regulated genes encoding diverse dehydrogenase, among them malate dehydrogenase (*mdh* and *mqo*), along with genes encoding enzymes involved in the polyamine biosynthesis (*potAD*). Both bacteria showed up-regulated genes encoding transporters from the multidrug resistance family (e.g. *emrB* and *acrAB*). *H. alvei* GB001 transcriptomic profile in the combined challenge seems to be an intermediate between the individual stresses whereas S*. equorum* Mu2 displayed a combined stress response very much alike gastric stress.

Highlights

- ✓ We performed a two-steps *in vitro* batch digestive stress experiment.
- ✓ We assessed transcriptomic response to *in vitro* digestive stress of a Gram-positive and a Gram-negative bacteria from cheese origin
- ✓ Gram-positive and Gram-negative bacteria differed in their resistance to digestive challenge.
- ✓ Gram-positive bacteria response to combined challenge looked like gastric challenge
- ✓ Gram-negative bacteria response to combined challenge was intermediate between the individual stress

Keywords

Smear-ripened cheese microbiota, *in vitro* digestive model, digestive stress, transcriptome, RNA-Seq

1. Introduction

Cheese is one of the oldest ways of conserving milk: in Northern Europe, evidence of cheese-making activity has been found at sites dating from the sixth millenium BC (Salque *et* al, 2012).
At present, Europe produces around 9000 thousand tons of cheese per annum (Eurostat, 2013), and Europeans eat between 25 and 30 kg of cheese per capita per annum. Given that a gram of cheese contains 10^8 to 10^9 live microorganisms on average (Beresford *et* al. 2001), the annual intake of viable cells can be estimated at 10^{13} to 10^{14} per capita per annum. Thus, a fermented food product like cheese is an important source of diverse microorganisms in the human diet. However, few studies have investigated the survival of the cheese microflora in the gastrointestinal tract. A review of the literature shows that most of the research in this field has focused on Lactobacilli, Bifidobacteria and Propionibacteria (Cousin et al. 2011, Saarela et al. 2000) with a view to find new probiotics or using cheese as a carrier for known probiotics (Gardiner et al. 1999, Saxelin et al. 2010).
It has been reported that pH is the major stress factor in the gastric compartment, whereas the presence of enzymes has a negligible effect on the microorganisms (Sumeri et al. 2012). The impact of the stomach's hydrochloric acid (HCl) on both Gram-positive and Gram-negative bacteria has been well characterized (mainly potential probiotics or pathogens) (Krulwich et al. 2011). In contrast, the impact of bile has been less documented (Begley et al. 2005) and has focused on food-borne pathogens (e.g. *Escherichia coli* and *Salmonella typhimurium* (Merrit et al. 2009)) or probiotic candidates such as Bifidobacteria and Lactobacilli (Ruiz et al. 2013). One of the few studies related to cheese-ripening bacteria found that the genus *Corynebacterium* survived passage through the gastrointestinal tract in human microbiota-associated rats (Lay et al. 2004). Cheese-ripening yeasts , such as *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Geotrichum candidum*, were found to be able to survive *in vitro* challenges with acid and bile (Kumura et al. 2004, Lay et al. 2004, Psomas et al. 2001). However, to date, no study has been focusing on the response to digestive stress of ripening microorganisms, using high throughput molecular tools such as next generation sequencing.
Given our previous work related to the ability of a selection of ripening microorganisms to withstand digestive stress along with the characterization of their basic immunomodulatory properties (Adouard et al., 2014 accepted, see Chap. 1; Adouard et al. unpublished data, see Chap.2), we selected for this study a Gram-positive bacterium (*Staphylococcus equorum* Mu2) and a Gram-negative bacterium (*Hafnia alvei* GB001) which are of importance in the ripening process of surface-ripened cheese (Irlinger et al., 2009) and whose genome has been sequenced (Irlinger, *personal communication* ; Irlinger et al., 2012).

We implemented a slightly modified version of the three-step *in vitro* digestive batch method used in the previous works (Adouard et al., unpublished data, see Chap.2), consisting of (i) a gastric-like challenge, (ii) a duodenal-like challenge and (iii) a gastric-like followed by a duodenal-like challenge (Adouard et al. 2014, submitted). We used a global transcriptomic analysis based on RNA sequencing to study the transcriptional response of both strains to each of the three *in vitro* stress conditions.

2. Materials & Methods

2.1. Microorganisms

Hafnia alvei GB001 and *Staphylococcus equorum* Mu2 are part of the GMPA strain collection and are both able to grow in cheese environment (Plé et al. 2014).

2.2. Growth and plate count media

Both bacteria were cultured in 100 mL of Brain Heart Infusion broth (BHI: Biokar Diagnosis, Beauvais, France) in 500 mL Erlenmeyer flasks at 25°C, with shaking at 200 rpm. Prior to use in the experiments described below, all strains were grown until they reached the same growth phase (the late stationary phase, as defined in prior growth kinetics experiments; data not shown). Microorganisms were counted on the BHI agar. Prior to plating, cultures were diluted in Maximum Recovery Diluent (MRD, 9 g/L) (Difco,Pessac, France).
The same incubation temperatures were used as for broth cultures.

2.3. *In vitro* gastric and duodenal batch challenges

The stress conditions and the stress medium's composition were adapted from our previous work (Adouard et al 2014, Chap.2). All digestive juice components were purchased from Sigma (Saint-Quentin-Fallavier, France) and diluted in MRD. Pepsin (P6887, EC 3.4.23.1, activity: 3300 U/mg of protein, calculated using hemoglobin as a substrate), pancreatin (P1750), and bile (B8631) were of porcine origin. The “gastric lipase” was a recombinant enzyme produced in the fungus *Rhizopus oryzae* (80612, EC 3.1.1.3, activity: ≥30 U/mg). Simulated gastric and duodenal juices were made fresh daily. Gastric juice consisted in pepsin (0.025 g/L or 1.10^6U/mL), lipase from *R. oryzae* (0.2 g/L or 6.10^4U/L) and NaCl (2,75 g/L) suspended in a 0.020 M glycine-HCl buffer pH 4 at 37°C prior to experiments.

Duodenal juice was prepared by suspending pancreatin (9 g/L), bile (30 g/L) and NaCl (7 g/L) in 0.1 M phosphate buffer pH 6.5 at 37°C, prior to the experiments. In a 1000 mL sterile Erlenmeyer, 15 or 20 milliliters of a late-stationary phase culture were added to 135 mL or 130 mL of either gastric or duodenal juice for respectively *S. equorum* Mu2 or *H. alvei* GB001. The pH was checked again after inoculation and (if necessary) re-adjusted to either pH 4 or pH 6.5 using HCL 1M. Compared to the previous stress conditions (Chap.2), the pH of gastric-like conditions was changed from pH 3 for 1h to pH 4.5 for 45 min in such a way *S. equorum*'s Mu2 remaining viability was suitable for RNA extraction and further RNAseq analysis.The incubation times were respectively 45 min and 2 h for gastric and duodenal juices at 37°C, with moderate shaking (100 rpm). The serial stress condition (*i.e*. gastric stress followed by duodenal stress) was performed by adjusting the pH of 45-min gastric juice incubation to 6.5 with 1M Na_2CO_3, and then adding bile and pancreatin under sterile conditions for the subsequent 2 h duodenal incubation.

Control samples were made by incubating each species in MRD instead of the stress media, all else being equal (i.e. incubating time of 45 min, 120 min or 165 min, 37°C, final volume 150 mL, 100 rpm shaking table). Control and stressed samples were repeated 4 times. To assess cell survival, strains were counted on the corresponding agar-based media before and after each stress condition.

2.4. RNA extraction

Cells were harvested by centrifugation at 5000 x g for 5 min and pellets were immediately resuspended into 1.5 ml (*S. equorum* Mu2) or 2.1 ml (*H. alvei* GB001) of RLT Buffer containing β-mercaptoethanol (1% V/V) (Qiagen). RNA extraction was performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. DNase treatment was performed on 10 µg of total RNA using the TURBO DNA-free kit (Ambion, Grand Island, NY, USA) according to the manufacturer's instructions. Finally, RNA was purified using the RNA cleanup protocol of the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Purified RNA was quantified at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The quality of the RNA was analyzed with a 2100 Bioanalyzer (Agilent, Palo Alto, CA,USA) using RNA 6000 NANO chips.

2.5. RNA sequencing and differential analysis

RNA samples were provided to Genome Quebec and McGill University Innovation Centre (Montréal, Québec, Canada) for an additional RNA quality control and for library preparation and sequencing. Briefly, cDNA libraries were prepared using the TruSeq stranded mRNA sample preparation kit and sequenced (100 bp single-end reads) on a HiSeq 2000 instrument (Illumina, San Diego, CA, USA) following the manufacturer's protocol. The quality of sequencing reads was checked with FastqC software V0.10.1 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimming, including the removal of adaptators and quality filtering, was performed using Trimmomatic V0.32 (Bolger et al., 2014) with the following parameters: crop = 85, headcrop = 10, leading = 10, trailing = 10, slidingwindow = 4:20, minlen = 60. Then, reads were mapped onto the sequenced genome of *H. alvei* GB001 (total coding DNA sequence (CDS) : 4692 ; accession number PRJEB6257) or *S. equorum* Mu2 (total coding DNA sequence (CDS) : 2932 ; accession number CAJL01000001-CAJL01000030) using the Bowtie mapping software (Langmead et al., 2009), allowing up to one mismatch in alignments. The number of reads uniquely mapped to each gene was determined using HTSeq (Anders et al., 2014).

Differential expression analysis was performed by comparing the number of mapped reads for each gene in the four replicates from two different conditions (stress versus control). First, the count table was filtered to select only data corresponding to CDS features exhibiting more than 200 mapped reads on average. Then, data were normalized by using R and the package DESeq2 (Love et al., 2014).

Finally, differentially expressed genes were identified according to the false discovery rate adjusted Benjamini Hochberg *p*-value (Benjamini and Hochberg, 1995) implemented within the DESeq2 package and using a cutoff of adjusted *p*-value < 0.05. Genes showing an adjusted *p*-value less than 0.05 were considered to have significantly different transcript levels between the stress and control conditions. Functional classification of the transcriptome datasets were performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations (Kanehisa et al., 2012).

3. Results and discussion

3.1. Survival analysis

We assessed the resistance to in vitro batch digestive stress of *H. alvei* GB001 and S. *equorum* Mu2 two surface-ripened cheese isolated microorganisms. The incubation under gastric-like stress conditions took place for 45 min with pepsin, at 37°C and pH 4.5 ; duodenal-like stress incubation conditions were 2 h with bile and pancreatic enzymes, at 37°C and pH 6.5. Incubation under gastric-like conditions followed by duodenal-like conditions was also performed.

The strain *H. alvei* GB001 displayed a loss of viability of 0.29 log CFU/mL, 0.51 log CFU/mL and 0.38 log CFU/mL under gastric, duodenal and combined challenge conditions, respectively (Table 1). *S. equorum* Mu2 showed a loss of viability of 3.06 log CFU/mL following exposure to gastric stress, 0.60 log CFU/mL after duodenal challenge and 3.15 log CFU/mL following the combination of stress. Overall, *S. equorum* Mu2 proved to be more sensitive to gastric stress than *H. alvei* GB001, which is consistent with previous works (Adouard et al., unpublished data, Chap.2).

Table 1: Viability of *Hafnia alvei* GB001 and *Staphylococcus equorum* Mu2 before and after *in vitro* batch digestive stress

			Viability (log CFU/mL)
***Hafnia alvei* GB001**	**G-like stress**	Control	9.40 ± 0.15
		Stress	9. 11 ± 0.12
	D-like stress	Control	9.32 ± 0.13
		Stress	8.81 ± 0.12 **
	Combined stress	Control	9.80 ± 0.08
		Stress	9.42 ± 0.14 **
***Staphyloccocus equorum* Mu2**	**G-like stress**	Control	9.56 ± 0.12
		Stress	6.50 ± 0.08 ***
	D-like stress	Control	9.65± 0.09
		Stress	9.05 ± 0.14 *
	Combined stress	Control	9.49 ± 0.09
		Stress	6.31 ± 0.06 ***

Results are shown as the mean ± SD, n=3. Viable counts (log CFU/mL) of each strain were compared with a control sample incubated at 37°C in Maximum Recovery Diluent for the same length of time i.e. 45 min, 120 min or 165 min for respectively G-like, D-like and Combine stress.
G-like stress : Gastric challenge ; D-like stress : Duodenal challenge and Combined stress : Gastric followed by duodenal challenge.
The statistical significance of differences between controls and challenged groups was established using a two-tailed Student's t-test. Results are indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

3.2. Overview of the transcriptomic dataset

The bacterial transcriptome modifications induced by gastric-like stress (G), duodenal-like stress (D) or a combination of both (C), were determined for a Gram-positive bacterium (*S. equorum* Mu2) and a Gram-negative bacterium (*H. alvei* GB001) by whole transcriptome sequencing (RNA-Seq). To this end, 48 RNA-Seq libraries (24 per bacterial species) were generated and sequenced for a total of >1 billion reads. Sequencing statistics are provided in (Table 2). The number of raw sequence reads per library varied between 16,990,785 and 34,458,232. Most sequences (>90%) passed the quality filtering and mapped the reference genomes.

Table 2: Sequencing statistics and expression data.

	H. alvei GB001	***S. equorum Mu2***
Total number of reads	651,524,345	524,935,363
Average number of reads/library	27,146,848	21,872,307
Average number of filtered reads/library	25,006,194	20,894,352
Average number of unique mapped reads	24,683,814	20,425,363
Average expression (reads per CDS)	3,164	4,733

Overall, the differential expression analysis allowed to identify 3168 genes for *H. alvei* GB001 (68% of the predicted CDS) and 2456 genes for *S. equorum* Mu2 (84% of the predicted CDS) whose expression was significantly different (adjusted *p*-value < 0.05) from the control in at least one stress condition (G, D or C). Such variations indicate dramatic metabolic changes under stress conditions for these two microorganisms. A hierarchical clustering (Single Linkage method) of the samples was performed using the correlation matrix built from the expression data corresponding to those genes (Figure 1). This analysis revealed a perfect clustering of biological replicates (4 per stress condition or the corresponding controls) and a clear segregation between stress conditions. Furthermore, it emphasizes the importance of using a set of specific controls for each stress conditions. Indeed, each stress condition had its set of specific controls, namely a sample in which microorganisms were kept in a minimal medium for as long as the stressed was performed in the sample group. The clustering of controls made for each stress condition points out the necessity to use control that are different from a sample taken just before the stress and that does not take into account the period of time during which microorganisms are stressed.

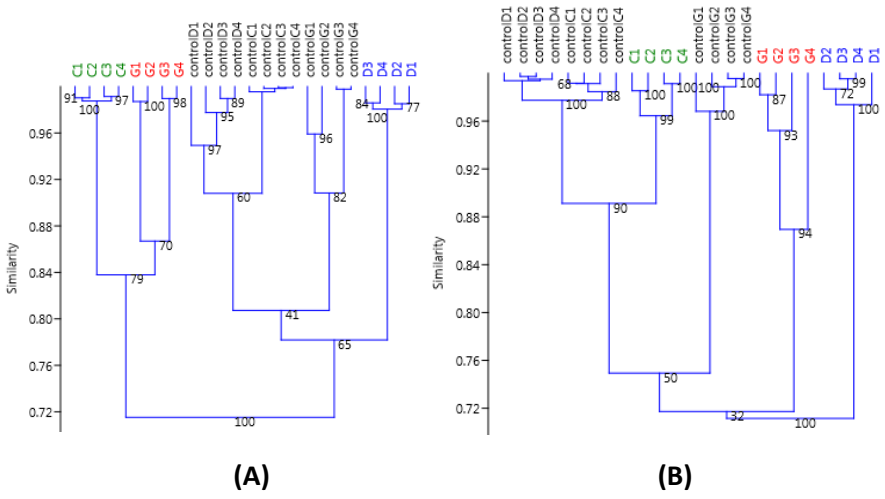


Figure 1 : Hierarchical clustering (Single Linkage method) of (A) *H. alvei* GB001 and (B) *S. equorum* Mu2 samples.
G : gastric-like stress; D: duodenal-like stress; C: combined stress.
Control with corresponding letters. n=4 in each stress and control group. Performed using the correlation matrix built from the expression data corresponding to those genes

3.3. Transcriptional response of *Hafnia alvei* GB001 and *Staphyloccocus equorum* Mu2 to *in vitro* batch digestive stress

3.3.1.Overall response

In order to determine which genes were involved in bacterial Gram-negative and Gram-positive response to gastric-like and/or duodenal-like batch digestive stress, we focused on pair-wise comparisons performed between stressed groups and controls. Overall, Table 3 exhibits 1730, 1761 and 2308 genes whose expression was significantly modified in response respectively to gastric-like,duodenal-like and combined *in vitro* batch stress (*p*-value <0.05) among a total of 4692 CDS in *H. alvei* GB001. Pair-wise comparisons performed between stressed groups and controls for the *S. equorum* Mu2 gave 573, 796 and 510 genes whose expression was significantly modified in response to G-like, D-like and combined *in vitro* batch stress, respectively (*p*-value<0.05); for a total of 2932 CDS in *S. equorum* Mu2 (Table 3). It is of note that, regarding *S. equorum* Mu2, around 50% to 60% of the differentially expressed genes encode for putative or unknown proteins. Such a lack of information makes the search of complete metabolisms very difficult to carry on.

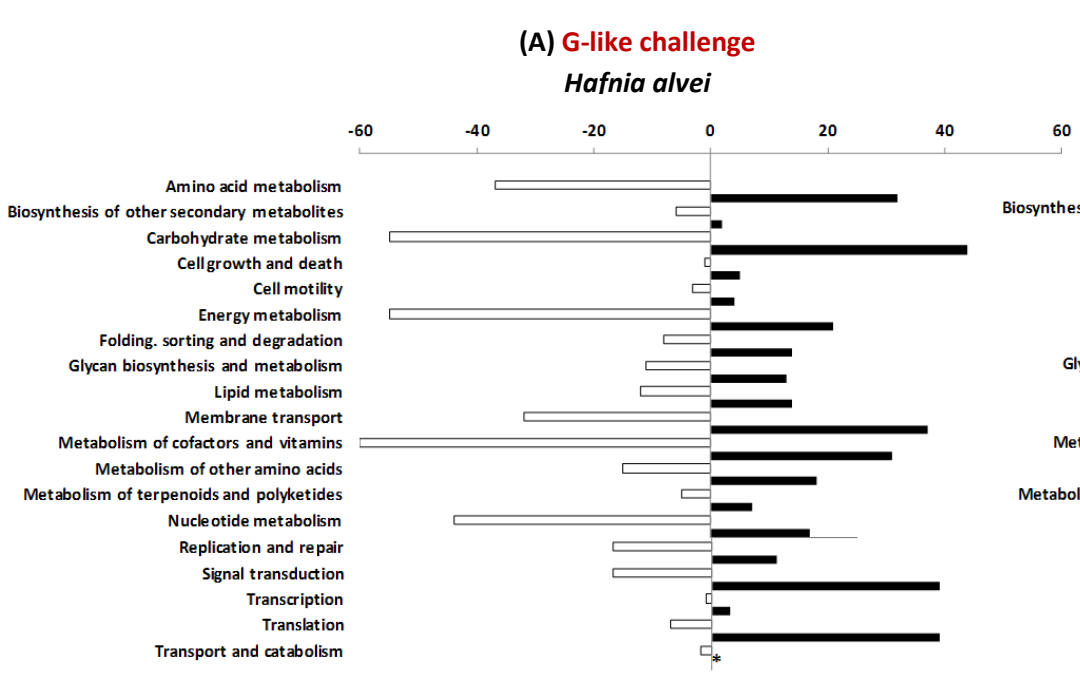
Table 1: General view of up-regulation and down-regulation (adjusted p-value < 0.05) of Coding DNA Sequence (CDS) in *Hafnia alvei* GB001 and *Staphylococcus equorum* Mu2 when exposed to (i) G-like, (ii) D-like and (iii) G-like followed by D-like – i.e. combined- stress

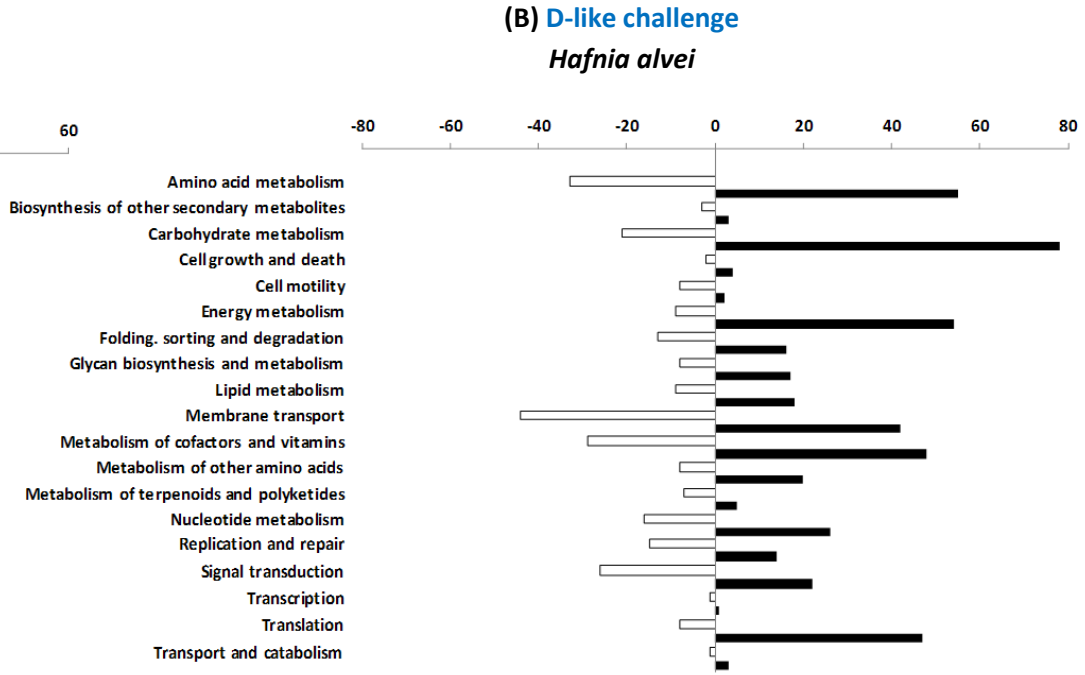
	***Hafnia alvei* GB001**[(1)] Total genome : 4692 CDS			***Staphylococcus equorum* Mu2**[(1)] Total genome : 2932 CDS		
	Up-regulated CDS	**Down**-regulated CDS	%Total CDS (up+down)	**Up**-regulated CDS	**Down**-regulated CDS	%Total CDS (up+down)
Gc-like stress	838	892	37	721	558	44
Dl-like stress	827	934	38	899	906	62
Combined stress	1174	1132	49	634	668	44

(1) Coding DNA Sequence of the […] bacterial strain. Genomes available under accession number PRJEB6257 for *H. alvei* GB001 and CAJL01000001-CAJL01000030 for *S. equorum* Mu2.

Figure 2 and Figure 3 show the number of up- and down-regulated genes after each of the three stress conditions for respectively *H. alvei* GB001 and *S. equorum* Mu2. The global expression pattern was evaluated by classifying differentially-expressed genes according to KEGG annotations (Kanehisa et al. 2012). Overall, even though the ranking was slightly different among each stress and bacterial strain, the main functional categories with differentially expressed genes were carbohydrates metabolism, amino acid metabolism, metabolism of cofactors and vitamins, membrane transport, energy metabolism, signal transduction and translation.

From a global perspective, Table 4 and Table 5 showed that, respectively for *H. alvei* GB001 and *S. equorum* Mu2, general stress response genes were significantly up-regulated for the three stress conditions. Indeed, genes such as those encoding molecular chaperons (*e.g. dnaK, clpB, hscAB, htpG*) were up-regulated. Their role is to address and stabilize proteins, by preventing and reversing their aggregation or by degrading irreversibly damaged materials (Hartl et al. 1996). Heat-shock and cold-shock proteins coding genes (namely *ibpA* and *cspE* for *H. alvei GB001* and *cspA* for *S. equorum* Mu2) were up-regulated as well. They are reported to collaborate with the previously mentioned chaperones to achieve protein sorting, repairing and ultimately degrading, if necessary (Thomas and Baneyx, 1998).





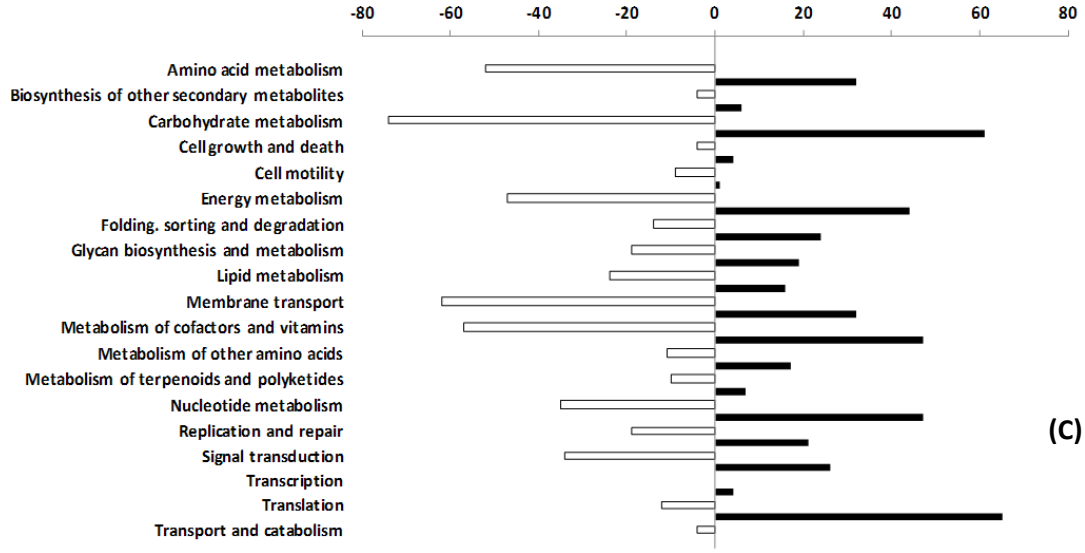
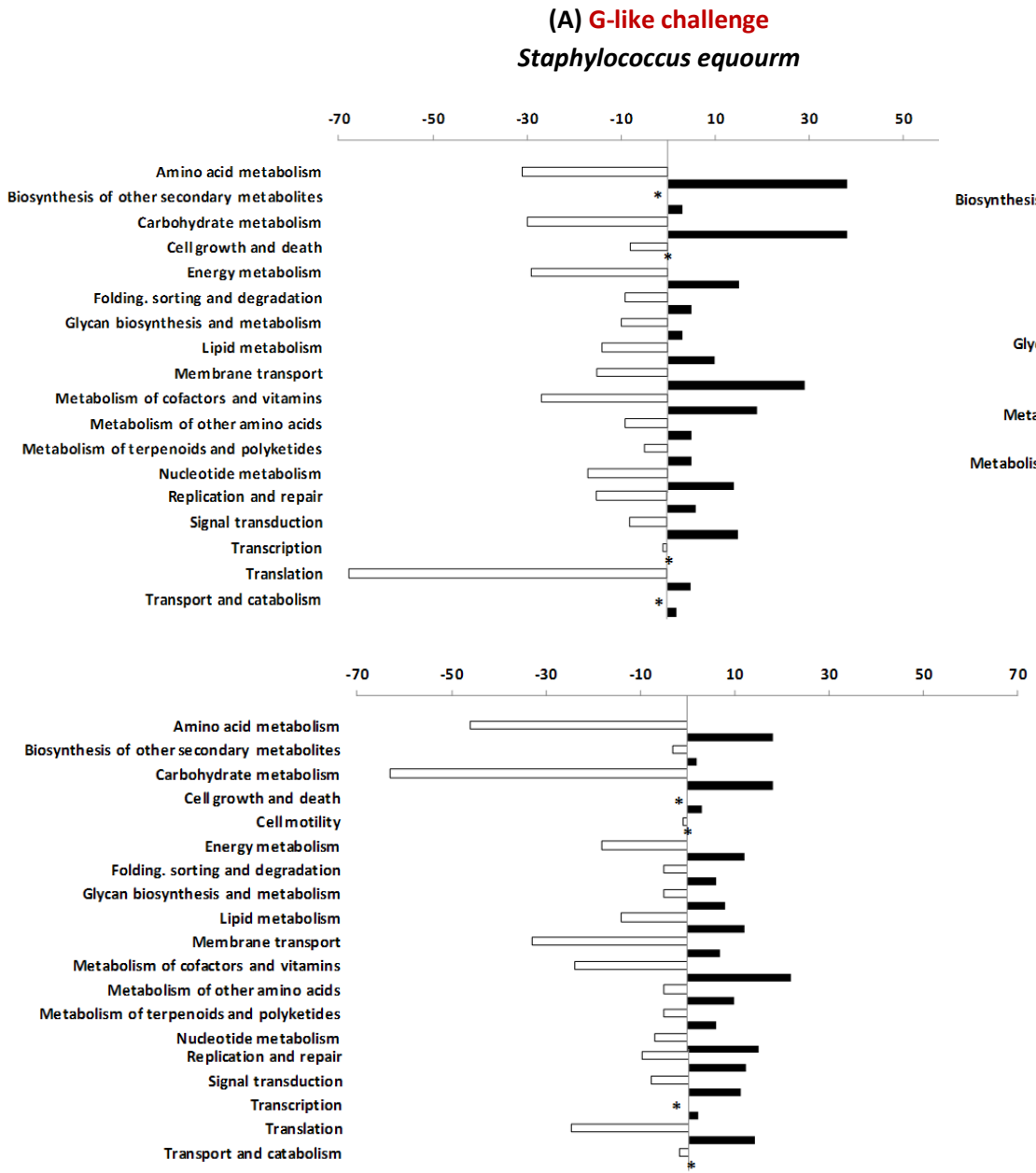


Figure 2: Functional classification of differentially expressed genes for *Hafnia alvei* GB001 after (A) gastric-like (B) duodenal-like and (C) Combined *in vitro* batch stress, , compared to control groups.
Dark bars : amount of up-regulated genes ;
Light bars amount of downregulated genes - in each condition

(C) Combined challenge
Hafnia alvei



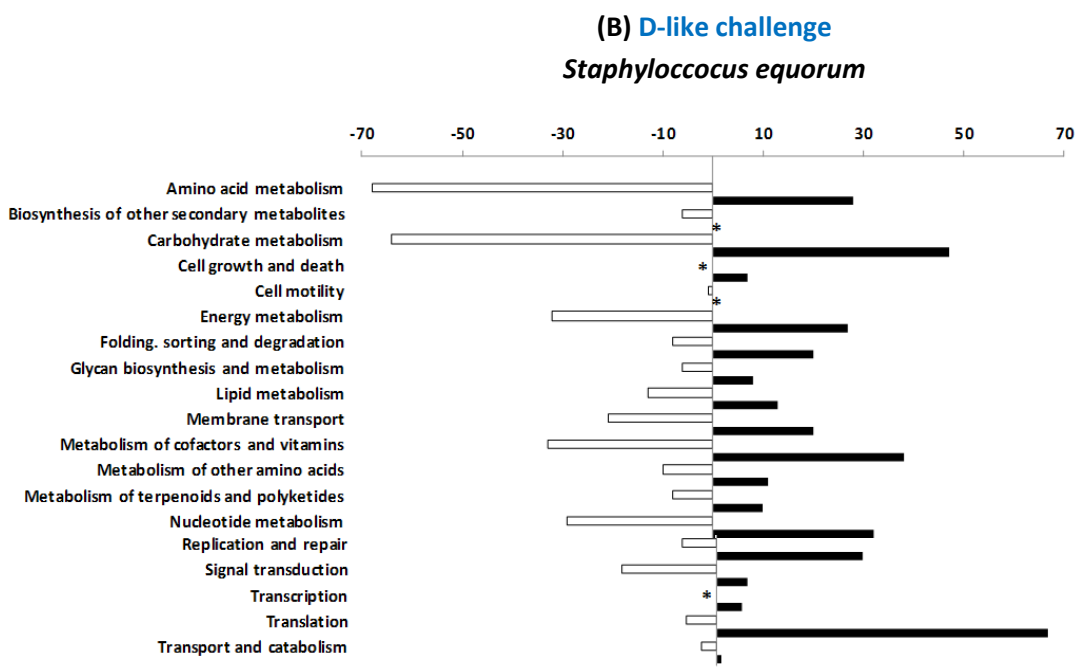


Figure 3: Functional classification of differentially expressed genes for *Staphyloccocus equorum* Mu2 after (A) gastric-like (B) duodenal-like and (C)Combined stress, alone or in combination, compared to control groups.
Dark bars : amount of up-regulated genes ;
Light bars amount of downregulated genes - in each conditions

3.3.2.Gastric-like stress

Figure 2 (A) and Figure 3 (A) show the number of up- and down-regulated genes per functional categories for *H. alvei* GB001 and *S. equorum* Mu2, respectively, after the gastric-like challenge compared to controls.

As mentioned in the previous section, numerous genes involved in the general stress response were up-regulated (Table 4 and Table 5). For example, the gene encoding the cold shock protein (*cspA*) was up-regulated in both microbial strains. In the particular case of *H. alvei* GB001, the phage shock protein complex (PSP-complexe; encoded by *pspABCD* genes) was also highly up-regulated. It has been reported that this complex helps preventing membrane damage in *Escherichia coli,* and in Gram-negative bacteria in general, in response to a wide range of environmental stresses (Darwin et al., 2006). Interestingly, Darwin et al. demonstrated a PSP-complex induction after the proton motrice force (PMF) was dissipated in the cell, and they reported that PSP-complex to be induced when a defect occurred in the assembly of cytochrome O oxidase and F_1-F_0ATPsynthase complex. A closer look to the energy metabolism of *H. alvei* GB001 showed that most of the oxidative phosphorylation main genes were repressed - *e.g*. F_1-F_0ATPsynthase complex (encoded by genes *atpABEH)*, cytochrome O oxidase complex (encoded by genes *cyoBCDE)* and NADH dehydrogenase complexe (encoded by genes *nuoCEFGHKN*). *cyoBCDE* and *nuoCEFGHKN* genes have already been reported to be up-regulated in *E. coli* when submitted to acid stress (Krulwich et al. 2011). *H. alvei* is genetically and metabolically close to *E. coli* (Janda *et* al., 2002) but does not seem to be able to use its Cyo and Nuo complex in the same way when exposed to an acidic environment. Therefore, as mentioned in Darwin's review conclusion (2006), we can speculate that PSP-complex may have been up-regulated in order to counteract the dysfunctions related to PMF defect and functioned as a signal transduction-like system. As a whole, and for both microorganisms, genes involved in carbohydrates metabolism were generally repressed. As shown in Table 4 and Table 5, major genes coding for enzymes of the TCA cycle (*e.g. sdhAB*, *sucABCD*, *frdABC*) and glycolysis (*e.g*. *pgi, pgk, pgm*, *acs*, *fba*, *fbp*) were significantly down-regulated, clearly suggesting that the cells were not using the carbon resources as efficiently to produce energy. Along with the repression of oxidative phosphorylation related genes already mentioned for *H. alvei* GB001 and that occurred as well for *S. equorum* Mu2, we can state that both bacteria tend to limit the use of aerobic respiration in response to gastric-like stress conditions.

Table 4: Selection of genes (CDS) significantly up- or down-regulated in *H. alvei* GB001 after exposure to (i) G-like, (ii) D-like and (iii) G-like followed by D-like stress – i.e. combined- stress

		Log_2 expression ratio[(1)]		
Gene	**Product**	G-like stress	D-like stress	Combined stress
General stress proteins				
clpA	ATP-dependent Clp protease ATP-binding subunit	0,85	-	-0,58
clpB	ATP-dependent chaperone protein ClpB	2,80	1,07	2,09
clpP	ATP-dependent Clp protease proteolytic subunit	2,05	-	0,80
clpS	ATP-dependent Clp protease adaptor protein ClpS	2,44	-	-
clpX	ATP-dependent serine protease specificity subunit of ClpX-ClpP	1,51	-0,51	-
cspA2	Major cold shock protein	1,97	-	4,54
cspD	Cold shock-like protein CspD	2,96	-1,84	-
cspE	Cold shock protein	1,48	1,57	1,18
dnaJ	Chaperone protein dnaJ	1,62	1,05	1,12
dnaK	Chaperone hsp70, autoregulated heat shock protein	3,03	0,55	1,65
hfq	RNA chaperone Hfq	2,11	-	-
hscA	chaperone protein HscA	1,44	-	-
hscB	co-chaperone protein HscB	1,63	-	-1,89
hslO	Heat shock protein Hsp33	2,34	-0,40	1,94
hslR	Heat shock protein	1,94	-0,44	2,61
htpG	Chaperone protein HtpG	3,07	-	1,62
ibpA	Heat shock chaperone	5,42	1,33	1,90
ibpB	Heat shock chaperone	4,29	-0,75	2,26
pspA	Phage shock protein A	4,32	-0,42	-0,95
pspB	Phage shock protein B	3,93	-0,40	-1,45
pspC	Phage shock protein C	3,59	-0,76	-0,88
pspD	Phage shock protein D	2,80	-	-1,21
uspG1	Universal stress protein G	-1,04	2,17	-
uspG2	Universal stress protein G	-	2,15	-
uspA	Universal stress protein	-1,48	2,10	-
groEL	60 kDa chaperonin		2,65	0,53
Response to oxidative stress and reductase				
katE	Catalase	-1,72	1,89	-1,94
osmY1	Putative lipoprotein	-1,50	1,23	-2,95
sodA	Superoxide dismutase	-	-4,23	-
sodB	Superoxide dismutase	-	3,99	-
cbiJ	Cobalt-precorrin-6a reductase	-	2,06	-
cysH	phosphoadenosine phosphosulfate reductase	-	1,48	3,41
dmsC	Anaerobic dimethyl sulfoxide reductase, C subunit	-1,88	2,95	-
ghrA	Hydroxypyruvate reductase	-	1,38	0,49
metF	methylenetetrahydrofolate reductase (NAD(P)H)	0,87	1,75	1,52
nemA2	NADH:flavin oxidoreductase	0,75	1,15	1,50
nrdA	Ribonucleoside-diphosphate reductase	-0,82	1,54	-
nrdG	Anaerobic ribonucleoside-TP reductase activating protein	-1,45	2,01	0,79
torD1	Putative oxidoreductase component	-1,39	1,41	-
grdB	Glycine reductase complex component B	-	1,20	-

(1) Expression ratio was calculated by comparing the stressed condition against a reference condition (adjusted p-value<0.05) in which microorganisms were kept in a minimal media (MRD) other things being equal. White cell: up-regulated ; Black cell: down-regulated ;

Gene	Product	Log_2 expression ratio[1] G-like stress	D-like stress	Combined stress
TCA				
acnA	aconitate hydratase	-0,62	0,29	-
acnB	aconitate hydratase 2	-1,18	1,40	-2,16
frdA	fumarate reductase flavoprotein subunit	-0,64	-	1,62
frdB	fumarate reductase iron-sulfur subunit	-1,18	-	0,73
frdC	fumarate reductase subunit C	-1,54	-	-
gltA	citrate synthase	-1,42	0,60	-4,97
mdh	malate dehydrogenase	-1,72	0,95	-1,52
sdhA	succinate dehydrogenase flavoprotein subunit	-0,89	-	-
sdhB	succinate dehydrogenase iron-sulfur subunit	-1,85	-	-
sucA	2-oxoglutarate dehydrogenase E1 component	-1,53	1,92	-1,42
sucB	2-oxoglutarate dehydrogenase E2 component	-2,59	-	-1,23
sucC	succinyl-CoA synthetase beta subunit	-3,51	-	-
sucD	succinyl-CoA synthetase alpha subunit	-3,46	-	-
Glycolyse				
acs	acetyl-CoA synthetase	-1,23	0,56	-2,79
fba	fructose-bisphosphate aldolase. class II	-1,09	0,76	-1,16
fbaB	fructose-bisphosphate aldolase. class I	-2,18	1,44	-2,14
fbp	fructose-1.6-bisphosphatase I	-1,49	1,37	-1,10
glpX	fructose-1.6-bisphosphatase II	-0,40	-	1,01
gpmI	2.3-bisphosphoglycerate	-1,02	1,33	0,35
pfkB	6-phosphofructokinase 2	-0,34	1,15	-0,56
pgi	glucose-6-phosphate isomerase	-1,18	1,11	-0,98
pgk	phosphoglycerate kinase	-0,73	0,41	-1,04
pgm	phosphoglucomutase	-0,59	1,23	0,86
tpiA	triosephosphate isomerase (TIM)	-1,25	1,17	-
Oxydative phosphorylation				
atpA	ATP synthase subunit alpha	-0,51	-	-
atpB	ATP synthase subunit a	-0,52	-	-
atpE	ATP synthase subunit c	-0,66	-	-
atpH	ATP synthase	-0,41	-	-
atpI	ATP synthase F0, I subunit	-0,98	-	-
cydA1	Cytochrome D ubiquinol oxidase subunit I	-	1,79	-
cydB	Cytochrome D ubiquinol oxidase subunit II	-1,22	2,48	-
cyoB	Cytochrome o ubiquinol oxidase subunit I	-2,24	2,64	-
cyoC	Cytochrome o ubiquinol oxidase, subunit III	-2,39	-	-2,39
cyoD	Cytochrome o ubiquinol oxidase subunit IV	-2,69	-	-2,25
cyoE	Protoheme IX farnesyltransferase	-2,44	-	-1,68
nuoC	NADH:ubiquinone oxidoreductase, chain C,D	-0,66	1,62	-
nuoE	NADH dehydrogenase I chain E	-0,74	-	-
nuoF	NADH:ubiquinone oxidoreductase, chain F	-0,60	-	-
nuoG	NADH-quinone oxidoreductase	-0,53	-	-
nuoH	NADH:ubiquinone oxidoreductase, membrane subunit H	-0,49	-	-
nuoJ	NADH dehydrogenase I chain J	-	1,20	-0,42
nuoK	NADH:ubiquinone oxidoreductase, membrane subunit K	-0,96	1,34	-
nuoL	NADH dehydrogenase I chain L	-	1,52	-
nuoM	NADH:ubiquinone oxidoreductase, membrane subunit M	-	1,97	-
nuoN	NADH:ubiquinone oxidoreductase, membrane subunit N	-1,44	2,08	0,73

(1) Expression ratio was calculated by comparing the stressed condition against a reference condition (adjusted p-value<0.05) in which microorganisms were kept in a minimal media (MRD) other things being equal. White cell: up-regulated ; Black cell: down-regulated ;

Gene	Product	Log_2 expression ratio[1] G-like stress	D-like stress	Combined stress
Hydrogenases				
hyfF	Hydrogenase-4 component F	2,61	-	-
hyfG	Hydrogenase-4 component G	1,02	-	-
hyb0	*Hydrogenase-2 small chain*	-2,12	-	2,74
hybA	*Hydrogenase-2 operon protein HybA*	-3,19	-	1,96
hybB	*Ni/Fe-hydrogenase 2 B-type cytochrome subunit*	-3,70	-	-
fdhE	Formate dehydrogenase formation protein	-1,83	0,76	-0,99
fdoI	Formate dehydrogenase, cytochrome b556 protein	-2,13	-	-1,69
hybC	*Hydrogenase-2 large chain*	-3,48	-	-
hybD	*Hydrogenase expression/formation protein*	-1,87	-	-
hypB	*Hydrogenase nickel incorporation protein*	-1,75	-	-
hypC	*Putative hydrogenase formation protein*	-2,16	-	-
hycE	Formate hydrogenlyase, subunit E	1,29	-	0,99
Formate dehydrogenase				
fdoG	Aerobic formate dehydrogenase, alpha subunit	-1,05	1,98	-2,36
fdoH	Formate dehydrogenase-O, iron-sulfur subunit	-1,67	-	-
fdoG	Formate dehydrogenase-O, selenocysteine-containing	-1,72	2,05	-
fdoI	Formate dehydrogenase, cytochrome b556 protein	-2,13	-	-1,69
Glutamate/GABA metabolism				
gadB	Glutamate decarboxylase beta	2,84	-	1,02
aspB	Glutamate synthase [NADPH] large chain	0,57	0,95	-
aspB	Glutamate synthase [NADPH] small chain.	0,57	0,95	-
gdhA	Glutamate dehydrogenase	-0,68	-0,65	-
gabT	4-aminobutyrate aminotransferase, PLP-dependent	-1,27	-3,47	-
arg	Carbamoyl-phosphate synthase, large subunit	-0,84	-	-
carA	Carbamoyl-phosphate synthase, small subunit	-3,11	-	2,24
glmS	L-glutamine:D-fructose-6-phosphate aminotransferase	-0,36	1,44	1,58
Decarboxylase				
adi	arginine decarboxylase	1,35	-0,38	-1,96
Sulfur metabolism and transport				
cysA	sulfate/thiosulfate import ATP-binding protein cysA	4,20	-	-
cysP	thiosulfate-binding protein	6,47	-	-
cysU	sulfate transport system permease protein cysT	5,75	-	-
cysW	sulfate transport system permease protein cysW	5,42	-	-
cysC	adenylyl-sulfate kinase	4,41	-	2,61
cysD	sulfate adenylyltransferase subunit 2	7,07	-	-
cysI	sulfite reductase [NADPH] flavoprotein alpha-component	2,81	1,33	-
cysJ	sulfite reductase [NADPH] flavoprotein alpha-component	4,05	0,72	-
cysN	sulfate adenylyltransferase subunit 1	5,06	-	-
iscS	cysteine desulfurase	1,85	-	-1,43
sbp	sulfate-binding protein	4,09	-	-
Nitrate metabolism				
narK	Nitrite extrusion protein (MFS-family transporter)	1,68	-	-0,93
narH	Nitrate reductase 1, beta subunit	1,41	-	-
narG	Respiratory nitrate reductase 1 alpha chain	0,91	-	-

(1) Expression ratio was calculated by comparing the stressed condition against a reference condition (adjusted p-value<0.05) in which microorganisms were kept in a minimal media (MRD) other things being equal. White cell: up-regulated ; Black cell: down-regulated ; "-" no significant differential expression

Gene	Product	Log_2 expression ratio[1] G-like stress	D-like stress	Combined stress
Transporter (ABC, PTS, Others)				
	ABC transporter, substrate-binding component	7,25	-	-
	ABC transporter, inner membrane subunit	6,17	0,66	2,36
	ABC transporter, inner membrane subunit	4,00	0,77	1,79
malX	PTS system, maltose and glucose-specific IIabc component	2,07	-	3,99
fliY	Putative amino-acid ABC transporter	4,85	-	1,29
	Sigma-54 dependent transcriptional regulator/ABC transporter	2,77	-	-
yecS	Extracellular solute-binding protein-amino acid abc transporter	2,86	-2,14	-
H^+ co-transport				
kdpA	P-type ATPase, high-affinity potassium transport system, A chain	1,82	-	2,99
kdpB	Putative potassium-transporting ATPase B chain	2,79	-	2,09
kdpC	K+-transporting ATPase, C subunit	1,82	-	-
trkA	Potassium uptake protein	-0,43	-	-1,09
trkH	Trk system potassium uptake protein TrkH	-0,85	-	-
mgtB	Magnesium transport ATPase	2,53	-	1,84
mgtC1	Mg2+ transport ATPase	4,41	-	2,64
sapB	Putative membrane protein	0,53	-0,87	-2,31
Efflux pumps / Porines				
emrB	multidrug resistance protein B	0,87	2,35	2,59
tolC	Outer membrane protein TolC	-1,11	1,32	-
acrA	Acriflavin resistance protein A	-0,36	0,56	0,34
acrD	putative aminoglycoside efflux pump	2,36	-0,75	-1,60
sdeY	Multidrug resistance efflux pump	-	1,39	0,39
ybhR	ABC-type multidrug transport system, permease component	1,39	1,16	0,62
mdlB	Multidrug ABC transporter, permease/ATP-binding protein	-	1,02	1,61
ompH	Cationic 19 kDa outer membrane protein	-	1,40	1,40
mdlB	Outer membrane pore protein N, non-specific	-	1,02	1,61
ompA2	Putative exported protein	1,28	1,24	-
galF	UTP-glucose-1-phosphate uridylyltransferase, GalF protein	-	1,37	-
meoA	Outer membrane protein C, porin	-1,91	1,85	-2,39

(1) Expression ratio was calculated by comparing the stressed condition against a reference condition (adjusted p-value<0.05) in which microorganisms were kept in a minimal media (MRD) other things being equal. White cell: up-regulated ; Black cell: down-regulated ;

Gene	Product	Log_2 expression ratio[1]		
		G-like stress	D-like stress	Combined stress
Translation/ Traduction				
rrmJ	Ribosomal RNA large subunit methyltransferase J	2,49	-0,50	-
rpsQ	30S ribosomal protein S17	1,80	-	2,60
rpsS	30S ribosomal protein S19	1,72	-	4,96
rpsF	30S ribosomal protein S6	-	2,03	4,96
rpsJ	30s ribosomal subunit protein s10	0,96	2,07	6,37
rpsR	30s ribosomal subunit protein s18	-	2,41	4,85
rpsC	30S ribosomal subunit protein S3	1,77	-	3,96
rplP	50S ribosomal protein L16	1,85	-	3,63
rplS	50S ribosomal protein L19	1,74	-	3,39
rplB	50S ribosomal protein l2	1,74	-	5,29
rpmC	50S ribosomal protein L29	1,82	-	3,64
rplC	50S ribosomal protein L3	1,15	2,41	6,40
rpmE	50S ribosomal protein L31	1,80	1,55	2,12
rplE	50S ribosomal protein L5	-	1,70	2,11
rplD	50S ribosomal subunit protein L4	1,39	2,64	6,21
rplI	50S ribosomal subunit protein L9	-	2,64	3,83
fmt	Methionyl-tRNA formyltransferase	-1,08	-1,24	0,33
yibK	Predicted rRNA methylase	-0,63	-1,73	1,16
rluA	Ribosomal large subunit pseudouridine synthase A	1,07	2,58	-
rplA	Ribosomal protein L1	-	1,90	-
rpsN	Ribosomal protein S14p	-	1,80	-
yheL	Ribosomal RNA large subunit methyltransferase J	2,21	-0,93	-
rpoD	RNA chaperone Hfq	2,03	-0,59	-
trmD	RNA polymerase sigma factor	1,65	1,20	-
luxS	S-ribosylhomocysteine lyase	1,62	-	-
rpoC	tRNA (guanine-N(1)-)-methyltransferase	1,60	-	1,31
DNA synthethis and reparation				
mutM	DNA glycosylase	4,11	-0,43	1,89
dnaG	DNA primase	3,07	-	-
topA	DNA topoisomerase	1,81	-	1,89
hupB	DNA-binding protein HU-beta	-1,04	-	2,58
dnaQ	DNA polymerase III subunit epsilon	-1,05	-0,86	0,94
tus	Inhibitor of replication at Ter, DNA-binding protein	-1,14	-	-1,67
Dps	DNA-binding protein	-0,45	0,87	-3,13
holE	DNA polymerase III theta subunit	-1,32	0,60	-0,62
ogt	methylated-DNA--protein-cysteine methyltransferase	-1,34	-0,57	0,55
comEA	DNA uptake protein	-1,46	0,66	-0,95
rpoA	DNA-directed RNA polymerase, alpha subunit	0,74	1,55	2,93
recO	DNA repair protein RecO	0,49	-1,17	-
dinJ	DNA-damage-inducible protein J	0,51	-1,21	-1,48
yrdD	Putative DNA topoisomerase	-	-1,29	-2,37

(1) Expression ratio was calculated by comparing the stressed condition against a reference condition (adjusted p-value<0.05) in which microorganisms were kept in a minimal media (MRD) other things being equal. White cell: up-regulated ; Black cell: down-regulated ;

The metabolism of amino acids appeared to be of importance in both bacteria. Decarboxylation of amino acids to help bacteria fight against acid stress has been nicely described in the literature, especially in the *Enterobacteriacea* family (for review see Krulwich et al. 2001). A focus on the glutamate metabolism (Figure 4) show that *H. alvei* GB001 up-regulated the L-Glutamate conversion to GABA while down-regulating the other pathways involving glutamate as a substrate. It is of note that the gene encoding the GadC transporter (exporting GABA while importing Glutamate) was not found in the annotated genome of *H. alvei* GB001 and might be explained by the poor composition of the stress medium. Indeed, if no extra-cellular glutamate is available to import, the cell shall not waste its GABA pool and last molecule could be used in other part of the microorganism metabolism. The arginine decarboxylase encoding gene, *adi,* along with *fliY* and *yecS* encoding two amino acids transporters were up-regulated as well, which add some more proof of the ability of *H. alvei* GB001 to use decarboxylation of amino acid to increase its internal pH. Decarboxylation of amino acid was up-regulated as well in *S. equorum* Mu2 with the over-expression of a putative lysine/arginine/ornithine decarboxylase encoding gene (*unamed*). Such phenomenon has been documented in gram-positive bacteria like *Lactococcus lactis* (Cotter et al. 2003). We also observed the up-regulation of genes *potAD* involved in polyamines metabolism in *S. equorum* Mu2. In agreement with this, spermidine and putrescine have been identified as agent regulating pH homeostasis among other functions in the bacterial cell (Pegg and Casero, 2009).

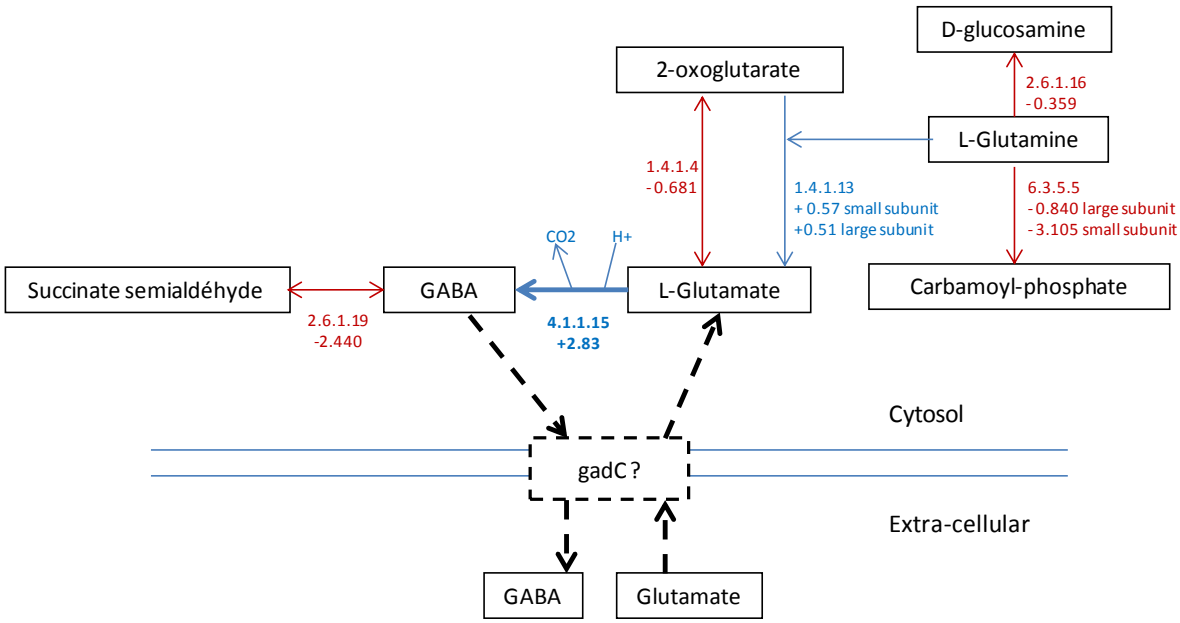


Figure 4 : Glutamate metabolism and conversion to GABA. Blue:Up-regulated genes. Red:down-regulated genes 1.4.1.13, glutamate synthase (NADPH) ; 1.4.1.4, glutamate dehydrogenase (NADP+) ; 2.6.1.16, glutamine-fructose-6-phosphate transaminase (isomerizing) ; 2.6.1.19, 4-aminobutyrate-2-oxoglutarate transaminase ; 6.3.5.5, Carbamoyl-phosphate synthase (glutamine-hydrolysing) ; 4.1.1.15, Glutamate decarboxylase A

Table 5: Selection of genes (CDS) significantly up- or down-regulated in *S. equorum* Mu2 after exposure to (i) G-like, (ii) D-like and (iii) G-like followed by D-like – i.e. combined- stress

		Log_2 expression ratio[(1)]		
Gene	**Product**	G-like stress	D-like stress	Combined stress
General stress proteins				
	universal stress family domain-containing protein	7,09	-1,57	1,60
hslO	33 kDa chaperonin	-	1,61	-
asp23	alkaline shock protein 23	3,43	-1,51	-
clpC	ATP-dependent Clp protease ATP-binding subunit	-	3,63	-
clpX	ATP-dependent Clp protease ATP-binding subunit ClpX	-2,74	3,63	1,81
clpP	ATP-dependent Clp protease proteolytic subunit ClpP	-	4,20	-
clpB	chaperone protein ClpB	-	4,52	-2,04
dnaJ	chaperone protein DnaJ	-	2,05	-1,46
dnaK	chaperone protein DnaK	-	1,81	-1,40
groEL	chaperonin GroEL	-1,79	3,33	-1,04
groES	co-chaperonin GroES	-2,10	3,69	-
cspA	cold shock protein CspA	4,36	-0,69	1,54
	general stress protein	3,76	-1,18	0,72
hfq	RNA chaperone Hfq	1,00	-	1,80
	universal stress protein	6,12	-3,63	-
Reductase / oxidoreductase				
panE	2-dehydropantoate 2-reductase	4,02	-0,58	-1,39
	aldehyde dehydrogenase	2,06	1,54	-
arsC	arsenate reductase	1,26	2,08	-1,15
cdr	coenzyme A disulfide reductase	2,41	0,71	0,97
guaC	GMP reductase	-	1,97	-
hisD	histidinol dehydrogenase	2,64	-1,90	-
guaB	IMP dehydrogenase	2,11	-1,98	-
mdh	**malate dehydrogenase**	2,49	0,37	-
mqo	**malate dehydrogenase (acceptor)**	3,09	-0,84	-
	malate dehydrogenase (oxaloacetate-decarboxylating)	1,71	1,27	-1,35
nfrA	NADPH-dependent oxidoreductase	5,75	-2,85	-
	nitroreductase family protein	4,06	-0,38	1,21
msrA	peptide-methionine (S)-S-oxide reductase	2,17	-0,48	1,66
putA	proline dehydrogenase	2,85	-0,35	-
dkgA	putative 2,5-didehydrogluconate reductase	1,52	1,72	0,68
	putative arsenate reductase	1,63	-1,42	1,28
	putative oxidoreductase	2,74	-	-
	putative thioredoxin reductase	-1,18	2,47	-
	putative Zn-dependent alcohol dehydrogenase	1,80	-3,18	-0,89
	pyridine nucleotide-disulfide oxidoreductase	3,47	-3,93	-
	short-chain dehydrogenases/reductases family protein	3,91	7,06	0,92
	short-chain dehydrogenases/reductases family protein	3,91	7,06	0,92
	short-chain dehydrogenases/reductases family protein	2,20	7,82	0,92
	short-chain dehydrogenases/reductases family protein	1,82	7,06	0,89
	short-chain dehydrogenases/reductases family protein	1,79	2,19	0,90
Response to oxidative stress				
sodA	superoxide dismutase	1,89	-	-

(1) Expression ratio was calculated by comparing the stressed condition against a reference condition (adjusted p-value<0.05) in which microorganisms were kept in a minimal media (MRD) other things being equal. White cell: up-regulated ; Black cell: down-regulated ; "-" no significant differential expression

Gene	Product	Log_2 expression ratio[1] G-like stress	D-like stress	Combined stress
Oxydative phosphorylation				
ubiE	demethylmenaquinone methyltransferase	-0,89	-	-
atpA	H(+)-transporting two-sector ATPase, alpha chain	-3,56	0,79	-
atpD	H(+)-transporting two-sector ATPase, beta chain	-3,38	0,62	-
atpE	H(+)-transporting two-sector ATPase, chain C	-4,00	-	-0,97
atpH	H(+)-transporting two-sector ATPase, delta chain fragment	-3,88	0,60	-
atpH	H(+)-transporting two-sector ATPase, delta chain fragment	-3,88	0,60	-
atpC	H(+)-transporting two-sector ATPase, epsilon chain	-4,23	0,71	-
atpG	H(+)-transporting two-sector ATPase, gamma chain	-3,88	0,64	-
ctaA/cyoB	heme A synthase	-2,41	-0,68	-
	probable NADH-quinone oxidoreductase subunit	-2,15	-	-
ctaB/cyoB	protoheme IX farnesyltransferase	-1,49	-0,59	-
qoxB	quinol oxidase subunit 1	-1,68	-1,96	-
qoxA	quinol oxidase subunit 2	-2,24	-2,00	-
sdhA	succinate dehydrogenase flavoprotein subunit	-1,18	-	-
sdhC	succinate dehydrogenase, cytochrome b558 subunit	-1,16	-0,64	-
TCA Cycle				
lpdA	dihydrolipoyl dehydrogenase	-1,46	1,45	-
pdhC	dihydrolipoyllysine-residue acetyltransferase	-2,31	1,55	-
pyc	pyruvate carboxylase	-1,72	0,44	1,23
pdhA	pyruvate dehydrogenase E1 component subunit alpha	-2,24	1,43	-
pdhB	pyruvate dehydrogenase E1 component subunit beta	-2,24	1,58	-
sdhA	succinate dehydrogenase flavoprotein subunit	-1,18	-	-
sdhC	succinate dehydrogenase, cytochrome b558 subunit	-1,16	-0,64	-
sucD	succinyl-CoA synthetase (ADP-forming) alpha subunit	-1,88	-0,48	-
sucC	succinyl-CoA synthetase beta subunit	-1,37	-0,46	-
Glycolyse				
acs	acetyl-CoA synthetase	-1,56	-	-
galM	aldose 1-epimerase	-0,76	-	-
pckA	phosphoenolpyruvate carboxykinase (ATP)	-2,51	-0,98	-1,67
pdhA	pyruvate dehydrogenase E1 component subunit alpha	-2,24	1,43	-
pdhB	pyruvate dehydrogenase E1 component subunit beta	-2,24	1,58	-
pdhC	pyruvate dehydrogenase E2 component	-2,31	1,55	-
pdhD	dihydrolipoamide dehydrogenase	-1,46	-	-
Amino acid decarboxylases				
	putative lysine decarboxylase fragment	1,77	0,53	-
	arginine/lysine/ornithine decarboxylase	1,15	-	-
Polyamines				
	spermidine/putrescine ABC transporter, inner membrane subunit	2,30	-2,80	-
potD	spermidine/putrescine ABC transporter, substrate-binding protein	2,24	-2,72	0,95
potA	spermidine/putrescine ABC transporter, ATP-binding subunit	2,01	-	-1,67
Sulfur metabolism				
cysK	putative cysteine desulfurase	-	1,77	0,97
	cysteine synthase	-	1,28	-1,66
	putative iron-sulfur cluster assembly accessory protein	-1,11	1,83	1,28

(1) Expression ratio was calculated by comparing the stressed condition against a reference condition (adjusted p-value<0.05) in which microorganisms were kept in a minimal media (MRD) other things being equal. White cell: up-regulated ; Black cell: down-regulated ; "-" no significant differential expression

Gene	Product	Log_2 expression ratio[1] G-like stress	D-like stress	Combined stress
Transporter (ABC, PTS, other)				
	ABC transporter, ATP-binding subunit	4,41	2,38	-1,12
	ABC transporter, inner membrane and binding protein subunit	1,73	-	-1,38
	ABC transporter, inner membrane subunit	5,34	2,42	-0,95
	amino acid ABC transporter, binding protein subunit	1,99	-	-1,93
	amino acid ABC transporter, inner membrane subunit	1,40	-	-1,69
	amino acid transporter	1,89	0,48	1,36
	BCCT family osmoprotectant transporter	-	2,01	-
gluA	glutamate ABC transporter, ATP-binding subunit GluA	2,06	-	-
	iron-siderophore ABC transporter, ATP-binding subunit	1,77	0,90	0,84
	iron-siderophore ABC transporter, substrate-binding protein	2,26	0,85	-0,48
metN	methionine ABC transporter, ATP-binding subunit MetN	1,82	-	-
	MFS superfamily transporter	1,73	3,23	-1,71
	glycine/carnitine/choline/L-proline ABC transporter	-1,84	1,94	1,03
	polar amino acid ABC transporter, ATP-binding subunit	1,47	0,84	-1,53
	possible betaine/carnitine/choline transporter	2,26	-2,93	-
	putative ABC transporter component	1,84	-	-1,52
	putative nitrate/nitrite transporter	1,66	-	-1,66
	putative transporter	3,09	1,69	0,70
	RHBT family amino acid transporter	3,03	-	-1,50
rbsA	ribose ABC transporter, ATP-binding subunit	1,93	-0,90	-2,30
sdcS	sodium-dependent dicarboxylate transporter SdcS	1,83	-1,73	-
	sugar transporter fragment	1,99	-2,04	-1,39
	TRAP dicarboxylate transporter, substrate-binding component	1,75	-0,90	-1,09
Ion H^+ co-transport				
trkA	Trk system potassium uptake protein TrkA	2,00	0,50	-
mnhC	Na(+)/H(+) antiporter subunit C	-1,20	-0,53	-1,83
mnhB	Na(+)/H(+) antiporter subunit B	-1,48	-0,63	-1,95
mnhG	Na(+)/H(+) antiporter subunit G	-1,50	-	-0,85
mnhA	Na(+)/H(+) antiporter subunit A	-1,51	-0,66	-1,41
mnhE	Na(+)/H(+) antiporter subunit E	-1,60	-	-1,26
mnhD	Na(+)/H(+) antiporter subunit D	-1,85	-0,33	-1,61
mnhF	Na(+)/H(+) antiporter subunit F	-1,89	-	-1,16
Metal transport				
	putative heavy metal/cadmium-transporting ATPase	8,09	-1,81	-
	iron-siderophore ABC transporter, ATP-binding subunit	1,77	0,90	0,84
	iron-siderophore ABC transporter, substrate-binding protein	2,26	0,85	-0,48
	iron-siderophore ABC transporter, substrate-binding protein	2,26	0,85	-0,48
	metal ion transporter	2,42	0,47	0,95
	metal-dependent hydrolase	2,84	-1,32	1,41
modA	molybdate ABC transporter, substrate-binding protein ModA	1,61	-	-
	putative metal uptake regulation protein	5,00	-1,86	2,18
	putative metal-dependent amidase/carboxypeptidase	1,69	0,50	0,64
Multi-drug resistance transporter				
	MFS superfamily transporter	1,73	3,23	-1,71
	putative MFS superfamily transporter	-1,24	3,66	-0,92
EmrB	EmrB/QacA subfamily drug resistance transporter	-	1,74	-
	putative drug exporter of the RND superfamily	1,24	2,79	-0,82
	putative drug resistance ATP-binding protein	1,09	3,91	-
	putative EmrB/QacA subfamily drug resistance transporter	-	1,21	-1,00
	quaternary ammonium compound-resistance protein	-	4,72	-

(1) Expression ratio was calculated by comparing the stressed condition against a reference condition (adjusted p-value<0.05) in which microorganisms were kept in a minimal media (MRD) other things being equal. White cell: up-regulated ; Black cell: down-regulated ; "-" no significant differential expression

Gene	Product	Log_2 expression ratio[1] G-like stress	D-like stress	Combined stress
Translation/ Traduction				
prmA	Ribosomal protein L11 methyltransferase	-1,24	1,96	-1,52
frr	ribosome-recycling factor	-1,56	1,34	0,86
rimM	16S rRNA-processing protein RimM	3,74	-1,08	-0,84
rpsJ	30S ribosomal protein S10	-2,97	2,39	-
rpsK	30S ribosomal protein S11	-3,06	1,51	-
rpsC	30S ribosomal protein S3	-3,15	1,68	-1,25
rpsD	30S ribosomal protein S4	-3,33	3,81	-
rpsE	30S ribosomal protein S5	-3,33	1,53	-1,09
rpsF	30S ribosomal protein S6	-4,44	3,02	-
rpsG	30S ribosomal protein S7	-3,99	0,94	-
rpsS	30S ribosomal protein S19	-3,52	1,91	-1,20
rpsH	30S ribosomal protein S8	-3,54	1,59	-1,27
rpsQ	30S ribosomal protein S17	-3,55	1,51	-1,37
rpsP	30S ribosomal protein S16	-3,67	3,16	-
rpmG	50S ribosomal protein L33	-2,59	2,06	0,63
rpmH	50S ribosomal protein L34	-4,35	2,96	1,11
rpsL	30S ribosomal protein S12	-4,41	1,11	-
rpmH	50S ribosomal protein L34	-4,35	2,96	1,11
rpmI	50S ribosomal protein L35	-3,67	2,72	-
rplD	50S ribosomal protein L4	-3,61	2,25	-
rplE	50S ribosomal protein L5	-3,16	1,58	-1,30
rplF	50S ribosomal protein L6	-3,45	1,40	-1,17
rplL	50S ribosomal protein L7/L12	-4,74	1,95	-
argS	arginine--tRNA ligase	-1,96	-	-
gatA	aspartyl/glutamyl-tRNA amidotransferase subunit A	-2,78	2,47	0,76
gatB	aspartyl/glutamyl-tRNA amidotransferase subunit B	-3,01	2,44	0,68
rpoA	DNA-directed RNA polymerase alpha chain	-3,16	1,55	-
glyQS	glycine--tRNA ligase	-3,35	-0,51	-
ileS	isoleucine-tRNA ligase	-1,89	0,82	-1,24
trmFO	methyltransferase	-2,05	2,04	-
tgt	queuine tRNA-ribosyltransferase	-3,43	1,84	1,28
rlmN	ribosomal RNA large subunit methyltransferase N	-1,59	0,83	-
queA	S-adenosylmethionine:tRNA ribosyltransferase-isomerase	-2,43	1,19	-
trmD	tRNA (guanine-N(1)-)-methyltransferase	3,32	-0,49	-0,75
miaA	tRNA isopentenyltransferase fragment	1,86	0,65	1,17
trpS	tryptophan--tRNA ligase	1,62	0,90	1,16
DNA synthethis and repair				
recA	RecA bacterial DNA recombination protein	2,74	1,39	0,87
	ImpB/MucB/SamB family DNA-damage repair protein	2,68	-	-0,64
mutY	putative A/G-specific DNA glycosylase	2,01	0,35	-0,54
dnaI	primosomal protein DnaI	1,99	0,25	-
mutL	DNA mismatch repair protein MutL	-1,20	-	-1,04
pcrA	ATP-dependent DNA helicase PcrA	-1,20	2,08	-0,82
recO	DNA repair protein RecO	-1,26	1,70	1,31
mutS	DNA mismatch repair protein MutS	-1,26	0,73	-
dnaB	replicative DNA helicase	-1,43	1,85	0,91
nth	DNA-(apurinic or apyrimidinic site) lyase	-1,49	2,00	0,44
ruvB	Holliday junction ATP-dependent DNA helicase RuvB	-1,72	0,84	-0,39
ruvA	Holliday junction ATP-dependent DNA helicase RuvA	-2,20	0,45	-
	DNA topoisomerase	1,22	1,17	0,91
recG	ATP-dependent DNA helicase RecG	-2,36	0,89	-
rpoA	DNA-directed RNA polymerase alpha chain	-3,16	1,55	-
hup	DNA-binding protein HU	-3,24	-1,58	-

(1) Expression ratio was calculated by comparing the stressed condition against a reference condition (adjusted p-value<0.05) in which microorganisms were kept in a minimal media (MRD) other things being equal. White cell: up-regulated ; Black cell: down-regulated ; "-" no significant differential expression

A detailed observation of *H. alvei* GB001 sulfur metabolism (Table 4) showed a clear up-regulation of the genes *cysND*, *cysC* and *cysIJ* respectively coding for a transferase, a kinase and a reductase which are involved in the conversion of sulfate into sulfide. These enzymes allow the conversion of a +6 oxidation state molecule (sulfate) to a -2 oxidation state molecule (sulfide). Aside from the putative role of the sulfide molecule itself, the reduction process is H (+) dependent and is likely to contribute to decrease the intracellular concentration of protons in the intracellular compartment of *H. alvei*. Genes involved in the sulfate uptake are up-regulated as well (*cysA, cysT* and *cysW* respectively coding for an ATP-binding protein and two permeases). It has been documented that acidophilic organisms can use sulfur as an energy source (Johnson et al., 2008). Based on the convergent up-regulation of both metabolism and sulfur uptake, it would appear that sulfur is a core molecule in *H. alvei* GB001 ability to withstand gastric stress and should be further investigated. To pursue with *H. alvei*'s metabolism that can be linked to reduction of molecules, the genes involved in nitrate metabolism were up-regulated, namely *narH* and *narK* coding respectively for a nitrate reductase and a nitrite extrusion protein; both participate to the reduction of a nitrogen source and the same hypothesis can be drawn regarding the link between reduction and H(+) consumption as in the sulfur metabolism.

In *S. equorum* Mu2, genes encoding reductases and dehybrogenases, e.g. *mdh* and *mqo* encoding malate dehydrogenases, were up-regulated. These families of enzymes are thus possibly involved in pH homeostasis in this bacterium. Indeed, dehydrogenases are very often NAD+ dependent and take one proton to complete their dehydrogenation. Oxidized coenzymes need to be subsequently reduced which might explain the co-functioning of hydrogenases and reductases during gastric stress. In addition, still in *S. equorum* Mu2, *sodA,* encoding superoxide dismutase A*,* was up-regulated in case of gastric challenge, which participated to reduce the oxidative consequences of that stress. In the case of *H. alvei* GB001, the hydrogenases complexes drew our attention. These enzymes, widely described among gram-negative bacteria (Noguchi et al. 2010), give the ability to either produce or degrade dihydrogen. The Hyd-1, Hyd-2, Hyd-3 and Hyd-4 complexes, have been described to use dihydrogen and produce H(+) and CO_2 and, conversely, this being linked to the aeration conditions (Hayes et al. 2006, Noguchi et al. 2010). Part of the genes coding for these complexes are either up- (hyfFG) or down-regulated (*hybABCO*, *hypBCD*). Therefore, we will need to further investigate which groups of genes are involved in the formation of which hydrogenase complex in order to draw hypothesis about the role of this family of enzymes in the resistance of *H. alvei* to gastric stress. Moreover, formate metabolism, especially formate dehydrogenase, is linked to Hyd-n complexes (Noguchi et al. 2010). Still in *H. alvei* GB001, The down-regulation of *fdoGHI* genes coding for such enzymes might be linked to intracellular pH regulation as well.