



**HAL**  
open science

# Identification and transcriptional profile of *Lactobacillus paracasei* genes involved in the response to desiccation and rehydration

Aurore Palud, Karima Salem, Jean-François Cavin, Laurent Beney, H  l  ne Licandro

## ► To cite this version:

Aurore Palud, Karima Salem, Jean-Fran  ois Cavin, Laurent Beney, H  l  ne Licandro. Identification and transcriptional profile of *Lactobacillus paracasei* genes involved in the response to desiccation and rehydration. *Food Microbiology*, 2020, 85, pp.103301. 10.1016/j.fm.2019.103301 . hal-02310935

**HAL Id: hal-02310935**

**<https://u-bourgogne.hal.science/hal-02310935v1>**

Submitted on 20 Jul 2022

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destin  e au d  p  t et    la diffusion de documents scientifiques de niveau recherche, publi  s ou non,   manant des   tablissements d'enseignement et de recherche fran  ais ou   trangers, des laboratoires publics ou priv  s.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 **Identification and transcriptional profile of *Lactobacillus paracasei* genes involved in the**  
2 **response to desiccation and rehydration.**

3 **Aurore Palud<sup>a#</sup>, Karima Salem<sup>a,b</sup>, Jean-François Cavin<sup>a</sup>, Laurent Beney<sup>a</sup> and Hélène**  
4 **Licandro<sup>a#</sup>**

5 <sup>a</sup>Université de Bourgogne Franche-Comté, AgroSup Dijon, PAM UMR A 02.102, Dijon,  
6 France

7 <sup>b</sup> Ecole polytechnique de Sousse, Sousse, Tunisia

8 Running title: *Lactobacillus* desiccation genes

9 # Address correspondence to Aurore Palud, [palud.aurore@gmail.com](mailto:palud.aurore@gmail.com) and Hélène Licandro,  
10 [helene.licandro@u-bourgogne.fr](mailto:helene.licandro@u-bourgogne.fr)

11

12 **Abstract**

13 *Lactobacillus paracasei* is able to persist in a variety of natural and technological  
14 environments despite physico-chemical perturbations, in particular alternations between  
15 desiccation and rehydration. However, the way in which it adapts to hydric fluctuations and  
16 the genetic determinants involved are not clearly understood. To identify the genes involved  
17 in adaptation to desiccation, an annotated library of *L. paracasei* random transposon mutants  
18 was screened for viability after desiccation (25% relative humidity, 25°C). We found 16  
19 genes that have not been described as being involved in this response. Most of them are linked  
20 to either the transport of molecules or to cell wall structure and function. Our screening also  
21 identified genes encoding DNA related enzymes and an alarmone necessary for *L. paracasei*  
22 survival. Subsequently, the expression of the identified genes was measured at five stages of

23 the dehydration-rehydration process to decipher the chronology of genetic mechanisms. They  
24 were classified into four different transcriptional profiles: genes upregulated during both  
25 desiccation and rehydration phases, genes upregulated during the desiccation phase only,  
26 genes downregulated during both desiccation and rehydration and genes downregulated only  
27 during the rehydration stage. Thus, genetic response to hydric fluctuations seems to occur  
28 during desiccation and can continue or not during rehydration. The genes identified should  
29 contribute to improve the stabilization of *Lactobacillus* starters in dry state.

30

31 **Keywords** *Lactobacillus paracasei*, transposon mutants, desiccation, rehydration, gene  
32 expression

33

34

35

36

37

38

39

40

41

42

43

44

## 45        **1. Introduction**

46        Water is essential for all living organisms as it contributes to the structure of cells, stabilizes  
47        proteins, lipids and nucleic acids, and maintains vital metabolic systems and chemical  
48        reactions (Potts, 1994). Desiccation leads to water exit from cells, induces structural  
49        modifications, and causes osmotic and oxidative stresses (Dupont et al., 2014; França et al.,  
50        2007; Potts, 1994). In addition, the rehydration phase could lead to membrane alterations  
51        (Mille et al., 2003). The capacity to return to life after hydric fluctuations is not only crucial  
52        for bacteria as a function of diurnal and seasonal cycles in natural environments but also  
53        during food industry processes such as conventional drying, freeze drying and spray drying  
54        (Billi and Potts, 2002; Esbelin et al., 2018; Pointing and Belnap, 2012; Potts, 1994). In many  
55        organisms, desiccation survival is correlated with the accumulation of protective molecules, in  
56        particular trehalose (Crowe et al., 1998). However, desiccation tolerance involves other  
57        mechanisms that are mostly unknown except for extreme desiccation tolerant bacteria  
58        (anhydrobiotes) such as such as cyanobacteria (Potts, 1999), yeast (Dupont et al., 2014),  
59        resurrection plants (Crowe, 2014) and microscopic animals (Welnicz et al., 2011).

60        In the last few years, progress has been made in understanding the mechanisms of bacteria  
61        tolerance to partial desiccation using transcriptome analysis, in particular for *Salmonella*, one  
62        of the most common foodborne pathogens able to survive for extended periods after  
63        desiccation (Chen and Jiang, 2017; Deng et al., 2012; Finn et al., 2013; Gruzdev et al., 2012;  
64        Li et al., 2012; Maserati et al., 2017). The comparison of these studies has revealed  
65        differences in identified genes that could be explained by the procedures used to dry bacteria  
66        (surface, drying medium, RH, desiccation periods). Several common genes were linked to  
67        amino acid transport and metabolism functions. Global transcriptional analyses were also  
68        performed for soil-residing bacteria (Cytryn et al., 2007; Higo et al., 2007; LeBlanc et al.,  
69        2008) and similar responses were identified such as compatible solute and heat shock protein

70 accumulation, reactive oxygen species neutralization, and DNA modification and repair.  
71 However, transcriptomics has limitations since genes essential for a function can be  
72 constitutively expressed and even inducible genes can be overexpressed during a very short  
73 period that does not overlap with that of the experiment. Recently, random transposon  
74 mutagenesis approaches were developed to identify genes for resistance to desiccation in  
75 *Listeria monocytogenes* (Hingston et al., 2015) and *Salmonella enterica* (Mandal and Kwon,  
76 2017). For *L. monocytogenes* screening, genes were involved in energy production, membrane  
77 transport, amino acid metabolism, fatty acid metabolism and oxidative damage control. In *S.*  
78 *enterica*, in comparison to previous transcriptional studies, several genes were related to  
79 amino acid metabolism and more than 20% encoded hypothetical proteins. Recently, changes  
80 in proteomic expression were investigated for *S. enterica* during desiccation and rehydration  
81 (Maserati et al., 2018). The proteins with higher expression levels in dried samples were  
82 mainly ribosomal proteins whereas flagellar proteins, membrane proteins, and export systems  
83 as well as stress response proteins were identified in rehydrated samples.

84 Although *L. casei/paracasei* is one of the most emblematic groups of lactic acid bacteria  
85 (LAB), the genetic mechanisms involved in desiccation resistance and adaptation are not fully  
86 understood, limiting prospects for improving and developing preservation processes.  
87 Comparative genomics has demonstrated that LAB are highly adaptable to various niches  
88 such as soil, foods (dairy, meat, and vegetable), as well as oral, vaginal and gastrointestinal  
89 cavities (Cai et al., 2010; Douillard et al., 2013; Duar et al., 2017). This is correlated with an  
90 ability to adapt and persist under diverse environmental stresses (Hosseini Nezhad et al.,  
91 2015). Thanks to this capacity, humans have succeeded in selecting and producing certain  
92 LAB strains as efficient starters for fermented foods (particularly hard cheese) and as  
93 probiotics. The adequate preservation of starter cultures is necessary though it is currently

94 only possible using advanced desiccation technologies and storage in frozen state  
95 (Santivarangkna et al., 2007)

96 Considering the importance of the *L.casei/paracasei* group for health and industrial  
97 applications, we compiled a non-redundant, annotated transposon mutant library of *L.*  
98 *paracasei* (Licandro-Seraut et al., 2014; Scornec et al., 2014) based on the P<sub>junc</sub>-TpaseIS<sub>1223</sub>  
99 system, a random mutagenesis tool specifically designed for the *Lactobacillus* genus  
100 (Licandro-Seraut et al., 2012). Recently, using this approach we determined five  
101 *Lactobacillus pentosus* mutants sensitive to olive brine due to multifactorial stress (Perpetuini  
102 et al., 2013). In addition, new genetic determinants were identified during the early stage of *L.*  
103 *paracasei* establishment in the gut (Licandro-Seraut et al., 2014) and during monofactorial  
104 perturbations of mild intensities (Palud et al., 2018). In the present work, the *L. paracasei*  
105 transposon mutant library was screened to identify genes involved in the adaptation of this  
106 LAB to desiccation and rehydration. The expression of corresponding genes was studied by  
107 RT-qPCR during the two-step process to draw a chronological transcriptional profile during  
108 hydric fluctuations.

109

## 110 **2. Materials and Methods**

### 111 **2.1. Strains and growth conditions**

112 Wild-type (WT) *L. paracasei* ATCC 334 (CIP 107868, Institut Pasteur Collection) and its  
113 corresponding mutants obtained by random transposon insertion mutagenesis (Licandro-  
114 Seraut et al., 2012) were grown statically at 37°C in MRS (Difco). 5 µg/mL erythromycin  
115 (Em) was used to select mutants. The mutants correspond to the 1287 genic and intergenic  
116 mutants already described (Palud et al., 2018) (Licandro-Seraut et al., 2014). For each mutant,  
117 the putative inactivated function was assigned thanks to the genome annotation of this strain  
118 (Cai et al., 2010). The mutant library was grown in 96-well plates (200 µl) for 48 h. Plates

119 were mixed (30 s, 700 rpm, Eppendorf MixMate) and 10  $\mu$ L of each well was used to  
120 inoculate 190  $\mu$ L of MRS in new 96-well plates. Individual mutants were grown in tubes for  
121 48h (2 ml) and vortexed to inoculate at a dilution of 1/100 in new tubes. Plates and tubes were  
122 incubated for 24h at 37°C to obtain cells in stationary phase (concentration between  $1.0 \times 10^9$   
123 and  $2.0 \times 10^9$  UFC/mL) because it was the most convenient to obtain the same growth phase  
124 for the 1287 mutants, and thus to compare viability after drying.

## 125 **2.2. Drying and rehydration conditions**

126 Convective drying in a ventilated chamber at room temperature with a relative humidity (RH)  
127 of 25% was selected to mimic the severe desiccation conditions that can occur in natural  
128 environments during drought in soil, on plants, and on animal skin, for example. This process  
129 consisted in passing a flow of dry air over the cell suspension approximately simulating the  
130 desiccation conditions that organisms can undergo in the natural environment. The drying  
131 chambers were hermetic plastic boxes (20 cm  $\times$  13 cm  $\times$  6 cm) containing 100 mL saturated  
132 potassium acetate (Sigma–Aldrich) solution to obtain 25% RH at 25°C. Samples were placed  
133 on a rack in the drying chamber to keep them above the salt solution and the atmosphere was  
134 maintained using a ventilator (Sunon, Radiospare, France). Temperature and RH were  
135 controlled using an EASY Log USB tool (Lascar Electronics). Cells were rehydrated with  
136 bromocresol purple (BCP) medium which contains 5 g/L of tryptone, pepsic peptone, yeast  
137 extract and sodium acetate, 2 g/L of ammonium citrate and dipotassium phosphate, 1g/L of  
138 glucose, 1 ml of tween 80, 0.20 g/L of magnesium sulfate, 0.17 g/L of bromocresol purple and  
139 0.05 g/L of manganese sulfate.

## 140 **2.3. Effect of several sugar solutions on the viability of *L. paracasei* after desiccation**

141 We selected three disaccharides related in the literature to lactic acid bacteria preservation, in  
142 particular during freeze drying (Carvalho et al., 2004; Costa et al., 2000; Jofré et al., 2015),  
143 and their corresponding monosaccharides. *L. paracasei* cultures in stationary phase (1 mL)  
144 were centrifuged (5 min, 4000 g, 25°C) and pellets were suspended by vortexing with one  
145 volume of sugar solution diluted in water. After incubation for 15 min, 10 µL of cell  
146 suspensions were placed onto a sterile polypropylene (PP) coupon of 15 mm × 10 mm × 2  
147 mm (Scientix, Fougères, France). The inoculated coupons were placed in the ventilated  
148 chamber in plastic petri dishes. For rehydration, 110 µL of BCP medium was deposited on the  
149 dried cells and the latter were resuspended by 15 successive cycles using a micropipette.  
150 Colony enumeration by plate counts was averaged and the viability percentage was obtained  
151 with the ratio of colony enumeration (in CFU/mL) before desiccation to that after desiccation.  
152 Lactose 50 g/L was selected because it corresponds to the composition of milk, thus a  
153 condition often encountered by *L. paracasei* in the natural and technological environments of  
154 cheese-making, for example.

#### 155 **2.4. Screening of the mutant library for viability after desiccation**

156 A screening method composed of easy and rapid steps to save time and reduce the risk of bias  
157 was developed. The whole strategy followed in this work is illustrated in figure 1. The  
158 mutant library was subjected to 24-h desiccation (pronounced desiccation) followed by rapid  
159 rehydration. Stationary growth phase cultures were mixed (1 min, 1000 rpm) and 50 µL of  
160 cells were spotted in a U bottom 96-well microplate and centrifuged (5 min, 4000g, 25°C).  
161 Pellets were suspended with 50 µL of 50 g/L lactose diluted in water (1 min, 1700 rpm) and  
162 incubated 15 min at 20°C. Then, only 10 µL of suspension were placed in each well and the  
163 plates were placed in the ventilated chamber. After 24 h, each well was rehydrated rapidly  
164 with 110 µL of BCP medium at 37°C. This specifically designed culture medium with pH  
165 indicator turns to yellow when bacteria metabolize sugars after rehydration. Thus, the



166 viability of the 1287 mutants after 24-h desiccation followed by rapid rehydration was  
167 evaluated in 96-well microplates by monitoring the acidification kinetics (due to sugar  
168 metabolism). Absorbance at 420 nm ( $A_{420\text{nm}}$ ) was measured in a plate reader for 7h at 37°C  
169 (Paradigm, Beckman Coulter) and was correlated to the quantity of metabolically active (or  
170 viable) bacteria. The viability of the mutants could be compared to that of the WT ( $A_{420\text{nm}}$  (7  
171 h) =  $1.91 \pm 0.08$ ). Mutants were considered as potentially sensitive when absorbance at 7 h  
172 was lower than 1.75, which is the mean value of the WT minus twice the standard derivation,  
173 for the two biological replicates. Mutant phenotypes were subsequently validated by  
174 individual drying on PP coupons and by determining viability by the plate count method in  
175 order to remove possible false positive mutants (for instance, metabolic deficiency mutants).  
176 Colony enumeration by plate counts was averaged and the viability percentage was obtained  
177 with the ratio of colony enumeration (in CFU/mL) before desiccation to that after desiccation  
178 (3 biological replicates). Mutants were classified into three categories according to their  
179 viability after rehydration: + (mean viability at least 1.5-fold less than WT), ++ (mean  
180 viability at least 2-fold less than WT) and +++ (mean viability at least 3-fold less than WT).

## 181 **2.5. Determination of the viability of selected mutants after drying with phosphate** 182 **buffer**

183 *L. paracasei* cultures in stationary phase (1 mL) were centrifuged (5 min, 4000 g, 25°C) and  
184 pellets were suspended by vortexing with one volume of drying solution composed of  
185 phosphate buffer (10 mM pH 6,5). After incubation for 15 min, 10  $\mu\text{L}$  of cell suspensions  
186 were placed onto a sterile PP coupon. Three coupons were prepared for each mutant. The  
187 inoculated coupons were placed in the ventilated chamber in plastic petri dishes. For rapid  
188 rehydration, 110  $\mu\text{L}$  of BCP medium at 37°C was deposited on the dried cells and the latter  
189 were resuspended by 15 successive cycles using a micropipette. Colony enumeration by plate

190 counts was averaged and the viability percentage was obtained with the ratio of colony  
191 enumeration (in CFU/mL) before desiccation to that after desiccation. Mutants exhibiting a  
192 viability percentage significantly lower than the WT (Student test,  $p < 0.05$ ) and at least 1.5-  
193 fold less were determined as sensitive.

## 194 **2.6. Determination of the viability of selected mutants after progressive and rapid** 195 **rehydration**

196 *L. paracasei* cultures in stationary phase (1 mL) were centrifuged (5 min, 4000 g, 25°C) and  
197 pellets were suspended by vortexing with one volume of drying solution composed of lactose  
198 50 g/L. After incubation for 15 min, 10  $\mu$ L of cell suspensions were placed onto a sterile PP  
199 coupon. Three coupons were prepared for each mutant. The inoculated coupons were placed  
200 in the ventilated chamber in plastic petri dishes. For rapid rehydration, 110  $\mu$ L of BCP  
201 medium was deposited on the dried cells and the latter were resuspended by 15 successive  
202 cycles using a micropipette. For progressive rehydration, dried cells were introduced into a  
203 hermetic chamber at 99% RH for 2 h at 25°C. Then, 110  $\mu$ L of BCP medium was deposited  
204 on the wet bacteria cells that were recovered by 15 successive cycles using a micropipette.  
205 Colony enumeration by plate counts was averaged and the viability percentage was obtained  
206 with the ratio of colony enumeration (in CFU/mL) before desiccation to that after desiccation.  
207 Mutants exhibiting a viability percentage significantly lower than the WT (Student test,  $p <$   
208 0.05) and at least 1.5-fold less were determined as sensitive.

## 209 **2.7. Bioinformatics**

210 The putative operon organization of corresponding genes was established using the Biocyc  
211 website. Then, genes were aligned using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>)  
212 against all bacteria to determine their specificity.

## 213            **2.8.RT-qPCR**

214    The transcriptional analysis was performed using mid-exponential growth phase cells (OD<sub>600</sub>  
215    between 0.5 and 0.6) which are the most metabolically active and which generally present the  
216    strongest and the fastest gene regulation. Cells were centrifuged and concentrated to an OD<sub>600</sub>  
217    of 20. Cell pellets were suspended with phosphate buffer (10 mM pH 6.5) supplemented with  
218    50 g/L lactose except for control. The suspension was incubated for 15 min at 25°C and 1 mL  
219    was deposited on a 0.22 µm polyvinylidene membrane in a glass Petri dish to prevent cell  
220    adhesion. Cells were dried in ventilated chambers and RNA extractions were performed at  
221    two different stages of desiccation: partial desiccation D1 (76% of water evaporated after 1 h)  
222    and pronounced desiccation D2 (96% of water evaporated after 2 h) which corresponded to  
223    completely dried cells (Figure 2). As rehydration kinetics is decisive for bacterial survival,  
224    rapid and progressive rehydration were applied for RNA extractions after 2 h. Rehydrated  
225    cells were subsequently detached from the membrane using a cell lifter. Total RNA isolation,  
226    cDNA synthesis and qPCR were performed as previously described (Licandro-Seraut et al.,  
227    2008) using TRI Reagent (Sigma Aldrich), DNase I (Roche), iScript™ Reverse Transcription  
228    Supermix (Bio-Rad) and SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad).  
229    Primers were designed by using Primer3Plus (Untergasser et al., 2007) (Table 1). Quantitative  
230    PCR were performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad)  
231    in triplicate, in a 20 µL-reaction mixture. Cq (threshold value) calculation was determined by  
232    a regression model of the CFX Manager™ Software. The relative transcript levels of genes  
233    were calculated using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001). In order to select  
234    appropriate reference genes, 10 potential housekeeping genes (*fusA*, *ileS*, *lepA*, *leuS*, *mutL*,  
235    *pcrA*, *pyrG*, *recA*, *recG* and *rpoB*) (Landete et al., 2010) were tested with all the experimental  
236    conditions and analyzed using the CFX Manager™ Software. The genes *fusA*, *lepA* and *rpoB*  
237    were selected as the references because they displayed the lowest M values (0.22) and

238 coefficients of variation (0.09), meaning that they have the most stable expression in the  
239 tested conditions.

240

241

242

### 243 **3. Results**

#### 244 **3.1. Selection of a protective drying solution for the screening**

245 A library containing a total number of 1287 *L. paracasei* mutants was screened for their  
246 capacity to resist a 24-h desiccation period followed by rapid rehydration in comparison with  
247 their parental strain (*L. paracasei* ATCC 334, named WT). The viability of *L. paracasei* WT  
248 after 24-h desiccation at 25% RH was determined with and without the protectors (Table 2).  
249 Viability was very low in the absence of sugar and with monosaccharides. On the contrary,  
250 with disaccharides, viability was between 19 and 45%, which was an adequate range for  
251 screening sensitive mutants.

#### 252 **3.2. Identification of genes involved in resistance to pronounced desiccation followed** 253 **by rapid rehydration.**

254 Screening of the 1287 transposon mutants resulted in the detection of 47 sensitive mutants  
255 ( $A_{420nm}$  at 7 h < 1.75 for 2 biological replicates). After the validation step by individual drying  
256 of the 47 mutants, 16 mutants displayed a significant decrease of survival at least 1.5-fold less  
257 compared to the WT ( $p < 0.05$ ) (Table 3). Identified genes were mostly involved in metabolism  
258 and transport, cell wall structure and function, regulation of stress response and DNA related  
259 enzymes. Moreover, three sensitive mutants containing transposon in a gene encoding a  
260 hypothetical protein were identified (LSEI\_0040, LSEI\_1045 and LSEI\_1316). The majority  
261 of mutants (12) were categorized as +. The two most sensitive mutants (+++) were disrupted

262 for a putative ribonucleotide diphosphate reductase (LSEI\_1468) and for a hypothetical  
263 protein (LSEI\_1316). Two mutants disrupted for a putative PTS (LSEI\_0178) and for a  
264 putative alpha/beta fold family hydrolase (LSEI\_0756) were categorized as ++. Nine genes  
265 out of the 16 belonged to putative operons (Table 4). Seven genes were specific to *L.*  
266 *paracasei* or related species, four to *Lactobacillus* genus and five were well conserved among  
267 Gram (+) bacteria. Two genes among the three encoding hypothetical proteins presented a  
268 transmembrane domain (LSEI\_0040 and LSEI\_1045). All hypothetical protein genes were  
269 specific to the *L. paracasei* group.

### 270 **3.3. Analysis of selected mutant after pronounced desiccation with phosphate buffer**

271 We chose to omit the protector in order to strictly analyze the impact of the drying process.  
272 The WT and the 16 sensitive mutants identified previously (viability reduction > 1.5 in  
273 comparison of the WT) were subjected to a desiccation for 24 h without protector. In these  
274 conditions, the viability of the WT was  $0.059 \pm 0.006$  %, which is considerably lower than  
275 that obtained for the pronounced desiccation with lactose 50 g/L. Among the 16 sensitive  
276 mutants identified, nine were also more sensitive to a drying without protector than the WT  
277 (Table 5). They are disrupted for the following genes: a putative diadenosine tetraphosphatase  
278 (LSEI\_0167), a PTS system (LSEI\_0178), a Pyridoxine 5'-phosphate oxidase (LSEI\_0363),  
279 an NAD (FAD)-dependent dehydrogenase (LSEI\_0397), a ribonucleotide reductase  
280 (LSEI\_1468), a sorbitol operon transcription regulator (LSEI\_2725) and hypothetical protein  
281 (LSEI\_0040, LSEI\_1045 and LSEI\_1316). As observed for drying with lactose, the most  
282 sensitive mutant was disrupted for LSEI\_1468.

### 283 **3.4. Analysis of selected mutant after pronounced desiccation followed by** 284 **progressive rehydration**

285 To assess if the identified genes were involved during dehydration whatever the rehydration  
286 process, the WT and the 16 sensitive mutants identified previously (viability reduction > 1.5

287 in comparison of the WT) were subjected to desiccation for 24-h followed by progressive  
288 rehydration (for 2 h) instead of rapid rehydration. In these conditions, the viability of the WT  
289 was  $98 \pm 3\%$ , which is considerably higher than obtained with a rapid rehydration. Among the  
290 16 sensitive mutants identified previously, only four were also sensitive after desiccation and  
291 progressive rehydration (Table 6). They encode a putative diadenosine tetraphosphatase  
292 (LSEI\_0167), an NAD (FAD)-dependent dehydrogenase (LSEI\_0397), a cation transport  
293 ATPase (LSEI\_0749) and a ribonucleotide reductase (LSEI\_1468). As observed for  
294 desiccation and rapid rehydration, the most sensitive mutant was ribonucleotide diphosphate  
295 reductase.

### 296 **3.5. Transcriptional analysis of the identified genes during a desiccation –** 297 **rehydration cycle**

298 Mutant library screening allowed the identification of 16 genes involved in the global  
299 perturbation which consisted in desiccation followed by rehydration. Thus, it was not possible  
300 to determine during which phase these genes were involved since rehydration was necessary  
301 to analyze mutant phenotypes. To determine the chronology of the genetic mechanisms during  
302 humidity fluctuations, transcriptional analysis of the identified genes was carried out on the  
303 WT.

304 As lactose was required to ensure good survival of bacteria during the desiccation/rehydration  
305 process it was reasonable to expect that some candidate genes could be differentially  
306 expressed in the presence of lactose. However, for the 16 genes, expressions after 15-min  
307 incubation in lactose were comparable to that after a 15-min phosphate buffer incubation  
308 (Figure 3). Differentially expressed genes were classified to obtain the most upregulated  
309 (Table 7, values in dark red) and downregulated (Table 7, values in dark blue) genes, with a  
310 mean expression value higher or lower than 2.0. Among the 16 genes studied, 13 were  
311 differentially expressed ( $p < 0.05$ ) for at least one condition (Table 7). The three genes

312 involved in hydric changes but not differentially expressed encoded a putative PTS  
313 transporter (LSEI\_0178), a reductase (LSEI\_0758), and hypothetical proteins (LSEI\_1045).  
314 Four genes were upregulated for all the hydric fluctuations tested. These genes encoded a  
315 putative diadenosine tetraphosphatase (LSEI\_0167), a polysaccharide transporter  
316 (LSEI\_0238), a pyridoxine 5'-phosphate oxidase (LSEI\_0363), and hypothetical proteins  
317 (LSEI\_0040). Also, LSEI\_0397 encoding a putative NAD dependent dehydrogenase was  
318 upregulated during desiccation and progressive rehydration. Conversely, LSEI\_0749  
319 encoding a putative cation transport ATPase was upregulated during desiccation and rapid  
320 rehydration. Interestingly, these genes were the most upregulated (> 2-fold change) for at  
321 least one condition. One gene, LSEI\_1754, encoding a putative SAICAR synthase was the  
322 most downregulated gene in these experiments (mean expression value < 2.0 for all the drying  
323 and rehydration conditions).

324 One gene, LSEI\_2725 (a putative sorbitol operon transcription regulator) was upregulated  
325 after only 2 h of desiccation and represent potential dry state biomarkers. LSEI\_1316  
326 encoding a putative hypothetical protein was upregulated after 1 h and 2 h of desiccation.  
327 Interestingly, no gene was specifically upregulated during subsequent rehydration.  
328 Conversely, four genes were downregulated specifically during rehydration: LSEI\_0756 (a  
329 putative hydrolase) LSEI\_1009 (a putative spermidine/putrescine-binding protein),  
330 LSEI\_1468 (a putative ribonucleotide reductase) and LSEI\_1659 (a putative glucokinase).

331

#### 332 **4. Discussion**

333 Since water is the fundamental component of all living organisms, desiccation and  
334 rehydration alternation is one of the most prevalent and severe stress for most  
335 microorganisms. Adaptation to this stress occurs via a combination of mechanisms which  
336 depend on the genetic background of the microorganism. In *L. paracasei*, we developed a

337 strategy to identify genes involved in the adaptation to hydric fluctuations using random  
338 transposon mutagenesis and targeted transcriptional analysis.

339 Sixteen desiccation-sensitive mutants were identified from the *L. paracasei* mutant library  
340 screening after desiccation followed by rapid rehydration. Contrary to our recent screening of  
341 this library for mild stress sensitivity (Palud et al., 2018), no mutants for putative promoters  
342 were identified as sensitive to desiccation. Seven of the identified genes were specific of the  
343 *L. casei/paracasei/rhamnosus* group. On the contrary, five genes were well conserved among  
344 Gram-positive bacteria. Interestingly, these genes, although present in *Listeria monocytogenes*  
345 genome, were not identified by the screening of *L. monocytogenes* random mutants for  
346 desiccation resistance (Hingston et al., 2015). Otherwise, this study of *L. monocytogenes*  
347 desiccation resistance shows the involvement of a gene encoding a putative glutathione  
348 peroxidase, known to prevent oxidative damages. This gene is present in the *L. paracasei*  
349 mutant library but was not identified as sensitive. These differences may result from the  
350 drying conditions applied such as the surface used for desiccation, the relative humidity, the  
351 drying medium or the rehydration conditions.

#### 352 **4.1. Metabolism and transport**

353 Two transporters are required for survival to hydric fluctuation including a putative  
354 phosphotransferase system (PTS, LSEI\_0178) and one cation transporter (LSEI\_0749).  
355 Several authors have reported a link between PTS systems and stress response for  
356 *Lactobacillus* genus. *L. plantarum* mutants with an impaired expression of the mannose PTS  
357 operon exhibited increased sensitivity to peroxide, probably due to a diminution of glucose  
358 capture and energy production (Stevens et al., 2010).

359 Next, we identified a membrane associated protein (LSEI\_1009) predicted to bind polyamines  
360 before their transport into the cell by the ABC transporters encoded by other genes of the  
361 LSEI\_1005-1009 operon. Polyamines have various important physiological roles during stress



362 response, including the modulation of gene expression, signal transduction, oxidative defense  
363 mechanism, and cell-to-cell communication (Miller-Fleming et al., 2015; Shah and Swiatlo,  
364 2008).

365 One identified gene encoded a glucokinase (LSEI\_1659), a key enzyme of glucose  
366 metabolism. In *S. aureus*, glucokinase is also involved in pathogenicity (biofilm formation,  
367 virulence factors, cell wall synthesis) (Kumar et al., 2014). In our case, glucokinase as well as  
368 PTS interruption could lead to energy diminution and then sensitivity to hydric fluctuations.  
369 Another gene, LSEI\_0363, encodes a putative enzyme that catalyzes the oxidation of  
370 pyridoxamine-5-P (PMP) and pyridoxine-5-P (PNP) to pyridoxal-5-P (PLP) in the vitamin B6  
371 pathway. Vitamin B6, an antioxidant molecule, has been implicated in defense against cellular  
372 oxidative stress in *Saccharomyces cerevisiae* (Chumnantana et al., 2005).

#### 373 **4.2. Cell wall function and structure**

374 Modification at the cell wall level appears to be essential for surviving hydric fluctuation,  
375 considering the three genes related to the cell wall and identified as essential for hydric  
376 fluctuation survival. The gene LSEI\_0238 encodes a polysaccharide transporter involved in  
377 the export of lipoteichoic acid (LTA), a major constituent of the Gram-positive cell wall (Cai  
378 et al., 2010). Interestingly, this gene was also needed for *L. paracasei* gut establishment  
379 (Licandro-Seraut et al., 2014). The mutant for LSEI\_2546, another polysaccharide transporter  
380 involved in LTA export, was not identified as sensitive.

381 Finally, two hypothetical proteins exhibited a transmembrane domain (LSEI\_0040 and  
382 LSEI\_1045). Interestingly LSEI\_0040 was involved in the adaptation of *L. paracasei* to  
383 thermal, ethanol and oxidative stresses (Palud et al., 2018). The present and previous results  
384 show that membrane proteins are important for *L. paracasei* adaptation to various  
385 environments and involved in general stress response.

### 386 4.3. Regulation of stress response

387 One gene involved in alarmone degradation is needed for *L. paracasei* survival during hydric  
388 fluctuations. Alarmones are putative chemical messengers produced during environmental  
389 changes. LSEI\_0167 encodes a putative diadenosine tetraphosphatase, an enzyme that  
390 hydrolyzes diadenosine tetraphosphate (Ap4A) into two molecules of adenosine diphosphate  
391 (ADP). Ap4A has been reported to be involved in heat stress response in *E. coli* (Lee et al.,  
392 1983). Moreover *apaH* (diadenosine tetraphosphatase) mutation causes Ap4A accumulation  
393 and sensitivity to thermal stress (Johnstone and Farr, 1991). Nevertheless, Despotović and  
394 collaborators wondered whether Ap4A was an alarmone or a damage metabolite because they  
395 observed that no signaling cascade was triggered by Ap4A and that its accumulation at high  
396 level was toxic for *E. coli* (Despotović et al., 2017).

397 LSEI\_2725 (*gutR*) has been identified as a determinant for surviving hydric fluctuations.  
398 Although it is annotated as a sorbitol operon transcription regulator, its predicted function  
399 should be reconsidered. Alcantara *et al.*, 2008 reported that in *L. paracasei* ATCC334, *gutR* is  
400 split into 3 ORFs (LSEI\_2728, LSEI\_2726 and LSEI\_2725) as the result of transposition  
401 events, and consequently this strain is unable to use sorbitol, contrary to *L. casei* BL23. In  
402 addition, the LSEI\_2724 (*gutM*) gene, encoding a sorbitol operon activator with *gutR*, is  
403 dispensable for desiccation resistance. In conclusion, we assume that LSEI\_2725 functions as  
404 a regulator but independently of the sorbitol operon.

### 405 4.4. DNA related enzymes

406 It was widely reported that desiccation induces DNA damage (Fredrickson et al., 2008; Potts,  
407 1994). Indeed, the mutant library screening identified two genes involved in nucleotide  
408 metabolism, LSEI\_1468 (a ribonucleotide reductase, RNR) and LSEI\_1754 (a SAICAR  
409 synthase). The RNR mutant was particularly sensitive (mean viability 10-fold less than WT).  
410 RNR is an essential enzyme since it mediates the synthesis of deoxyribonucleotides, the

411 precursors of DNA synthesis (Torrents, 2014), and we have previously reported that the RNR  
412 gene was involved in general response to mild stresses in *L. paracasei* (Palud et al., 2018).  
413 SAICAR synthase is involved in de novo purine biosynthesis (Nelson et al., 2005). These two  
414 genes could be involved in DNA repair. Recently, García-Fontana *et al.* (2016) reported that  
415 the DNA molecule was overproduced and acted as a protein protector in desiccation tolerant  
416 bacteria. This phenomenon could occur in *L. paracasei*.

#### 417 **4.5. Taking into account desiccation and rehydration kinetics**

418 The results indicate that rapid rehydration of WT strain cells was more detrimental than  
419 progressive rehydration. It has been reported that progressive rehydration could reduce the  
420 stress applied to bacteria as it promotes membrane integrity recovery (Lang et al., 2017; Zoz  
421 et al., 2016). We found nine common sensitive mutants after drying with or without protector  
422 and only four sensitive mutants whatever their rehydration kinetics. Identified genes were  
423 generally specific to the screening strategy applied for the mutant library. It is very likely that  
424 the modifications of drying conditions lead to the identification of different genes. Studies on  
425 *Salmonella* species exposed to desiccation stress showed a response specificity in function of  
426 methodologies used (Finn et al., 2013)

#### 427 **4.6. Transcriptional profiles**

428 Our findings are schematized in Figure 3. Among the genes essential for survival, three genes  
429 were expressed at the same level as the control whatever the hydration phase. This result  
430 again shows the strength of global reverse genetics, since some genes may be essential for a  
431 function, although expressed constitutively. Four transcriptional profiles were observed  
432 during hydric fluctuations: six genes were upregulated during both desiccation and  
433 rehydration, two genes were upregulated only during the desiccation stage, one gene was  
434 downregulated during both desiccation and rehydration, four genes were downregulated only  
435 during the rehydration stage. We assume that adaption to hydric fluctuations occurs during

436 dehydration and continues or not during rehydration. Up regulation of genes was particularly  
437 observed after 2 hours of drying (96% of water evaporated). These results suggest that this  
438 step could be the most stressful for bacteria. Our data highlighted the importance of cell wall  
439 structure as well as diadenosine tetraphosphate degradation since LSEI\_0238 and LSEI\_0167  
440 were up regulated for all dehydration and rehydration phases.

441

442 In conclusion, this work identified 16 genetic determinants for the resistance of *L. paracasei*  
443 to desiccation. Transcriptional analysis of the corresponding genes highlighted that six genes  
444 were upregulated during both desiccation and rehydration and two during desiccation only.  
445 Thus, our results show that desiccation is a critical phase for inducing stress response in *L.*  
446 *paracasei*. This analysis provides clues for developing genetic biomarkers to monitor the  
447 intensity of desiccation during industrial processes since we identified two genes  
448 overexpressed only when the cells are dried during 2 h at 25% RH (dry state). However, it  
449 was reported that drying conditions (surface used for desiccation, the drying medium, the  
450 rehydration conditions) may modify the genes involved in the desiccation stress. Indeed, gene  
451 expression of these two genes should be studied during industrial drying process before  
452 stating that they constitute genetic biomarkers. Some genes identified in this study have  
453 higher potential since they are not limited to *L. paracasei* genomes. From an applied point of  
454 view, these genes will be preferred targets for obtaining more efficient *Lactobacillus* starters.  
455 Transcriptional studies during freeze drying could bring complementary information. In a  
456 fundamental point of view, these findings provide novel insights into the genetic mechanisms  
457 involved in desiccation and rehydration adaptation in *L. paracasei*.

#### 458 **Conflict of Interest Statement**

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

461 **Author Contributions**

462 Study conception and design: AP, KS, HL, LB and JFC. Acquisition of data: AP, KS.  
463 Analysis and interpretation of data: AP, HL, LB and JFC. Drafting of manuscript: AP. Critical  
464 revision: AP, HL, LB and JFC. All authors read and approved the final manuscript.

465 **Acknowledgments**

466 This work was funded by the Regional Council of Bourgogne Franche-Comté and the Fonds  
467 Européen de Développement Régional (FEDER). AP was supported by a PhD fellowship  
468 from the Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation  
469 (MESRI). We thank the other members of the laboratory for their assistance, including, in  
470 particular, Christine Rojas for her skillful technical assistance. We thank the Pasteur Institute  
471 Microorganism Collection for the *L. paracasei* strain (CIP 107868).

472

473

474 **References**

- Alcantara, C., Sarmiento-Rubiano, L.A., Monedero, V., Deutscher, J., Perez-Martinez, G.,  
Yebra, M.J., 2008. Regulation of *Lactobacillus casei* Sorbitol Utilization Genes  
Requires DNA-Binding Transcriptional Activator GutR and the Conserved Protein  
GutM. *Appl. Environ. Microbiol.* 74, 5731–5740.  
<https://doi.org/10.1128/AEM.00230-08>
- Billi, D., Potts, M., 2002. Life and death of dried prokaryotes. *Res. Microbiol.* 153, 7–12.  
[https://doi.org/10.1016/S0923-2508\(01\)01279-7](https://doi.org/10.1016/S0923-2508(01)01279-7)
- Cai, H., Thompson, R., Budinich, M.F., Broadbent, J.R., Steele, J.L., 2010. Genome  
Sequence and Comparative Genome Analysis of *Lactobacillus casei*: Insights into

- Their Niche-Associated Evolution. *Genome Biol. Evol.* 1, 239–257.  
<https://doi.org/10.1093/gbe/evp019>
- Carvalho, A.S., Silva, J., Ho, P., Teixeira, P., Malcata, F.X., Gibbs, P., 2004. Relevant factors for the preparation of freeze-dried lactic acid bacteria. *Int. Dairy J.* 14, 835–847.  
<https://doi.org/10.1016/j.idairyj.2004.02.001>
- Chen, Z., Jiang, X., 2017. Thermal Resistance and Gene Expression of both Desiccation-Adapted and Rehydrated *Salmonella enterica* Serovar Typhimurium Cells in Aged Broiler Litter. *Appl. Environ. Microbiol.* 83. <https://doi.org/10.1128/AEM.00367-17>
- Chumnantana, R., Yokochi, N., Yagi, T., 2005. Vitamin B6 compounds prevent the death of yeast cells due to menadione, a reactive oxygen generator. *Biochim. Biophys. Acta BBA - Gen. Subj.* 1722, 84–91. <https://doi.org/10.1016/j.bbagen.2004.11.013>
- Costa, E., Usall, J., Teixidó, N., Garcia, N., Viñas, I., 2000. Effect of protective agents, rehydration media and initial cell concentration on viability of *Pantoea agglomerans* strain CPA-2 subjected to freeze-drying. *J. Appl. Microbiol.* 89, 793–800.  
<https://doi.org/10.1046/j.1365-2672.2000.01182.x>
- Crowe, J.H., 2014. Anhydrobiosis: an unsolved problem. *Plant Cell Environ.* 37, 1491–1493.  
<https://doi.org/10.1111/pce.12304>
- Crowe, J.H., Carpenter, J.F., Crowe, L.M., 1998. The Role of Vitrification in Anhydrobiosis. *Annu. Rev. Physiol.* 60, 73–103. <https://doi.org/10.1146/annurev.physiol.60.1.73>
- Cytryn, E.J., Sangurdekar, D.P., Streeter, J.G., Franck, W.L., Chang, W. -s., Stacey, G., Emerich, D.W., Joshi, T., Xu, D., Sadowsky, M.J., 2007. Transcriptional and Physiological Responses of *Bradyrhizobium japonicum* to Desiccation-Induced Stress. *J. Bacteriol.* 189, 6751–6762. <https://doi.org/10.1128/JB.00533-07>

- Deng, X., Li, Z., Zhang, W., 2012. Transcriptome sequencing of *Salmonella enterica* serovar Enteritidis under desiccation and starvation stress in peanut oil. *Food Microbiol.* 30, 311–315. <https://doi.org/10.1016/j.fm.2011.11.001>
- Despotović, D., Brandis, A., Savidor, A., Levin, Y., Fumagalli, L., Tawfik, D.S., 2017. Diadenosine tetraphosphate (Ap<sub>4</sub>A) – an *E. coli* alarmone or a damage metabolite? *FEBS J.* 284, 2194–2215. <https://doi.org/10.1111/febs.14113>
- Douillard, F.P., Ribbera, A., Jarvinen, H.M., Kant, R., Pietila, T.E., Randazzo, C., Paulin, L., Laine, P.K., Caggia, C., von Ossowski, I., Reunanen, J., Satokari, R., Salminen, S., Palva, A., de Vos, W.M., 2013. Comparative Genomic and Functional Analysis of *Lactobacillus casei* and *Lactobacillus rhamnosus* Strains Marketed as Probiotics. *Appl. Environ. Microbiol.* 79, 1923–1933. <https://doi.org/10.1128/AEM.03467-12>
- Duar, R.M., Lin, X.B., Zheng, J., Martino, M.E., Grenier, T., Pérez-Muñoz, M.E., Leulier, F., Gänzle, M., Walter, J., 2017. Lifestyles in transition: evolution and natural history of the genus *Lactobacillus*. *FEMS Microbiol. Rev.* 41, S27–S48. <https://doi.org/10.1093/femsre/fux030>
- Dupont, S., Rapoport, A., Gervais, P., Beney, L., 2014. Survival kit of *Saccharomyces cerevisiae* for anhydrobiosis. *Appl. Microbiol. Biotechnol.* 98, 8821–8834. <https://doi.org/10.1007/s00253-014-6028-5>
- Esbelin, J., Santos, T., Hébraud, M., 2018. Desiccation: An environmental and food industry stress that bacteria commonly face. *Food Microbiol.* 69, 82–88. <https://doi.org/10.1016/j.fm.2017.07.017>
- Finn, S., Händler, K., Condell, O., Colgan, A., Cooney, S., McClure, P., Amézquita, A., Hinton, J.C.D., Fanning, S., 2013. ProP Is Required for the Survival of Desiccated

- Salmonella enterica* Serovar Typhimurium Cells on a Stainless Steel Surface. *Appl. Environ. Microbiol.* 79, 4376–4384. <https://doi.org/10.1128/AEM.00515-13>
- França, M.B., Panek, A.D., Eleutherio, E.C.A., 2007. Oxidative stress and its effects during dehydration. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* 146, 621–631. <https://doi.org/10.1016/j.cbpa.2006.02.030>
- Fredrickson, J.K., Shu-mei, W.L., Gaidamakova, E.K., Matrosova, V.Y., Zhai, M., Sulloway, H.M., Scholten, J.C., Brown, M.G., Balkwill, D.L., Daly, M.J., 2008. Protein oxidation: key to bacterial desiccation resistance? *ISME J.* 2, 393–403.
- García-Fontana, C., Narváez-Reinaldo, J.J., Castillo, F., González-López, J., Luque, I., Manzanera, M., 2016. A New Physiological Role for the DNA Molecule as a Protector against Drying Stress in Desiccation-Tolerant Microorganisms. *Front. Microbiol.* 7. <https://doi.org/10.3389/fmicb.2016.02066>
- Gruzdev, N., McClelland, M., Porwollik, S., Ofaim, S., Pinto, R., Saldinger-Sela, S., 2012. Global Transcriptional Analysis of Dehydrated *Salmonella enterica* Serovar Typhimurium. *Appl. Environ. Microbiol.* 78, 7866–7875. <https://doi.org/10.1128/AEM.01822-12>
- Higo, A., Suzuki, T., Ikeuchi, M., Ohmori, M., 2007. Dynamic transcriptional changes in response to rehydration in *Anabaena* sp. PCC 7120. *Microbiology* 153, 3685–3694. <https://doi.org/10.1099/mic.0.2007/009233-0>
- Hingston, P.A., Piercey, M.J., Hansen, L.T., 2015. Insertional mutagenesis reveals genes associated with desiccation and osmotic stress in *Listeria monocytogenes*. *Appl. Environ. Microbiol.* AEM.01134-15. <https://doi.org/10.1128/AEM.01134-15>



- Hosseini Nezhad, M., Hussain, M.A., Britz, M.L., 2015. Stress Responses in Probiotic *Lactobacillus casei*. Crit. Rev. Food Sci. Nutr. 55, 740–749. <https://doi.org/10.1080/10408398.2012.675601>
- Jofré, A., Aymerich, T., Garriga, M., 2015. Impact of different cryoprotectants on the survival of freeze-dried *Lactobacillus rhamnosus* and *Lactobacillus casei/paracasei* during long-term storage. Benef. Microbes 6, 381–386. <https://doi.org/10.3920/BM2014.0038>
- Johnstone, D.B., Farr, S.B., 1991. AppppA binds to several proteins in *Escherichia coli*, including the heat shock and oxidative stress proteins DnaK, GroEL, E89, C45 and C40. EMBO J. 10, 3897–3904.
- Kumar, P.S., Kumar, Y.N., Prasad, U.V., Yeswanth, S., Swarupa, V., Vasu, D., Venkatesh, K., Srikanth, L., Rao, V.K., Sarma, P.V.G.K., 2014. Comparative Structural and Functional Analysis of *Staphylococcus aureus* Glucokinase with other Bacterial Glucokinases. Indian J. Pharm. Sci. 76, 430–436.
- Landete, J.M., Garcia-Haro, L., Blasco, A., Manzanares, P., Berbegal, C., Monedero, V., Zuniga, M., 2010. Requirement of the *Lactobacillus casei* MaeKR Two-Component System for L-Malic Acid Utilization via a Malic Enzyme Pathway. Appl. Environ. Microbiol. 76, 84–95. <https://doi.org/10.1128/AEM.02145-09>
- Lang, E., Iaconelli, C., Zoz, F., Guyot, S., Alvarez-Martin, P., Beney, L., Perrier-Cornet, J.-M., Gervais, P., 2017. Drying parameters greatly affect the destruction of *Cronobacter sakazakii* and *Salmonella Typhimurium* in standard buffer and milk. Food Microbiol. 62, 82–91. <https://doi.org/10.1016/j.fm.2016.10.005>
- LeBlanc, J.C., Goncalves, E.R., Mohn, W.W., 2008. Global Response to Desiccation Stress in the Soil Actinomycete *Rhodococcus jostii* RHA1. Appl. Environ. Microbiol. 74, 2627–2636. <https://doi.org/10.1128/AEM.02711-07>

- Lee, P.C., Bochner, B.R., Ames, B.N., 1983. AppppA, heat-shock stress, and cell oxidation. Proc. Natl. Acad. Sci. 80, 7496–7500. <https://doi.org/10.1073/pnas.80.24.7496>
- Li, H., Bhaskara, A., Megalis, C., Tortorello, M.L., 2012. Transcriptomic Analysis of *Salmonella* Desiccation Resistance. Foodborne Pathog. Dis. 9, 1143–1151. <https://doi.org/10.1089/fpd.2012.1254>
- Licandro-Seraut, H., Brinster, S., Guchte, M. van de, Scornec, H., Maguin, E., Sansonetti, P., Cavin, J.-F., Serror, P., 2012. Development of an Efficient In Vivo System (Pjunc-TpaseIS1223) for Random Transposon Mutagenesis of *Lactobacillus casei*. Appl. Environ. Microbiol. 78, 5417–5423. <https://doi.org/10.1128/AEM.00531-12>
- Licandro-Seraut, H., Gury, J., Tran, N.P., Barthelmebs, L., Cavin, J.-F., 2008. Kinetics and Intensity of the Expression of Genes Involved in the Stress Response Tightly Induced by Phenolic Acids in *Lactobacillus plantarum*. J. Mol. Microbiol. Biotechnol. 14, 41–47. <https://doi.org/10.1159/000106081>
- Licandro-Seraut, H., Scornec, H., Pédrón, T., Cavin, J.-F., Sansonetti, P.J., 2014. Functional genomics of *Lactobacillus casei* establishment in the gut. Proc. Natl. Acad. Sci. 111, E3101–E3109. <https://doi.org/10.1073/pnas.1411883111>
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the  $2^{-\Delta\Delta CT}$  Method. Methods 25, 402–408. <https://doi.org/10.1006/meth.2001.1262>
- Mandal, R.K., Kwon, Y.M., 2017. Global Screening of *Salmonella enterica* Serovar Typhimurium Genes for Desiccation Survival. Front. Microbiol. 8. <https://doi.org/10.3389/fmicb.2017.01723>

- Maserati, A., Fink, R.C., Lourenco, A., Julius, M.L., Diez-Gonzalez, F., 2017. General response of *Salmonella enterica* serovar Typhimurium to desiccation: A new role for the virulence factors sopD and sseD in survival. *PloS One* 12, e0187692.
- Maserati, A., Lourenco, A., Diez-Gonzalez, F., Fink, R.C., 2018. iTRAQ-Based Global Proteomic Analysis of *Salmonella enterica* Serovar Typhimurium in Response to Desiccation, Low Water Activity, and Thermal Treatment. *Appl. Environ. Microbiol.* 84. <https://doi.org/10.1128/AEM.00393-18>
- Mille, Y., Beney, L., Gervais, P., 2003. Magnitude and kinetics of rehydration influence the viability of dehydrated *E. coli* K-12. *Biotechnol. Bioeng.* 83, 578–582. <https://doi.org/10.1002/bit.10706>
- Miller-Fleming, L., Olin-Sandoval, V., Campbell, K., Ralser, M., 2015. Remaining Mysteries of Molecular Biology: The Role of Polyamines in the Cell. *J. Mol. Biol.* 427, 3389–3406. <https://doi.org/10.1016/j.jmb.2015.06.020>
- Nelson, S.W., Binkowski, D.J., Honzatko, R.B., Fromm, H.J., 2005. Mechanism of Action of *Escherichia coli* Phosphoribosylaminoimidazolesuccinocarboxamide Synthetase. *Biochemistry* 44, 766–774. <https://doi.org/10.1021/bi048191w>
- Palud, A., Scornec, H., Cavin, J.-F., Licandro, H., 2018. New Genes Involved in Mild Stress Response Identified by Transposon Mutagenesis in *Lactobacillus paracasei*. *Front. Microbiol.* 9. <https://doi.org/10.3389/fmicb.2018.00535>
- Perpetuini, G., Scornec, H., Tofalo, R., Serror, P., Schirone, M., Suzzi, G., Corsetti, A., Cavin, J.F., Licandro-Seraut, H., 2013. Identification of Critical Genes for Growth in Olive Brine by Transposon Mutagenesis of *Lactobacillus pentosus* C11. *Appl. Environ. Microbiol.* 79, 4568–4575. <https://doi.org/10.1128/AEM.01159-13>

- Pointing, S.B., Belnap, J., 2012. Microbial colonization and controls in dryland systems. *Nat. Rev. Microbiol.* 10, 551–562. <https://doi.org/10.1038/nrmicro2831>
- Potts, M., 1999. Mechanisms of desiccation tolerance in cyanobacteria. *Eur. J. Phycol.* 34, 319–328. <https://doi.org/10.1080/09670269910001736382>
- Potts, M., 1994. Desiccation tolerance of prokaryotes. *Microbiol. Rev.* 58, 755–805.
- Santivarangkna, C., Kulozik, U., Foerst, P., 2007. Alternative Drying Processes for the Industrial Preservation of Lactic Acid Starter Cultures. *Biotechnol. Prog.* 23, 302–315. <https://doi.org/10.1021/bp060268f>
- Scornec, H., Tichit, M., Bouchier, C., Pédrón, T., Cavin, J.-F., Sansonetti, P.J., Licandro-Seraut, H., 2014. Rapid 96-well plates DNA extraction and sequencing procedures to identify genome-wide transposon insertion sites in a difficult to lyse bacterium: *Lactobacillus casei*. *J. Microbiol. Methods* 106, 78–82. <https://doi.org/10.1016/j.mimet.2014.08.001>
- Shah, P., Swiatlo, E., 2008. A multifaceted role for polyamines in bacterial pathogens. *Mol. Microbiol.* 68, 4–16. <https://doi.org/10.1111/j.1365-2958.2008.06126.x>
- Stevens, M.J.A., Molenaar, D., de Jong, A., de Vos, W.M., Kleerebezem, M., 2010. Involvement of the Mannose Phosphotransferase System of *Lactobacillus plantarum* WCFS1 in Peroxide Stress Tolerance. *Appl. Environ. Microbiol.* 76, 3748–3752. <https://doi.org/10.1128/AEM.00073-10>
- Torrents, E., 2014. Ribonucleotide reductases: essential enzymes for bacterial life. *Front. Cell. Infect. Microbiol.* 4. <https://doi.org/10.3389/fcimb.2014.00052>
- Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., Leunissen, J.A.M., 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res.* 35, W71–W74. <https://doi.org/10.1093/nar/gkm306>

Welnicz, W., Grohme, M.A., Kaczmarek, Ł., Schill, R.O., Frohme, M., 2011. Anhydrobiosis in tardigrades—The last decade. *J. Insect Physiol., Dormancy and Developmental Arrest in Invertebrates* 57, 577–583. <https://doi.org/10.1016/j.jinsphys.2011.03.019>

Zoz, F., Iaconelli, C., Lang, E., Iddir, H., Guyot, S., Grandvalet, C., Gervais, P., Beney, L., 2016. Control of Relative Air Humidity as a Potential Means to Improve Hygiene on Surfaces: A Preliminary Approach with *Listeria monocytogenes*. *PLoS ONE* 11. <https://doi.org/10.1371/journal.pone.0148418>

## Tables

**Table 1. Primers designed for RT-qPCR.**

Primers	Sequence (5' → 3')	Primers	Sequence (5' → 3')
0040-F	CCACAAATGAGTCAAACCATCT	0040-R	GGTCGTTATCGCACCTAATGA
0167-F	ATGCCGACTGCTGTTGAGTT	0167-R	CGGACCCACATTCGATTA
0178-F	TAGGCAACATTCGCAGTCAA	0178-R	ATAATCGGCAACGGCTTTC
0238-F	CGGATCGCTTCCCTTATTCT	0238-R	CCAACATCTGTGAGCCAGAC
0363-F	GGTCCCAAGGGTTCACTACA	0363-R	GCCAAATGGTTGTTGAGAG
0397-F	GGCACGACGCAGGATTAT	0397-R	CCACGCATCAAGAAGACATT
0749-F	ATGACTGGTGACGGTGTGAA	0749-R	AACGAAATCCGCAATAGAGC
0756-F	CACTGACAGCCACGATCAAA	0756-R	CTTGCTGCCGTGAATAAACA
0758-F	TGATTCAGGCACAACGATTT	0758-R	GCGTCACGTCGGTTTCTTTA
1009-F	CAGGTGAAGCCAGTGAGATG	1009-R	CCGTCTTAGGGATGACCAAGT
1045-F	CAATACACAGACCGCCAGTG	1045-R	ATCGTTGGCTTTGTGCTCTT
1316-F	CGCCGCATGAAGTATGTTTA	1316-R	GAGGGCTCAACACGTTTAACA
1468-F	CAATGGTTTCGTGCTTTGACTT	1468-R	TGCTGTGAGCCTGGTGATTA
1659-F	TGGATGAAGGCAGTCACATC	1659-R	CCCATACCGATACCGACAAA
1754-F	CCGCCAATCCACGAATACTA	1754-R	GCACTTGCTTGAGTTGTTTCAG
2725-F	CAGATCGACCATTGACACACA	2725-R	GCCGATGCCCTTACCTTAGT
fusA-F	CCTGAAACTGGCGAAACATT	fusA-R	CCAACCTTAGCAGCAACCTT
ileS-F	GCAACGGTTGACTCTTCCTC	ileS-R	GCTACCATAAACCGCATCGT
lepA-F	AAGAGCAGCACGAAGGGATA	lepA-R	AGGATCGCCATTAAGCAAGA
leuS-F	GCTGGATGCTGGTATTGCTT	leuS-R	ATCACGCAGTTTGCCTTCAT
mutL-F	CCTGCCAGTGTTGTCAAAGA	mutL-R	CGTTATCGCTAACCCGAATC
pcrA-F	GCAACACAAGCGTTGAGAAG	pcrA-R	ACCAGCAATCGGACTGAGTT
pyrG-F	GAAGCGTGATGTTGGTTCG	pyrG-R	TGTGCTGAGTTGGTTTCGTC
recA-F	TTGGCAACCGATAAAGACAA	recA-R	TGAGATGCGTTGACAAGTCC
recG-F	CGTAGCGTGATTCTGGTGAC	recG-R	TTCCAAAGATTGCTGCTTCA
rpoB-F	AATACAAGGCAGCCCATGAC	rpoB-R	ATCCAAGGCACCATCTTCAC

**Table 2. *L. paracasei* ATCC 334 viability after a pronounced desiccation period followed by rapid rehydration with or without saccharides used as protectors.**

<b>Condition of drying</b>	<b>Concentration (g/L)</b>	<b>Viability percentage after desiccation/rehydration</b>
No protector (distilled water)		0.001 ± 0.000
<b>Monosaccharides</b>		
Glucose	25	0.13 ± 0.09
	50	0.01 ± 0.01
Fructose	25	0.01 ± 0.01
	50	0.04 ± 0.02
Galactose	25	0.05 ± 0.02
	50	0.09 ± 0.04
<b>Disaccharides</b>		
Lactose	25	21 ± 2
	50	35 ± 7
Trehalose	25	41 ± 9
	50	19 ± 7
Sucrose	25	27 ± 9
	50	45 ± 0

Bacterial cells in stationary growth phase suspended in various protective solutions were air dried for 24 h at 25% RH and 25°C on PP coupons. Survival was determined after suspending dried cells in BCP medium. Viability was measured by plate count (CFU/ml).

**Table 3. Viability of *L. paracasei* sensitive mutants after a pronounced desiccation period followed by rapid rehydration**

Disrupted gene	Predictive function	A <sub>420nm</sub> at 7 h	Viability %	Sensitivity level
WT		1.91 ± 0.08	41 ± 2	
LSEI_0040	Hypothetical protein	1.56 / 1.69	23 ± 4**	+
LSEI_0167	Diadenosine tetraphosphatase	1.71 / 1.64	22 ± 2**	+
LSEI_0178	PTS	1.73 / 1.59	18 ± 9*	++
LSEI_0238	PST transporter	1.63 / 1.66	23 ± 7**	+
LSEI_0363	Pyridoxine 5'-phosphate oxidase	1.74 / 1.75	26 ± 6*	+
LSEI_0397	NAD (FAD)-dependent dehydrogenase	1.34 / 1.40	25 ± 5**	+
LSEI_0749	Cation transport ATPase	1.74 / 1.75	23 ± 3**	+
LSEI_0756	Alpha/beta fold family hydrolase	1.62 / 1.75	18 ± 1**	++
LSEI_0758	Aldo/keto reductase related enzyme	1.45 / 1.48	22 ± 3**	+
LSEI_1009	Spermidine/putrescine-binding protein	1.63 / 1.45	26 ± 1**	+
LSEI_1045	Hypothetical protein	1.72 / 1.69	23 ± 4**	+
LSEI_1316	Hypothetical protein	1.30 / 1.27	14 ± 5**	+++
LSEI_1468	Ribonucleotide diphosphate reductase	1.10 / 1.11	4 ± 1**	+++
LSEI_1659	Glucokinase	1.65 / 1.66	25 ± 8*	+
LSEI_1754	SAICAR synthase	1.75 / 1.46	23 ± 4**	+
LSEI_2725	Sorbitol transcription regulator	1.74 / 1.70	27 ± 5*	+

Cells in stationary growth phase were air dried for 24 h at 25% RH and 25°C with 50 g/L lactose solution in microplates or on PP coupons. Sensitive mutants were determined by monitoring the A<sub>420nm</sub> of dried cells suspended in BCP medium (2 biological replicates) and confirmed by individual drying on PP coupons and plate count (3 biological replicates). Mutants were classified into three categories according to their viability: + (mean viability at least 1.5-fold less), ++ (mean viability at least 2-fold less) and +++ (mean viability at least 3-fold less than WT).

\*, significant changes in viability (p<0.05) compared to the WT strain (3 biological replicates).

\*\*, significant changes in viability (p<0.01) compared to the WT strain (3 biological replicates).



**Table 4. Operon organization and specificity of the 16 genes involved in adaptation to pronounced desiccation followed by rapid rehydration.**

Gene	Predictive function	Putative operon	DNA strand	Specificity
LSEI_0040	Hypothetical protein	No	(+)	<i>L.casei/paracasei/rhamnosus</i>
LSEI_0167	Diadenosine tetraphosphatase	No	(-)	<i>L.casei/paracasei/rhamnosus</i>
LSEI_0178	PTS	0178-0180	(+)	<i>L.casei/paracasei/rhamnosus</i>
LSEI_0238	PST transporter	0238-0240	(+)	<i>L.casei/paracasei/rhamnosus</i>
LSEI_0363	Pyridoxine 5'-phosphate oxidase	No	(-)	<i>Lactobacillus</i>
LSEI_0397	NAD (FAD)- dehydrogenase	No	(-)	<i>Lactobacillus</i>
LSEI_0749	Cation transport ATPase	No	(-)	<i>Lactobacillus</i>
LSEI_0756	Alpha/beta fold family hydrolase	No	(+)	<i>Lactobacillus</i>
LSEI_0758	Aldo/keto reductase related enzyme	0757-0758	(-)	Gram +
LSEI_1009	Spermidine/putrescine-binding protein	1005-1009	(+)	Gram +
LSEI_1045	Hypothetical protein	No	(-)	<i>L.casei/paracasei/rhamnosus</i>
LSEI_1316	Hypothetical protein	1314-1319	(+)	<i>L.casei/paracasei/rhamnosus</i>
LSEI_1468	Ribonucleotide reductase	1467-1470	(+)	Gram +
LSEI_1659	Glucokinase	1658-1661	(-)	Gram +
LSEI_1754	SAICAR synthase	1746-1756	(-)	Gram +
LSEI_2725	Sorbitol transcription regulator	2720-2726	(-)	<i>L.casei/paracasei/rhamnosus</i>

**Table 5. Viability of selected *L. paracasei* mutants after a pronounced desiccation without drying protector**

<b>Disrupted gene</b>	<b>Predictive function</b>	<b>Viability %</b>
<b>WT</b>		<b>0,059 ± 0,006</b>
LSEI_0040	Hypothetical protein	0.034 ± 0,002**
LSEI_0167	Diadenosine tetraphosphatase	0.032 ± 0.003**
LSEI_0178	PTS	0.037 ± 0.003**
LSEI_0363	Pyridoxine 5'-phosphate oxidase	0,035 ± 0,005**
LSEI_0397	NAD (FAD)-dependent dehydrogenase	0,034 ± 0,000**
LSEI_1045	Hypothetical protein	0,017 ± 0,001**
LSEI_1316	Hypothetical protein	0,026 ± 0,002**
LSEI_1468	Ribonucleotide diphosphate reductase	0,002 ± 0,000**
LSEI_2725	Sorbitol operon transcription regulator	0,021 ± 0,003**

Cells in stationary growth phase were air dried for 24 h at 25% RH and 25°C with phosphate buffer on PP coupons. Viability percentages were determined after rapid rehydration by suspending dried cells with 110 µL of BCP medium at 37°C (15 successive cycles).

\*, significant changes in viability ( $p < 0.05$ ) compared to the WT strain (3 biological replicates).

\*\*, significant changes in viability ( $p < 0.01$ ) compared to the WT strain (3 biological replicates).

**Table 6. Viability of *L. paracasei* selected mutants after a pronounced desiccation followed by a progressive rehydration**

Disrupted gene	Predictive function	Viability %
WT		98 ± 3
LSEI_0167	Diadenosine tetraphosphatase	60 ± 0**
LSEI_0397	NAD (FAD)-dependent dehydrogenase	41 ± 6**
LSEI_0749	Cation transport ATPase	57 ± 4**
LSEI_1468	Ribonucleotide diphosphate reductase	14 ± 2**

Cells in stationary growth phase were air dried for 24 h at 25% RH and 25°C with 50 g/L lactose solution on PP coupons. Progressive rehydration was performed in a closed chamber with RH adjusted at 99% for 2 h. Viability percentages were determined by suspending dried cells with 110 µL of BCP medium at 37°C (15 successive cycles).

\*, significant changes in viability ( $p < 0.05$ ) compared to the WT strain (3 biological replicates).

\*\*, significant changes in viability ( $p < 0.01$ ) compared to the WT strain (3 biological replicates).

**Table 7. Relative transcript level of *L. paracasei* genes after desiccation (1 h and 2 h) and rehydration (rapid or progressive) for the 16 genes identified after library mutant screening.**

Gene	Predictive function	LAC	D1	D2	RR	PR
LSEI_0040	Hypothetical protein	1.0 ± 0.1	1.7 ± 0.6*	2.6 ± 0.3*	2.1 ± 0.2*	1.9 ± 0.4
LSEI_0167	Diadenosine tetraphosphatase	1.0 ± 0.1	1.8 ± 0.4*	2.3 ± 0.3*	1.6 ± 0.2	2.1 ± 0.4*
LSEI_0178	PTS	1.0 ± 0.2	-1.1 ± 0.2	-1.1 ± 0.1	-1.3 ± 0.2	-1.3 ± 0.3
LSEI_0238	PST family polysaccharide transporter	1.0 ± 0.1	1.3 ± 0.5*	2.2 ± 0.3*	1.6 ± 0.1*	1.9 ± 0.3*
LSEI_0363	Pyridoxine 5'-phosphate oxidase	1.0 ± 0.1	1.4 ± 0.4*	1.9 ± 0.2*	1.6 ± 0.2*	1.9 ± 0.3*
LSEI_0397	NAD(FAD)-dependent dehydrogenase	1.0 ± 0.2	1.6 ± 0.4*	1.8 ± 0.2*	1.7 ± 0.2*	-1.3 ± 0.2
LSEI_0749	Cation transport ATPase	1.1 ± 0.1	2.1 ± 0.2*	2.1 ± 0.2*	1.3 ± 0.3	1.6 ± 0.1*
LSEI_0756	Alpha/beta fold family hydrolase	1.0 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	-1.4 ± 0.2*	-1.7 ± 0.3*
LSEI_0758	Aldo/keto reductase	-1.1 ± 0.2	-1.4 ± 0.4	-1.1 ± 0.1	-1.1 ± 0.1	1.0 ± 0.2
LSEI_1009	Spermidine/putrescine-binding protein	-1.1 ± 0.1	-1.3 ± 0.2	-1.4 ± 0.1	-2.0 ± 0.4*	-2.0 ± 0.4*
LSEI_1045	Hypothetical protein	-1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	-1.3 ± 0.2	-1.4 ± 0.2
LSEI_1316	Hypothetical protein	-1.1 ± 0.1	1.7 ± 0.2*	1.6 ± 0.2*	1.0 ± 0.1	-1.1 ± 0.2
LSEI_1468	Ribonucleotide diphosphate reductase	1.0 ± 0.2	1.5 ± 0.1	1.2 ± 0.1	-1.3 ± 0.2*	-1.7 ± 0.3*
LSEI_1659	Glucokinase	1.1 ± 0.1	-1.4 ± 0.4	-1.3 ± 0.2	-1.4 ± 0.2*	-1.4 ± 0.2
LSEI_1754	SAICAR synthase	-1.1 ± 0.1	-2.5 ± 0.6*	-2.5 ± 0.6*	-2.5 ± 0.6*	-2.5 ± 0.6*
LSEI_2725	Sorbitol operon transcription regulator	-1.1 ± 0.1	1.3 ± 0.3	1.5 ± 0.2*	1.2 ± 0.1	1.4 ± 0.3
<b>Total of genes differentially expressed</b>		<b>0</b>	<b>10</b>	<b>13</b>	<b>13</b>	<b>11</b>

LAC (cells incubated 15 min with lactose), D1 (cells dehydrated for 1 h), D2 (cells dehydrated for 2 h), RR (rapid rehydration with 1 mL of distilled water), PR (progressive rehydration in a closed chamber with RH adjusted at 99% for 2 h). RTL were calculated using  $2^{-\Delta\Delta C_t}$  method. For the phosphate buffer control condition, a gene expression value of 1.0 was attributed and RTL of genes in stress condition were calculated as a function of this value. Positive values (> 1.0) represent upregulation and negative values (< 1.0) represent downregulation. \*, significant changes in gene expression (p<0.05) compared to the phosphate buffer condition (4 biological replicates). Values in light red correspond to

upregulation and in dark red to upregulations  $> 2.0$ ; values in light blue correspond to downregulation and in dark blue to down regulation  $< -2.0$ .

### **Figure legends**

**Figure 1. Organizational chart of the strategy developed in this work to identify genetic determinants of *L. paracasei* resistance to desiccation and rehydration.** PPC, polypropylene coupons.

**Figure 2. Evolution of the cell suspension mass during desiccation (orange curve) and rehydration (blue curve) on a polyvinylidene membrane.** Sampling times for RNA extractions are represented by the grey boxes: PB (1 mL of cells incubated 15 min with phosphate buffer control), LAC (1 mL of cells incubated 15 min with phosphate buffer supplemented with 50 g/L lactose), D1 (cells dried 1 h, partial desiccation), D2 (cells dried 2 h, pronounced desiccation), RR (rapid rehydration with 1 mL of distilled water), PR (progressive rehydration in a closed chamber with RH adjusted at 99% for 2 h and addition of water to obtain the mass before drying).

**Figure 3. Schematic representation of genes differentially transcribed in *L. paracasei* during desiccation and rehydration.** Gene expressions are represented by color boxes in light red for upregulation, dark red for upregulations  $> 2.0$ , light blue for downregulation, dark blue for downregulation  $< -2.0$  and in white for constitutive regulation. For each gene, boxes from left to right correspond to: lactose incubation (LT), desiccation for 1h (D1), desiccation for 2h (D2), rapid rehydration (RR) and progressive rehydration (PR).

**Mutant Library – 1287 mutants**  
(1110 genic mutants + 177 intergenic mutants)

**24 h air drying in 96 well plates**  
25 % RH – 25°C – lactose 50 g/L

**Absorbance at 420 nm in 96 well plates**  
Rehydration with BCP medium – 37°C

**Sensitive mutants**  
 $A_{420}$  at 7h < 0.75  
(2 biological replicates)

**WT absorbance**  
 $A_{420}$  at 7h  
=  $0.91 \pm 0.08$

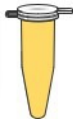
**Validation of mutants on PPC**  
Percentage viability ( $p < 0.05$ )  
(3 biological replicates)

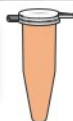
**WT survival on PPC**  
Percentage survival  
=  $41 \pm 2 \%$

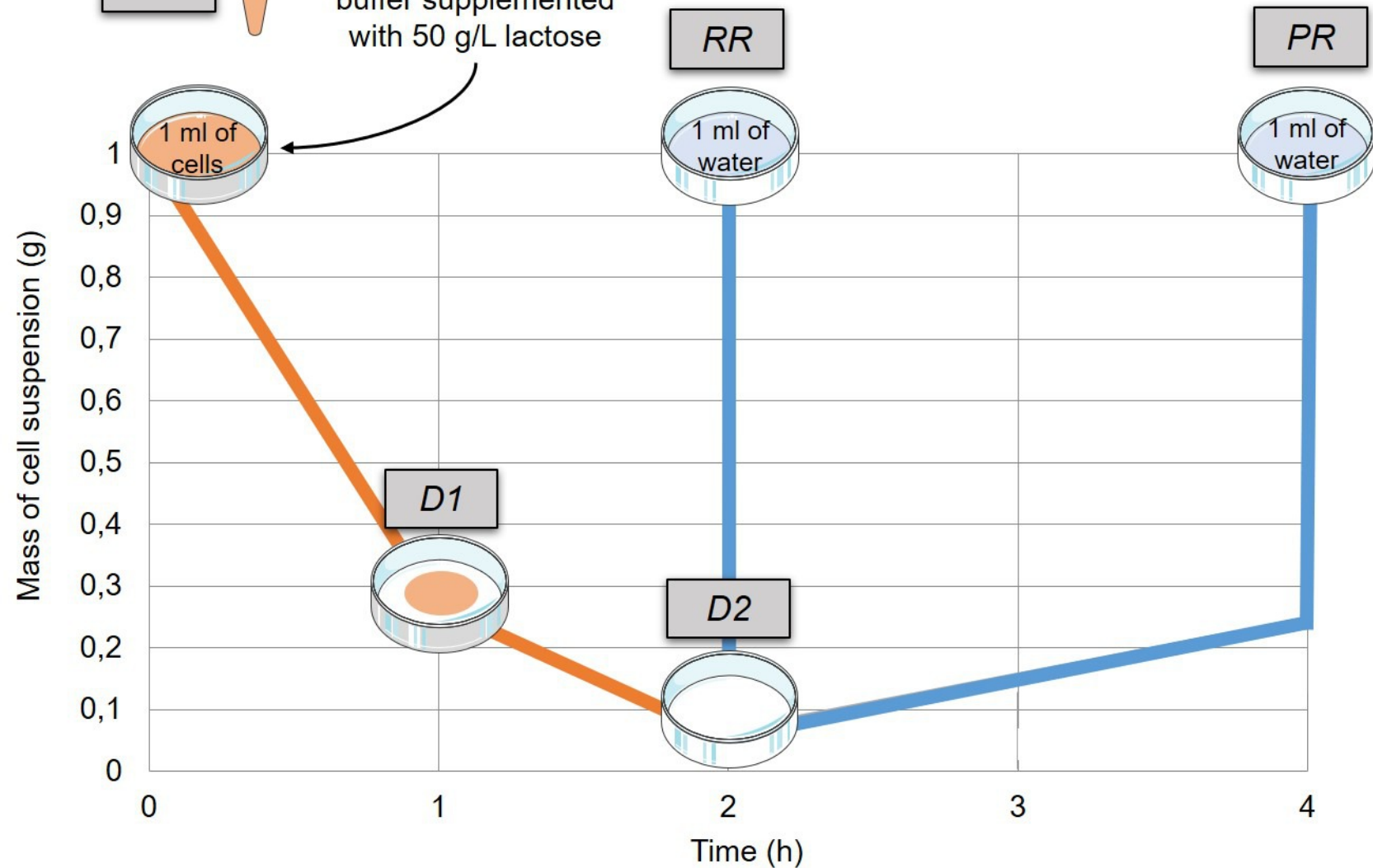
**Identification of genes**  
Involved in dehydration and  
rehydration phases

**WT**  
RNA extraction in the  
different phases

**RT-qPCR**  
Expression of genes in the  
different phases

**PB**  15 min in phosphate buffer control

**LAC**  15 min in phosphate buffer supplemented with 50 g/L lactose



LT | D1 | D2 | RR | PR

