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1 # Assessment of stress tolerance acquisition in the heat-tolerant
2 # derivative strains of *Bifidobacterium animalis* subsp. *lactis* BB-
3 # 12 and *Lactobacillus rhamnosus* GG

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16 Running title: Heat adaptation and response in LGG and BB-12

ABSTRACT

Aims: The purpose of this study was to investigate the heat shock response at molecular level in *L. rhamnosus* GG, *B. animalis* subsp. *lactis* BB-12 and their heat tolerant derivatives and to characterize the changes that make the derivatives more robust in terms of heat stress.

Methods and results: The study strains were exposed for 2 hours to a heat shock treatment, *B. animalis* subsp. *lactis* BB-12 and its derivative at 50 °C and the *L. rhamnosus* GG and its derivative at 60 °C. Protein synthesis before and after heat shock was examined using proteomics and RT-qPCR. The analysis revealed that the regulation of seven proteins in both strains and their derivatives was modified during heat treatment. The comparison of wild-type strains and the heat-tolerant derivatives suggests that the acquisition of heat-tolerance in the *B. animalis* subsp. *lactis* BB-12 derivative is due to a slightly increased constitutive level of chaperones whilst in *L. rhamnosus* GG derivative the main reason seems to be a higher ability to induce the production of chaperones.

Conclusions: This study revealed possible markers of heat-tolerance in *B. lactis* and *L. rhamnosus* strains.

Significance and Impact of study: This study increases our knowledge on how *Lactobacillus* and *Bifidobacterium* strains may acquire heat tolerance. These findings may be useful for improving the heat tolerance of existing probiotic strains as well as screening new heat tolerant strains.

Keywords: proteomics, probiotics, 2D-DIGE, *Lactobacillus rhamnosus* GG, *Bifidobacterium animalis* subsp. *lactis* BB-12, heat shock

37 ## INTRODUCTION

38 Probiotics are defined as ‘live microorganisms which when administered in adequate amounts,
39 confer a health benefit on the host’ (FAO/WHO 2002). At present, lactobacilli and bifidobacteria are
40 the most common bacteria used as probiotics and their properties are strain-dependent and strain-
41 specific. In order to exert their beneficial effects probiotic bacteria must retain the characteristics
42 and properties originally used for their selection (Tuomola et al. 2001).

43 During different manufacturing and storage stages, in order to survive probiotic bacteria have to
44 respond rapidly to stress. Heat is among the hardest stress conditions that may be present during
45 manufacturing of different food products, such as bread and chocolate, in which probiotics could be
46 applicable. The poor heat-tolerance of probiotics has precluded their inclusion in such products and
47 limited their application to, fermented milks and similar products where heat-stress is not present
48 during manufacturing processes. Challenge with high temperatures leads to denaturation of proteins
49 and their subsequent aggregation. Heat also destabilizes macromolecules such as ribosomes and
50 RNA and alterations of membrane fluidity have been reported as well (van de Guchte et al. 2002).
51 The mechanisms underlying heat shock adaptation have been described earlier in different bacterial
52 species, most notably in *Escherichia coli* and *Bacillus subtilis* (Barreiro et al. 2005, De Angelis and
53 Gobbetti 2004, Gao et al. 2004, Helmann et al. 2001, Rezzonico et al. 2007, Richmond et al. 1999,
54 Stewart et al. 2002, Weiner et al. 2003).

55 In this study we used two-dimensional differential in gel electrophoresis (2D-DIGE) coupled with
56 mass spectrometry, together with reverse transcriptase quantitative PCR (RT-qPCR), to assess the
57 heat-response in the two most commonly used probiotic strains, *Bifidobacterium animalis* subsp.
58 *lactis* BB-12 and *Lactobacillus rhamnosus* GG (ATCC 53013), and to compare with that of their
59 previously obtained heat-tolerant derivatives (BB-12 HS and LGG HS) (du Toit et al. 2013). Our

60 aim was to determine the molecular basis of the heat-stress response in these strains and to
61 characterize the changes that make the derivative strains more robust in terms of heat stress.

62 MATERIALS AND METHODS

63 Bacterial strains and growth

64 Bacterial strains used in this study were *B. animalis* subsp. *lactis* BB-12 and its heat tolerant
65 derivative (BB-12 HS) and *L. rhamnosus* GG (ATCC 53013) and its heat-tolerant derivative (LGG
66 HS) (du Toit et al. 2013). Strains were routinely grown in de Man-Rogosa-Sharpe broth (De Man et
67 al. 1960) (MRS, BD Diagnostic Systems) supplemented with 0.05% L-cysteine (w/v) (Sigma
68 Chemical Co) (MRSC) in an anaerobic cabinet (MG500, Don Whitley Scientific, Yorkshire, UK)
69 with an atmosphere of 10% CO_2 - 10% H_2 - 80% N_2 at 37ºC. 0.5 ml of these pre-cultures was
70 inoculated to 50 ml of MRSC medium and grown over-night at 37 °C in the same conditions. After
71 16 hours biomass was harvested. The cultures were centrifuged at 15 000 × g and re-suspended in
72 50 ml of fresh medium. The heat treatment was performed by incubating the *B. animalis* subsp.
73 *lactis* BB-12 and BB-12 HS cultures at 50 °C and those of *L. rhamnosus* GG at 60 °C for 2 hours
74 (Figure 1). Samples were taken at 0 hours and after the heat-treatment, the cultures were centrifuged
75 at 15 000 × g and washed twice with PBS and then the bacterial pellets were frozen at -20 °C until
76 protein extraction. For RT-qPCR, after the heat treatment and washing of the cultures, 100 µl of the
77 bacterial suspension were diluted in 400 µl of PBS buffer and 1000 µl of RNA Protect Bacteria
78 Reagent (Qiagen) were added to stabilise the RNA according to the manufacturer's instructions. The
79 stabilised pellets were stored at -80ºC until RNA extraction.

80 **Protein extraction**

81 The cells were broken by ultrasonic treatment (Sonics & materials, Vibra-cell, model VC600, 20
82 kHz) by sonicating the cells three times for one minute and keeping them on ice for one minute in
83 between the sonications. Next, unbroken cells and cell debris were removed by centrifugation at (15
84 000 × g at 4 °C, 5 minutes). The proteins were precipitated by methanol-chloroform (3:1, vol/vol)
85 according to the method described by Wessel and Flugge (1984) and the protein concentration of the
86 extract was determined using Pierce® BCA Protein Assay Kit (Thermo Scientific) according to the
87 manufacturer's instructions. The protein extracts were frozen at -20 °C until further use.

88 **2D Electrophoresis**

89 The 2D electrophoresis was done with two different dyeing techniques. First the electrophoresis was
90 visualized using Gelcode™ Blue Safe Protein Stain (Thermo scientific) to obtain reference maps of
91 the proteins in the different extracts. Additionally, these gels were used for spot picking and mass
92 spectrometry analysis. The same protein extracts were also analysed with Amersham CyDye DIGE
93 Fluors (minimal dyes) for Ettan DIGE (GE Healthcare). For the 2D electrophoresis, 500 µg of
94 protein was used for the reference map gels and 50 µg for the gels on which the samples were
95 labelled with fluorescent dyes.

96 For the reference map gels, the protein samples were solubilized in 450 µl Destreak Rehydration
97 Solution (GE Healthcare, Little Chalfont, United Kingdom) supplemented with 0.5 % IPG Buffer
98 (pH 4-7, GE Healthcare). The solutions were then used to rehydrate Immobiline™ Drystrips (pH 4-
99 7, 24 cm, GE Healthcare) for 12 h at constant voltage of 50 V at 20 °C using an Ettan IPGphor
100 device (Amersham Biosciences, Amersham, United Kingdom) and focused at 60 000 Vh. The
101 focused strips were then equilibrated for 15 minutes in a buffer (1 mol l^{-1} Tris-HCl, pH 6.8,

102 containing 6 mol l^{-1} urea, 30% [vol/vol] glycerol) and 1% (wt/vol) sodium dodecyl sulfate)

103 supplemented with 0.83% (wt/vol) dithiothreitol in the first equilibration step and with 7.5%

104 (wt/vol) iodoacetamide in the second one. The second-dimension separation was performed by

105 sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12.5 % polyacrylamide gels and

106 carried out with Ettan DALTsix electrophoresis unit (Amersham Biosciences) at a constant current

107 of 16 mA per gel over night at 4 °C. After the electrophoresis, the gels were stained with Gelcode™

108 Blue Safe Protein Stain (Thermo scientific) according to the manufacturer's instructions and finally,

109 the gels were scanned with Imagescanner using Labscan software (Amersham Biosciences).

110 **2D-DIGE with Amersham CyDye DIGE Fluors (minimal dyes) for Ettan DIGE (6905-6919GE**

111 **Healthcare)**

112 As earlier mentioned, protein spots were also visualized with fluorescent dyes. After the

113 precipitation the samples were solubilized in 20 µl of lysis buffer (30 mmol l^{-1} Tris, 7 mol l^{-1} urea, 2

114 mol l^{-1} thiourea and 4 % (w/v) CHAPS at pH 8.5. Each sample was labelled with different

115 Amersham CyDye DIGE Fluors (minimal dyes) for Ettan DIGE (GE Healthcare). Briefly, 333 pmol

116 of dye was used for 50 µg of protein and the samples were incubated on ice for 30 minutes in the

117 dark. After the incubation 1 µl of 10 mmol l^{-1} lysine was added to stop the reaction and the samples

118 were incubated on ice in the dark for 10 minutes. Twenty µl of 2× Sample buffer (8 mol l^{-1} Urea,

119 130 mmol l^{-1} DTT, 4 % (w/v) CHAPS, 2 % (v/v) IPG Buffer, pH 4-7, GE Healthcare) was added to

120 the samples and again, they were incubated on ice in the dark for 10 minutes. Samples for the same

121 gels were pooled together and 330 µl of rehydration buffer (8 mol l^{-1} Urea, 4 % (w/v) CHAPS, 1 %

122 (v/v) IPG Buffer, pH 4-7 and 13 mmol l^{-1} DTT) was added to the pooled samples.

123 Immobiline™ Drystrips (pH 4-7, 24 cm, GE Healthcare) were rehydrated with the protein solutions
124 and both first-dimensional isoelectric focusing and second dimension separation were carried out as
125 described earlier for the protein reference maps.

126 Ultimately, the gels were scanned in a Typhoon 9400 scanner (GE Healthcare) at a resolution of 100
127 µm. Spots showing clear difference between the time-points and strains were chosen for further
128 analysis. Spot detection and normalized volume quantization were carried out with ImageMaster 2D
129 Platinum (version 6.0 DIGE, GE Healthcare). The effect of heat shock on protein production was
130 analysed by spot matching across gels, normalized volume ratio computing (0 vs 2 h). Differences
131 between the spot normalized volumes were analysed statistically with the Student's t test, at a
132 significance level of $P<0.05$. Spots showing statistical differences among conditions were excised
133 from the 2D-PAGE gels visualized with Gelcode™ stain and sent to "Unidad de Proteómica-
134 Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III" (Madrid, Spain) for in-
135 gel trypsin digestion and identification by matrix-assisted laser desorption/ionization coupled to time
136 of flight mass spectrometry (MALDI-TOF) analysis. The in-gel trypsination, mass spectrometry
137 analysis and database searching was performed as previously described by Dominguez et al. (2009).
138 All searches were performed against the database for *Bifidobacterium animalis* subsp. *lactis* BB-12
139 (Garrigues et al. 2010) and *Lactobacillus rhamnosus* GG (Morita et al. 2009)
140 (http://www.ncbi.nlm.nih.gov/genome).

141 **Verification and quantitation of gene-expression by reverse transcriptase quantitative PCR**

142 Primers for different genes identified as differentially expressed in the proteomic analysis, were
143 designed using Primer Express 2.0 Software (Applied biosystems). The primers used are listed in
144 Table 1.

145 The RNA was isolated from the earlier obtained culture samples with the Rneasy mini kit (Qiagen)
146 according to the manufacturer's instructions. Rnase free Dnase set (Qiagen) was used for DNA
147 removal. RNA was quantified at 260 and 280 nm with Biotek Take 3 apparatus. One µg of RNA
148 was then used for cDNA synthesis. The cDNA synthesis was done by using the High-Capacity
149 cDNA Archive kit (Applied biosystems) following the provided protocol. The cDNA samples were
150 stored at -80 °C for further analyses.

151 The quantitative PCR studies were carried out on an ABI Prism 7500 Fast Real-Time PCR System
152 (Applied Biosystems) using SYBR® Green master mix (Applied Biosystems). Each reaction
153 mixture (25 µl) composed of SYBR® Green master mix, 0.2 µM of primers and 1 µl of cDNA.
154 Thermal cycling consisted of an initial cycle of 10 min at 95°C followed by 35 cycles of 15 s at
155 95°C and 1 min at 60°C. Expression levels were determined by relative quantification using the
156 ΔΔCt method, as described by Livak and Schmittgen (2001), in which the expression level in the
157 control culture (time zero or original strain culture, depending on the comparison) is arbitrarily set to
158 1 and the expression levels in the other samples are calculated relative to that control. The 16S
159 rRNA was used as an endogenous control. Samples were analysed in duplicate in two independent
160 PCR runs.

161 **RESULTS**

162 Several changes were observed when a proteomic approach was used to detect proteins potentially
163 involved in the heat adaptation and response of *B. animalis* subsp. *lactis* BB-12, *L. rhamnosus* GG
164 and their heat-tolerant derivative strains (Figure 1). In addition to characterizing the heat response in
165 general, our goal was to define how the heat-tolerant derivatives of the study probiotic strains have
166 acquired their heat-tolerance. To this end, the protein expression profiles of these strains, as well as

167 that of previously obtained heat-tolerant derivative strains of *L. rhamnosus* GG and *B. animalis*
168 subsp. *lactis* BB-12 (du Toit et al. 2013), were determined at time zero and after two hours of
169 exposure to heat-stress. Moreover, RT-qPCR was used to evaluate changes in the expression of the
170 genes coding the identified proteins. This study focused on cytosolic proteins within a pI range from
171 4 to 7.

172 As a response to heat stress, the regulation of seven spots was modified in the *B. animalis* subsp.
173 *lactis* strains and another seven spots in the *L. rhamnosus* strains (Table 2). These spots were
174 excised from the gels and subjected to peptide mass fingerprint analysis in order to be identified. For
175 spot BL3 no protein could be identified whilst for spot BL5, two different proteins were detected
176 and thus no conclusions can be made from the data concerning these spots. Proteins affected by heat
177 were chaperones and proteins involved in carbohydrate metabolism and translation.

178 **Response of *B. animalis* subsp. *lactis* strains to heat**

179 With regard to the response to heat (Table 2) we found that after 2 hours of heat-treatment, on *B.*
180 *animalis* subsp. *lactis* BB-12 WT, proteins identified as GroEL, GroES, thioredoxin peroxidase and
181 transketolase were upregulated and elongation factor Tu downregulated in the proteomic analysis.

182 In the heat-tolerant derivative strain of *B. animalis* subsp. *lactis* BB-12, the heat shock response was
183 otherwise similar but transketolase was downregulated. When the gene expression levels obtained
184 after the heat-treatment for each strain were compared with those obtained at time zero (Table 2) a
185 lower production of transketolase was observed in the heat tolerant strain which is in accordance
186 with the 2D-DIGE results. Additionally, a slightly higher induction of elongation factor Tu due to
187 heat treatment was seen in the original than in the heat tolerant derivative strain, in which the
188 expression of the gene coding this protein remained stable compared to the starting point.

189 A direct comparison of the wild type and the heat-tolerant strains (table 3) revealed that the levels of
190 proteins identified as GroEL, GroES and thioredoxin peroxidase were higher before and after the
191 heat shock in the heat-tolerant derivative strain according to both 2D-DIGE and RT-qPCR.

192 **Response of *L. rhamnosus* strains to heat**

193 According to the proteomic analysis, in *L. rhamnosus* GG WT five proteins were upregulated
194 (GroEL, DnaK, L-lactate dehydrogenase, and cell division trigger factor, peptidyl-prolyl cis-trans
195 isomerase) and two downregulated (Glucosamine-fructose-6-P-aminotransferase and phosphocarrier
196 protein Hpr) after two hours of heat-treatment when compared to baseline (Table 2). However, the
197 RT-qPCR results suggest that the induction rate of the genes coding the proteins identified as GroEL
198 and DnaK was lower at the two hour time point.

199 The heat shock response in the heat-tolerant derivative of *L. rhamnosus* GG differed slightly from
200 the original strain. The main differences between the derivative and the original strain were that the
201 regulation of the cell division trigger factor remained stable throughout the experiment and
202 elongation factor G was downregulated following the heat treatment (table 2). However, the RT-
203 qPCR results indicated that genes coding the chaperones GroEL and DnaK were highly expressed
204 after two hours. In addition, glucosamine-fructose-6-phosphate aminotransferase, and to a lesser
205 extent L-lactate dehydrogenase, were also induced by the heat-treatment on the derivative strain
206 according to the RT-qPCR analysis.

207 When the protein expression profiles of the *L. rhamnosus* strains before the heat-treatment were
208 compared (Table 3), higher levels of GroEL, DnaK, glucosamine--fructose-6-phosphate
209 aminotransferase and L-latate dehydroganese and lower levels of protein Hpr, trigger factor and
210 elongation factor G were detected in the heat-tolerant derivative strain. However, these results were

211 not confirmed by the RT-qPCR results which indicated a slight underexpression of the genes coding
212 GroEL, DnaK, glucosamine--fructose-6-phosphate aminotransferase and L-lactate dehydrogenase
213 while phosphocarrier protein Hpr, cell division trigger factor and elongation factor G were
214 overexpressed in the derivative strain with regard to the original strain (Table 3). After the heat-
215 treatment, proteomic results revealed that *L. rhamnosus* GG HS still had higher levels of GroEL,
216 glucosamine--fructose-6-phosphate aminotransferase, L-lactate dehydrogenase and lower levels of
217 trigger factor and elongation factor G than the wild type strain whilst both strains had comparable
218 levels of DnaK and the protein Hpr was induced at a higher level in the derivative strain (Table 3).
219 However, these results were not confirmed by the RT-qPCR which indicated a higher activity of all
220 the genes that were measured at two hours.

221 **DISCUSSION**

222 **Heat shock response in *B. animalis* subsp. *lactis* strains**

223 According to proteomic studies, chaperones GroEL, and GroES, which are known to work together
224 as a complex (van de Guchte et al. 2002), were upregulated by heat in both *B. animalis* subsp. *lactis*
225 BB-12 strains. However, no difference could be seen in the expression level of the gene coding
226 GroES. Chaperones are proteins that facilitate the folding of newly synthesized proteins and refold
227 denatured ones and thus protect the host cells from stresses. In lactobacilli and bifidobacteria,
228 chaperones have been reported as one of the major stress response systems against heat stress
229 (Sugimoto et al. 2008). GroEL and GroES are known as one of the classical heat shock proteins but
230 they are also known to be a part of the general stress response in bifidobacteria and lactobacilli
231 (Kilstrup et al. 1997, Sanchez et al. 2005, Ventura et al. 2005, Prasad et al. 2003) and many stress
232 response mechanisms have been described to be dependent on the GroEL and GroES activity in a

233 model for the stress regulatory network for bifidobacteria (Zomer et al. 2009). Although the
234 induction rate of the chaperones was similar between both of the *B. animalis* subsp. *lactis* strains, in
235 the end the heat-tolerant derivative of BB-12 harboured higher levels of both GroEL and GroES
236 before and after the treatment. The higher basal level of these chaperones in the heat-tolerant
237 derivative strain might be one of the factors responsible for the enhanced heat tolerance in the
238 derivative strain.

239 Transketolase, a protein involved in carbohydrate metabolism, appeared upregulated in the original
240 strain but in the heat-tolerant derivative it was downregulated. This protein is an important enzyme
241 for the formation of xylulose 5-phosphate which is one intermediate in the so-called bifidobacterial
242 shunt, which is a unique pathway that bifidobacteria use to utilize hexoses (Sanchez et al. 2005).

243 One translational protein was also identified, elongation factor Tu (EF-Tu), which was
244 downregulated in both *B. animalis* subsp. *lactis* BB-12 strains, although RT-qPCR results indicated
245 that the gene coding the protein was slightly upregulated in the wild type strain. EF-Tu has been
246 reported to be induced by acid in *Propionibacterium freudenreichii* (Leverrier et al. 2004), by bile in
247 *B. longum* (Sanchez et al. 2005) and by acid stress in *Streptococcus mutans* (Wu et al. 2011).
248 Elongation factors are involved in the delivery of aminoacyl-tRNA to the ribosome (Stark et al.
249 2002) and they have as well been described as chaperones on unfolded and denatured proteins in *E.*
250 *coli* (Caldas et al. 2000). Hence, the over-expression of the gene during heat shock would be logical,
251 but the reason why the particular proteins appear to be downregulated remains unknown.
252 Interestingly, it has been noticed that EF-Tu might appear in different isoforms after environmental
253 stress, most likely due to post-translational modifications such as phosphorylation (Leverrier et al.
254 2004, Wilkins et al. 2002). If EF-Tu appears in different isoforms having different functions, it can
255 also appear as more than one spot on the 2D-PAGE/DIGE -gels. Thus, we might be looking at only

256 one of the isoforms in our proteomic studies. Elongation factors may also appear extracellular or in
257 cell walls (Izquierdo et al. 2009) but we only analysed the cytoplasmic proteins which could lead to
258 underestimation of the regulation by our methods.

259 An antioxidant, thioredoxin peroxidase, was identified likewise as upregulated in *B. animalis* subsp.
260 *lactis* BB-12 strains according to the proteomic studies and it also appeared at higher levels in the
261 heat-tolerant derivative before and after the heat-treatment. This protein has been described as one
262 of the main cellular enzymes for the detoxification of H_2O_2 and as a specific inducer of thioredoxin
263 and thioredoxin reductase gene expression in response to oxidative stress in *Saccharomyces*
264 *cerevisae* (Ross et al. 2000). In addition, thioredoxin peroxidase has been suggested to be involved
265 in stress response in *B. animalis* subsp. *lactis* (Sanchez et al. 2007) and *Bac. subtilis* (Scharf et al.
266 1998). Thus we believe that his protein may help the heat-tolerant derivative of *B. animalis* subsp.
267 *lactis* BB-12 to survive under heat shock.

268 **Heat shock response in *L. rhamnosus* strains**

269 Likewise to *B. lactis* strains, the regulation of two proteins identified as chaperones, GroEL and
270 DnaK, was affected by the heat-treatment. Though, the high induction rate of DnaK was only
271 detected by RT-qPCR in the heat-tolerant derivative. It is possible that our proteomic assay
272 underestimates the amount of DnaK because the surface proteins were excluded from the analysis.
273 For example, DnaK has been reported to be induced by bile in *B. animalis* subsp. *lactis* when the
274 cell wall proteome was examined (Candela et al. 2010). The ability to induce chaperones (GroEL
275 and DnaK) at higher concentrations in the heat-tolerant derivative *L. rhamnosus* GG HS strain seem
276 to contribute to the increased heat resistance of the derivative strain with regard to the original one.
277 One mechanism responsible for the overexpression of DnaK has been described in a heat-tolerant

278 *Bifidobacterium longum* strain which was obtained in a similar fashion to our strains (Berger et al.
279 2010). Berger and coworkers found point mutations in the *hspR* gene which codes a negative
280 regulator of *DnaK* and *clpB*.

281 A protein involved in glutamate metabolism (glucosamine-fructose-6-phosphate aminotransferase,
282 isomerizing) was found downregulated in the *L. rhamnosus* strains during heat stress. However, the
283 gene coding this protein was over-expressed in the heat tolerant derivative according to the RT-
284 qPCR results. This enzyme might help the membrane recovery after damage caused by
285 environmental stresses (Wu et al. 2011, Milewski 2002).

286 Two proteins involved in carbohydrate metabolism were affected by the heat-treatment in *L.*
287 *rhamnosus* srains: phosphocarrier protein HPr and L-lactate dehydrogenase. The phosphocarrier
288 protein HPr, a component of the phosphoenolpyruvate-dependent sugar phosphotransferase system
289 (van Nuland et al. 1995), was upregulated according to proteomic studies, although the RT-qPCR
290 results did not show any change. It is known that HPr may also exist in a phosphorylated form
291 (Schmidl et al. 2010) so it is possible that the observed upregulation in the proteomic studies is
292 caused by a difference in the phosphorylation status of the protein. The HPr protein is known to
293 negatively regulate the protease production and has been described to help *Bacillus subtilis* survive
294 from oxidative stress (Dowds and Hoch 1991). Earlier, HPr has been revealed upregulated during
295 heat and osmotic stress in *L. rhamnosus* HN001 (Prasad et al. 2003). In addition, there is evidence
296 that the phosphotransferase system might play a role in cold shock response of *L. casei,* although the
297 mechanism implied remains to be elucidated (Monedero et al. 2007). In addition to HPr, another
298 protein involved in carbohydrate metabolism, L-lactate dehydrogenase was upregulated after 2 hours
299 of heat-treatment in the *L. rhamnosus* strains according to the proteomic results. However, the gene
300 expression was induced at a higher extent in the heat-tolerant derivative. The lactate dehydrogenase

301 has been reported to be induced by bile exposure in *B. longum* (Sanchez et al. 2005), various
302 stresses in *Enterococcus faecalis* (Giard et al. 2001), by heat in *Lactobacillus gasseri* (Suokko et al.
303 2008) and help *Neisseria gonorrhoeae* to survive under oxidative stress (Fu et al. 1989). The
304 cytoplasmic L-lactate dehydrogenase is NAD-dependent and it catalyzes the conversion of pyruvate
305 to lactate, which is a major end product of carbohydrate fermentation in lactic acid bacteria and
306 bifidobacteria (Sanchez et al. 2005, Fu et al. 1989).

307 According to proteomic studies the regulation of two translational proteins was modified in the *L.*
308 *rhamnosus* strains following the heat-treatment. A protein identified as elongation factor G (EF-G)
309 was slightly upregulated in the original *L. rhamnosus* GG and downregulated in the heat-tolerant
310 derivative following the heat-treatment EF-G has been reported to be induced by acid stress in
311 *Streptococcus mutans* (Wu et al. 2011). The second translational protein was identified as a cell
312 division trigger factor. This protein was upregulated in the original strain and slightly downregulated
313 in the derivative strain after two hours of heat-treatment in the proteomic studies, although the
314 expression of the gene encoding the protein barely changed according to the RT-qPCR results. This
315 protein has been reported to work together with enzymes involved in protein folding (Gothel and
316 Marahiel 1999, Schonbrunner and Schmid 1992).

317 **Conclusions**

318 In our study, some discrepancies were observed between the between protein and gene expression
319 analyses. These may be related to the different sensitivities of the techniques. Moreover, RT-qPCR
320 quantifies the mRNA while proteomic techniques quantify the protein in the cell and due to post-
321 translational regulation and modifications, the two techniques may occasionally give contradicting
322 results (Hegde et al. 2003). Similar discrepancies have been reported by other authors as well when

323 stress response in lactic acid bacteria has been examined using proteomics and RT-qPCR (Wu et al.
324 2011). Also, it must be kept in mind that we only analysed the cytoplasmic proteins which may
325 affect the outcome since some of the proteins analysed have been reported to appear in the cell wall
326 as well.

327 Our results characterise potential molecular mechanisms involved in both the response to heat-
328 treatments and the acquisition of heat-tolerance in the two most commonly used probiotic strains. At
329 the same time they raise the question how to further increase the tolerance to heat stress in the study
330 strains for allowing their inclusion in new probiotic-food categories. This could be achieved by
331 emphasizing the factors that increase the production of the identified chaperones. For instance,
332 identifying growth conditions in which a larger amount of the identified chaperones are produced, so
333 that the strain produced under those growth conditions harbours more chaperones to be better
334 prepared to cope with heat stress. Similarly, some small heat shock proteins have been proposed as
335 possible biomarkers for screening and selecting robust *L. acidophilus* strains (Capozzi et al. 2011).
336 Furthermore, a comparison to bacteria already able and adapted to survive at high temperature
337 conditions may improve the understanding on how to achieve a better heat tolerance to further
338 develop processes and products allowing the inclusion of specific probiotic strains. Nevertheless, the
339 verification of the presence of the original probiotic properties in the developed heat-tolerant
340 derivative strains should be confirmed to enable their use in functional foods and in products aimed
341 at health benefits for humans. Moreover, the impact of the processing conditions, production
342 processes and carrier vehicle, either food, food component or pharmaceutical component, on the
343 probiotic properties should also be routinely evaluated (Grzeskowiak et al. 2011).

344 Taken together, we conclude that the comparison of wild-type strains and the heat-tolerant
345 derivatives suggest that the acquisition of heat-tolerance may be associated with at least two

346 different mechanisms, a slightly increased constitutive level of chaperones in *B. lactis* and an
347 increased ability to induce their production in *L. rhamnosus*. Similar changes have been observed
348 before during various stresses, so the acquisition of heat-tolerance might affect the tolerance to other
349 stress factors as well. Though, this may happen for better or for worse as described in
350 *Bifidobacterium longum* for example (Mozzetti et al. 2013). All in all, it should be kept in mind that
351 the acquisition of heat or any other tolerance might also lead to some unexpected and maybe
352 unwanted genetic or other type of changes that may affect the probiotic potential of the strain.

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356 **CONFLICT OF INTEREST**

357 No conflict of interest declared.

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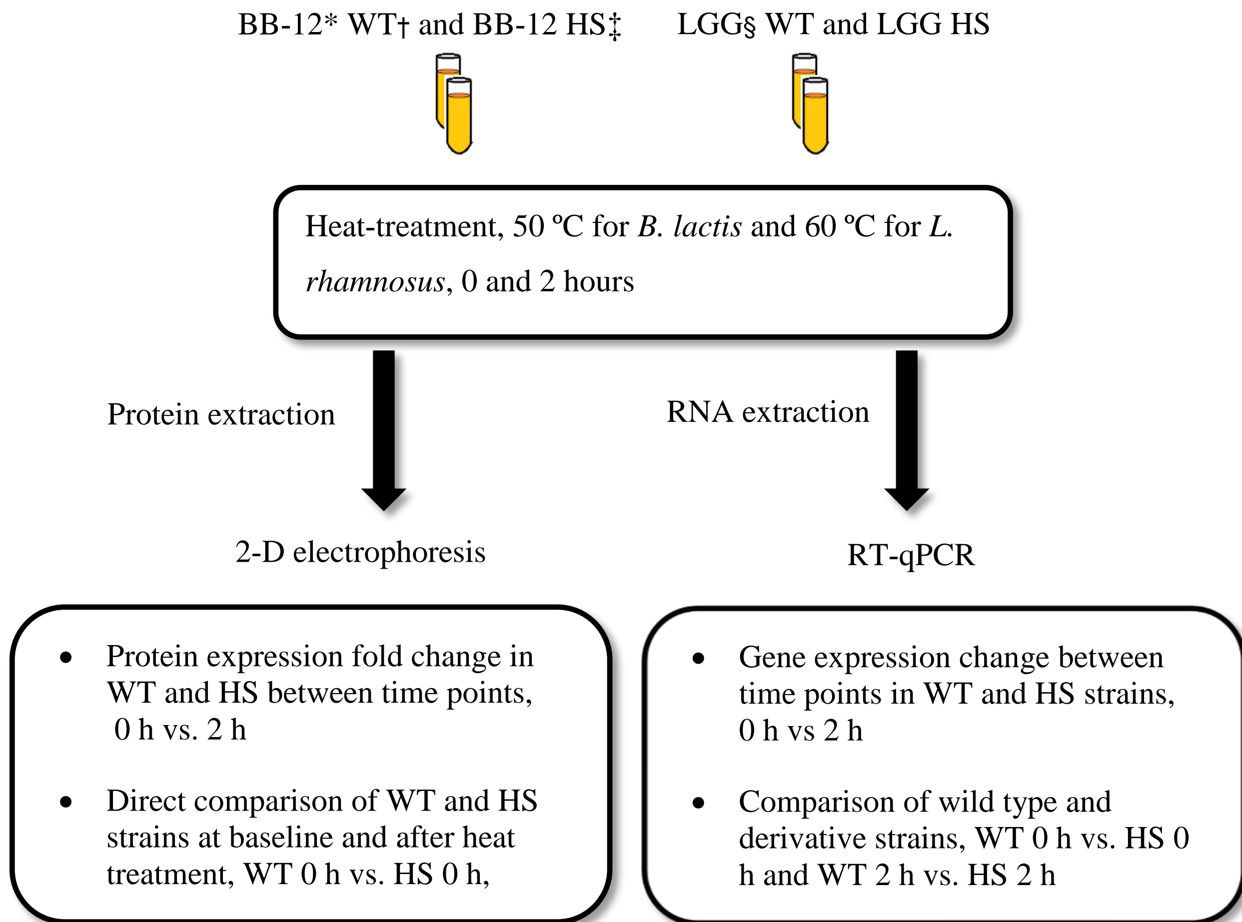
491 **SUPPORTING INFORMATION**

492 **Figures and figure legends**



493

494 Figure 1, A schematic workflow of the study

495 *BB-12 = Bifidobacterium animalis subsp. lactis BB-12

496 †WT = wild type strain

497 ‡HS = heat-tolerant derivative strain

498 §LGG = Lactobacillus rhamnosus GG

499 **Tables**

500 Table 1, Specific primers used for RT-qPCR analyses

Spot	Protein	Locus tag	Gene	Primer	Sequence	Amplicon Length
BL1	GroEL	BIF_00400		BL1F	TCGTCACCAATGCGGAAGAC	66 bp
				BL1R	TGCTCGAGGTGAGCAGAATGT	
BL2	GroES	BIF_00675		BL2F	AGGTTTCCTTGCCGTCCTTCT	62 bp
				BL2R	GTTCACCGTGCACAAGCTGTAC	
BL4	thioredoxin peroxidase	BIF_00644		BL4F	AGGTTTCCTTGCCGTCCTTCT	62 bp
				BL4R	GTTCACCGTGCACAAGCTGTAC	
BL5	elongation factor G	BIF_00045		BL5F	CGGCATCATGGCACACATC	79 bp
				BL5R	CTTGTAGTTCTTACCGGTGTAGAA CAGA	
BL6	transketolase	BIF_0093		BL6F	TCTTTCTGGCGGTCATGCA	71 bp
				BL6R	CCAGCGTCAGGCCATAACC	
BL7	elongation factor Tu	BIF_01972		BL7F	TCCAAGGTTCTGCACGATGAG	70 bp
				BL7R	GAGCGGCATCGATCTGGTT	
LR1	chaperonin GroEL	LRHM_2152		LR1F	CCAAAGCCAGGTGCTTTAACG	71 bp
				LR1R	CTGCCGACCTTGGTTCTGAA	
LR2	molecular chaperone, DnaK	LGG_01604	dnaK	LR2F	ACAAGCCGCTGGAACTCTTG	65 bp
				LR2R	ATCGGCAAAGGACCTTGGTACT	
LR3	glucosamine-fructose-6-phosphate aminotransferase, isomerizing	LGG_00983	glmS	LR3F	GACGCTAACCGCTGATGCA	64 bp
				LR3R	CGGCTTGCGATCTTCTACCTT	
LR4	L-lactate dehydrogenase	LGG_02523	ldh	LR4F	TCGTGACGCCGCTTATGAA	65 bp
				LR4R	GGGCAGTTGCGATACCATAGAA	
LR5	phosphocarrier protein HPr	LGG_01821	ptsH	LR5F	ACTTGCTTGCTGCCTGTACCA	60 bp
				LR5R	TTGCAGAAACCGGGATCCA	
LR6	trigger factor	LGG_01351	tig	LR6F	TGACCGTCCCTGGATTTCG	68 bp
				LR6R	GGCATCACCATACATCCGTTTG	
LR7	elongation factor G	LGG_02493	fusA	LR7F	AGTCCGGTGGTAAAGGTCAATATG	68 bp
				LR7R	GCCCTTGCCTTCTTCGTTTG	

501

502 Table 2, Proteins identified in mass spectrometry analysis and regulation of the proteins after the two hour heat-treatment according to
503 proteomic studies. Ratios represent increases/decreases of normalized spots volumes after 2 h of heat shock exposure in the wild type
504 (WT) and heat-shock resistant (HS) strains (0<ratio<1 → protein down-regulation; 1<ratio → protein up-regulation

						Fold difference 2 h/0 h			
						2D-DIGE		**RT-qPCR**	
Strain	**Spot**	**Protein**	**Functional category**	**Accession code**	**MM ***	**WT†**	**HS‡**	**WT**	**HS**
BB-12§	BL1	GroEL	chaperone	YP_005576691	56456	1.89	1.78	5.96 ± 3.58	3.55 ± 0.84
	BL2	GroES	chaperone	YP_005576393	7043	3.54	2.20	1.04 ± 0.91	0.65 ± 0.83
	BL3	unidentified							
	BL4	thioredoxin peroxidase	antioxidant	YP_005576917	18567	5.88	2.44	0.73 ± 0.38	0.81 ± 1.06
	BL5	unidentified							
	BL6	transketolase	carbohydrate metabolism and transport	YP_005576867	80150	1.33	0.63	0.85 ± 0.27	0.20 ± 0.03
	BL7	elongation factor Tu	translation	YP_005576655	44281	0.81	0.35	3.08 ± 0.78	0.57 ± 0.41
LGG¶	LR1	chaperonin GroEL	chaperone	YP_005866644	55809	4.56	4.41	0.10 ± 0.01	7.47 ± 3.36
	LR2	molecular chaperone DnaK	chaperone	YP_003171350	67179	1.26	0.74	0.23 ± 0.01	11.36 ± 9.39
	LR3	glucosamine--fructose-6-phosphate aminotransferase, isomerizing	glutamate metabolism	YP_003170729	65788	0.68	0.45	0.65 ± 0.25	2.68 ± 2.71
	LR4	L-lactate dehydrogenase	carbohydrate metabolism and transport	YP_003172269	35508	2.38	1.78	1.25 ± 0.79	3.58 ± 2.61
	LR5	phosphocarrier protein Hpr	carbohydrate metabolism and transport	YP_003171567	9248	0.44	1.85	1.16 ± 0.40	0.86 ± 0.33

LR6	trigger factor	chaperone	YP_003171097	49751	2.05	0.91	1.01 ± 0.63	1.68 ± 1.24
LR7	elongation factor G	translation	YP_003172239	76872	1.22	0.62	1.14 ± 0.25	1.83 ± 1.30

505

506 *MM = theoretical molecular mass (Da)

507 †WT = wild type

508 ‡HS = heat-tolerant derivative

509 §BB-12 = *B. animalis* subsp. *lactis* BB-12

510 ¶LGG = *L. rhamnosus* GG

511 Table 3, Induction ratios of the identified proteins and the genes coding them in the heat-tolerant

512 derivatives of *B. animalis* subsp. *lactis* BB-12 and *L. rhamnosus* GG with regard to the wild-type

513 strains before and after the two hour heat-treatment. For example, when the fold difference is higher

514 than one, the induction rate of the gene or the protein is higher in the heat-tolerant derivative than in

515 the wild-type strain.

Strain	**Spot**	**Protein**	**Fold difference HS*/WT†**			
			0 hours		***2 hours***	
			2D-DIGE	*RT-qPCR*	*2D-DIGE*	*RT-qPCR*
BB-12‡	BL1	heat shock protein 60, GroEL	1.6	2.9	1.6	3.2
	BL2	GroES	2.2	2.6	1.4	2.6
	BL4	thioredoxin peroxidase	3.2	1.8	1.9	3.0
	BL6	transketolase	1.4	2.3	0.7	2.6
	BL7	elongation factor Tu	0.9	2.9	0.4	1.8
LGG§	LR1	chaperonin GroEL	2.3	0.6	2.3	25.2
	LR2	molecular chaperone, DnaK	1.9	0.7	1.1	13.5
	LR3	glucosamine--fructose-6-phosphate aminotransferase, isomerizing	3.2	0.6	2.2	11.5
	LR4	L-lactate dehydrogenase	1.7	0.8	1.2	2.3
	LR5	phosphocarrier protein Hpr	0.7	1.7	3.1	1.7
	LR6	cell division trigger factor, peptidyl-prolyl cis-trans isomerase	0.7	3.1	0.3	4.1
	LR7	elongation factor G	0.9	4.8	0.5	4.4

516

517 *HS = heat-tolerant derivative

518 †WT = wild type

519 ‡BB-12 = *Bifidobacterium animalis* subsp. *lactis* BB-12

520 §LGG = *Lactobacillus rhamnosus* GG