TITLE: Response of *Lactobacillus casei* BL23 to phenolic compounds

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ABSTRACT

Aims: To determine the inhibitory effect of phenolic compounds on *Lactobacillus casei* BL23, the role of two component signal transduction systems (TCS), and the response of *Lact. casei* BL23 to *p*-coumaric acid.

Methods and Results: Growth of *Lact. casei* BL23 and 17 derivative strains defective in each TCS harboured by this strain in the presence of *p*-coumaric acid, ferulic acid, caffeic acid or methyl gallate was monitored. Furthermore, changes in the protein content of *Lact. casei* BL23 when exposed to *p*-coumaric acid were evaluated by 2D-SDS PAGE. Eleven proteins differentially expressed in the presence of *p*-coumaric acid were detected. Six of them could be identified: ClpP and HtrA, involved in protein turnover and folding, acetyl-CoA carboxylase, involved in lipid metabolism, and an arginyl-tRNA synthetase were more abundant whereas PurL and PurN, involved in purine biosynthesis, were less abundant.

Conclusions: No significant differences were observed between the parental strain and the TCS-defective mutants. *p*-coumaric acid elicited a response against membrane and cytoplasmic damages.

Significance and Impact of the Study: The inhibitory effect of phenolic compounds on *Lact. casei* BL23 has been determined. For the first time, cytoplasmic proteins presumably involved in the response of *Lact. casei* BL23 against *p*-coumaric acid have been identified.

Keywords

Lactobacillus casei; phenolic compound; inhibition of growth; ClpP; HtrA; purine metabolism

INTRODUCTION

The term “phenolic compound” describes a great variety of molecules that possess on their structure a benzenic ring substituted by, at least, one hydroxyl group. Phenolic compounds are ubiquitous in plant foodstuffs and therefore they are a significant component of human diet. In addition, they influence sensorial food properties such as flavour, astringency, and colour. Notwithstanding, phenolic compounds have been traditionally considered as undesirable components of the diet since the protein binding and metal chelating activity of some of these compounds can reduce the nutritional value of food (Bravo 1998). However, a renewed interest on phenolic compounds of both research and industry has arisen after the recognition of their antioxidant properties and their probable role in the prevention of various diseases associated with oxidative stress (Bravo 1998; Manach et al. 2004; Rodríguez et al. 2009). Furthermore, some phenolic compounds are toxic for numerous bacteria, specially Gram positive, although lactobacilli usually are more resistant compared to other bacterial groups (Hervert-Hernández and Goñi 2011).

Due to their abundance in plant materials, studies on the influence of phenolic compounds in lactic acid bacteria (LAB) has focused on LAB associated to vegetable fermentations, particularly *Lactobacillus plantarum* (Rodríguez *et al*. 2009). However, some studies have also addressed the influence of phenolic compounds on LAB associated to gut microbiota (Hervert-Hernández and Goñi 2011). For example, *Lactobacillus acidophilus* was resistant to tea phenolic extracts (Almajano *et al.* 2008). In another study focused on the antimicrobial properties of phenolic compounds isolated from berries, a number of *Lactobacillus* strains of human origin were assayed, showing that most phenolic compounds tested did not inhibit their growth under the experimental conditions used (Puupponen-Pimiä *et al.* 2001). Some studies have even shown that

phenolic compounds can stimulate the growth of some LAB (Hervert-Hernández and Goñi 2011; Rodríguez et al. 2009). Phenolic acids, such as ferulic, *p*-coumaric, and caffeic acids are toxic for gram-positive bacteria under acidic conditions and are able to specifically induce the expression of phenolic acid decarboxylases (Barthelmebs et al. 2000; Cavin et al. 1997; Tran et al. 2008).

This study addresses the effect of some phenolic acids on the growth of *Lactobacillus casei*. *Lact. casei* is a facultative heterofermentative lactic acid bacterium of interest for food industry as a starter culture for milk fermentation and for maturation of some types of cheeses. Furthermore, some strains of *Lact. casei* have received considerable attention for their probiotic properties (de Vrese and Schrezenmeir 2008). Probiotics are defined as living microorganisms which, upon ingestion in certain numbers, exert health benefits beyond that of inherent basic nutrition (Guarner and Schaafsma 1998); therefore, in order to exert its beneficial effect, probiotic microorganisms must survive the transit through the gastrointestinal tract where they will be in contact with phenolic compounds present in food. In contrast to other LAB, no putative gene encoding a protein displaying a significant similarity to a phenolic acid decarboxylase enzyme can be found in *Lact. casei* BL23 (Mazé *et al.* 2010). Hence, this study aims to determine the antimicrobial activity of some phenolic compounds against *Lact. casei* BL23, to analyze the response of *Lact. casei* BL23 to the presence of a phenolic acid (*p*-coumaric acid) and to determine whether any of the two component systems (TCS) encoded by *Lact. casei* BL23 is involved in the response to phenolic acids since these systems have been shown to be involved in the response to diverse environmental stress conditions (Alcántara *et al.* 2011).

MATERIAL AND METHODS

Strains, media and phenolic compounds

Strains used in this study are listed in Table 1. Insertional mutants were obtained by recombination with pRV300 derivative plasmids containing internal DNA fragments of each response regulator-encoding gene (Alcántara et al. 2011). TC17 strain harbouring a complete deletion of the RR17 gene was obtained as previously described (Landete et al. 2010a). *Lact. casei* strains were routinely grown in MRS broth (Difco) at 37 ºC. Agar was added to 1.8 % (w/v) for plates. When required, erythromycin was added to a final concentration of 5 µg ml^{-1} (see Table 1). For the inhibition assays, RPMmod10 medium was used. RPMmod10 is a modification of RPM (Rozès and Peres 1998) containing glucose (2 g l^{-1}), trisodium citrate dihydrate (0.5 g l^{-1}), D-L-malic acid (5 g l^{-1}), casamino acids (1g l^{-1}), yeast nitrogen base without amino acids (6.7 g l^{-1}) and tryptone (1 g l^{-1}). Culture media were adjusted to pH 6.5 before sterilisation at 121 ºC for 20 min. The phenolic compounds used in this study; *p*-coumaric acid, ferulic acid, caffeic acid and methyl gallate were purchased from Sigma Chemical Co. Stock solutions of *p*-coumaric acid (0.66 mol l^{-1}) and methyl gallate (1 mol l^{-1}) were prepared with absolute ethanol, ferulic acid (0.75 mol l^{-1}) and caffeic acid (0.4 mol l^{-1}) were prepared with methanol.

Determination of the minimal inhibitory concentration (MIC)

For MIC determinations, *Lact. casei* BL23 was inoculated on an MRS agar plate and a single colony was used to inoculate 10 ml of MRS medium. Cells were grown at 37 ºC overnight and harvested by centrifugation (5000 × *g*, 5 min., 4 ºC). The pellet was washed twice with one volume of sterile distilled water and finally resuspended to an OD (595 nm) of 10. The cell suspension was used to inoculate, at an initial OD (595 nm) of 0.05, 5 ml of RPMmod10 supplemented with serial dilutions of each phenolic

compound ranging from 0 to 50 mmol l^{-1}. The tubes were incubated in darkness without shaking, at 37 ºC for 24 hours. As controls, an additional series of tubes of RPMmod10 supplemented with the same volumes of ethanol or methanol as those added with the phenolic compounds were incubated in the same conditions. The MIC was defined as the lowest concentration of the compound where absence of growth was recorded. The assay was repeated three times from independent cultures.

Inhibition of growth of *Lact. casei* BL23 and TCS-defective mutants by phenolic compounds

The effect on the growth of *Lact. casei* BL23 and 17 derivative strains defective in each TCS encoded by this strain (Table 1) was determined as follows: cells were grown and inoculated as described above (with addition of erythromycin 5 µg ml^{-1} to TC01-16 strains) for MIC determinations in 5 ml of RPMmod10 supplemented with each phenolic compound assayed to a final concentration of 20 mmol l^{-1} or without any supplementation (control). No antibiotics were added for these growth assays. Loss of erythromycin resistance in the insertional mutants was evaluated by comparing viable cell countings on MRS agar plates and MRS supplemented with erythromycin (5 µg ml^{-1}). The cultures were incubated as described above and growth was estimated by measuring the OD (595 nm.) after 24 hours. Five independent replicates were assayed for each strain. To determine whether the responses of the mutant strains to each phenolic compound assayed were significantly different from that of the wild type, pairwise two-way ANOVA analyses were performed, testing the growth of *Lact. casei* BL23 and that of each mutant strain under the reference condition and each of the different phenolic compounds. Levene's test was used to assess the equality of error variances. We considered a significant difference to be detected if the analysis estimated

that both the strain variable and interaction were below *P* values of 0.01. Statistical analyses were carried out with GraphPad Prism 4.00.

Two-dimensional SDS PAGE analysis of the response of *Lact. casei* BL23 to *p*-coumaric acid

The inoculum of *Lact. casei* BL23 for the proteomic analysis was obtained as described above. *Lact. casei* BL23 was subsequently grown in RPMmod10 at 37 ºC without shaking to an OD (595 nm.) of 0.26 after which *p*-coumaric acid was added to a final concentration of 15 mM and incubation was continued for one hour. As controls, two cultures were used: *Lact. casei* BL23 grown with no additions, and a second culture supplemented with the same volume of ethanol added to the culture supplemented with *p*-coumaric acid (2.3 % v/v). Cells were harvested by centrifugation, washed twice, resuspended in 20 mmol l^{-1} sodium phosphate (pH 7.5) 140 mmol l^{-1} NaCl and disrupted with 100 µm diameter glass beads (1 g ml^{-1}) in a Mini-BeadBeater (Biospec). Unbroken cells and cell debris were removed by centrifugation (12,000 × *g* 5 min at 4 °C). In order to improve the resolution of soluble proteins in 2D-SDS PAGE, the supernatants were collected and centrifuged again (100,000 × *g* 60 min at 4 °C) to remove membranes. The supernatants were collected, concentrated with Amicon® Ultra-4 (3000 Da; Millipore) and stored at -80 ºC until use. The protein concentration of the cell-free extracts was measured using the Bradford Microassay (Bio-Rad).

2D-SDS PAGE was performed using the immobiline-polyacrylamide system, essentially as described by Blomberg (Blomberg 2002). The same amount of protein (100 µg) was resuspended in rehydration buffer (7 mol l^{-1} urea, 2 mol l^{-1} thiourea, 0.2% (w/v) dithiothreitol (DTT), 1% (w/v) nonidet P-40, 1% (v/v) pharmalyte 3-10 and a few grains of bromophenol blue) and incubated at room temperature for 30 min. The

162 samples were then loaded on IPG-strips providing a non-linear 3-10 pH gradient (Bio-
163 rad) and allowed to rehydrate overnight. Isoelectric focusing was carried out by using an
164 Ettan IPGPhor II (GE Healthcare) system. The isoelectric focusing was performed using
165 the following program: voltage was increased from 0 to 500 V during the first 5 h,
166 maintained at 500 V for 5 h, increased from 500 V to 8000 V in 9 h, and fixed at 8000
167 V for 5 h. After isoelectrofocusing, the strips were first equilibrated for 20 min in a
168 buffer containing 6 mol l^{-1} urea, 30% (w/v) glycerol, 2% (w/v) SDS, 0.05 mol l^{-1} Tris-
169 HCl pH 8.8, and 1% (w/v) DTT (reduction step), and subsequently for 20 min in a
170 buffer containing 6 mol l^{-1} urea, 30%(w/v) glycerol, 2% (w/v) SDS, 0.05 mol l^{-1} Tris-
171 HCl pH 8.8, and 2.5% (v/v) iodoacetamide (alkylation step). The strips were then
172 deposited onto a 12.5 % (w/v) polyacrylamide-SDS gels and run at 1 W/gel for 16 h in
173 an Ettan DALTsix Large Vertical System (GE Healthcare). Silver staining was
174 performed as described by Blomberg (Blomberg 2002).
175 Six biological replicates were obtained for each growth condition. Gels were scanned
176 using a HP ScanJet 5100C (300 dpi, 12-bit image) and the images analysed with the
177 PDQuest 8.0 software (Bio-Rad). Spot detection was performed using the PDQuest
178 automated spot detection algorithm. Normalization was performed automatically using
179 the local regression model implemented in the software to compensate image
180 differences caused by variations in experimental conditions (e.g. protein loading or
181 staining). A master gel image was automatically generated by the software and spots in
182 the master gel were then matched across all gels. Matching was then visually inspected
183 and corrected when required. Subsequently, gels were divided into three different
184 groups: untreated samples, ethanol-supplemented samples and *p*-coumaric-
185 supplemented samples. Matching features of the software were used to relate and
186 compare the sets of gels. A protein was considered to be under- or overproduced when,

after image analysis and subsequent computing of the normalized spot volumes, the means from at least four gels coming from independent cultures were 1.5-fold different among the conditions tested at a significance level of $P < 0.05$ (Student's *t* test for paired samples).

Protein identification

Selected spots were excised from 2D gels and transferred to polypropylene tubes containing ultrapure water. Proteins were identified by peptide mass fingerprinting after trypsinolysis and MALDI-TOF/TOF at the Proteomic Unit of Centro Nacional de Investigaciones Cardiovasculares (CNIC; Madrid, Spain). MALDI-MS and MS/MS data were combined through the BioTools 3.0 program (Bruker Daltonik) to search a nonredundant protein database (NCBInr or SwissProt) using the Mascot software (Matrix Science, London, UK) (Perkins *et al*. 1999). Annotations were made according to the cluster of orthologous groups of proteins classification (COGs; www.ncbi.nlm.nih.gov/COG/). KEGG database (http://www.genome.jp/kegg/) was used for metabolic pathway identification.

RESULTS

Determination of the minimal inhibitory concentration (MIC)

Lact. casei BL23 grew very poorly in RPM medium, therefore a number of modifications in the composition of the medium were assayed in order to improve the growth of *Lact. casei* (results not shown). Finally, RPMmod10 was chosen as basal medium for testing the inhibitory effect of phenolic compounds on this strain. To this end, the MIC values for *p*-coumaric acid, ferulic acid, caffeic acid and methyl gallate were determined. The results obtained showed that all four compounds inhibited the

212 growth of *Lact. casei.* The MIC values determined were 25 mmol l^{-1} for *p*-coumaric
213 acid and 40 mmol l^{-1} for the other phenolic compounds assayed.
214
215 **Inhibition of growth of *Lact. casei* BL23 and TCS-defective mutants by phenolic**
216 **compounds**
217 Next, we tested whether TCS play a role in the response to phenolic compounds since
218 these systems had been previously shown to be involved in the tolerance to a number of
219 stress conditions in *Lact. casei* (Alcántara *et al.* 2011). To this end, 17 TCS-defective
220 mutants and the parental strain *Lact. casei* BL23 were grown in presence of *p*-coumaric
221 acid, ferulic acid, caffeic acid and methyl gallate. No significant loss of erythromycin
222 resistance in the insertional mutants was observed under the experimental conditions
223 used (data not shown) thus indicating that reversion to the wild-type phenotype was
224 negligible. The results obtained are shown in Table S1 in Supplemental Information.
225 Since some mutants showed significant differences with the parental strain in
226 RPMmod10 (TC06 and TC12; determined by the Student's *t* test after assessing
227 equality of variances by the Levene's test), pairwise two-way ANOVA analyses,
228 considering treatment (control *versus* phenolic compound) and strain (BL23 *versus* each
229 mutant strain) as variables, were performed in order to determine whether the
230 differences observed were due to the phenolic compound (in this case a significant
231 interaction should be detected). Addition of phenolic compounds resulted in significant
232 inhibition of all strains (Table S1), however in no case significant differences were
233 observed between the parental strain and any of the mutants (data not shown) thus
234 indicating that TCS do not play a significant role in the response against phenolic
235 compounds of *Lact. casei* BL23. However, involvement of TCS cannot be ruled out
236 under the experimentaldifferent growth conditions used here.

237

238 **2D-PAGE analysis of the response of *Lact. casei* BL23 to *p*-coumaric acid**

239 A proteomic approach was used to characterize the adaptation to *p*-coumaric acid of
240 *Lact. casei* BL23. Our study focused on the cytosolic proteins in a pI range of 3.0 to
241 10.0. For this experiment, a concentration of 15 mM of *p*-coumaric acid was chosen
242 since a significant decrease on growth rate was observed after addition of *p*-coumaric
243 acid but not a complete arrest of growth (Fig. 1). The addition of an equivalent volume
244 of ethanol did not result in a significant change in growth rate under our experimental
245 conditions (Fig. 1).

246 Fig. 2 shows representative gels resulting from the analysis of the total proteins. No
247 significant differences were detected between the control samples and the samples
248 supplemented with ethanol (data not shown). When the samples supplemented with *p*-
249 coumaric acid were compared with the control samples, eleven spots showed statistical
250 differences according to the criteria established (see Materials and Methods). Table 2
251 summarizes the quantitative data of the spots identified as differentially expressed. In
252 response to *p*-coumaric acid, six proteins were upregulated and five downregulated in
253 *Lact. casei* BL23. Six spots could be identified by mass spectrometry, the other five did
254 not render reliable mass spectra. Among the six identified proteins, two of them were
255 significantly less abudant in the presence of *p*-coumaric acid (Table 2): a putative
256 phosphoribosylformylglycinamidine synthase II and a folate-dependent
257 phosphoribosylglycinamide formyltransferase (LCABL_19700 and LCABL_19670,
258 respectively). Both proteins catalyze consecutive steps in the purine biosynthetic
259 pathway. Among the upregulated proteins, two of them are involved in protein turnover:
260 the Clp protease proteolytic subunit and the trypsin-like serine protease HtrA
261 (LCABL_10770 and LCABL_30080, respectively). In addition, an acetyl-CoA

carboxyltransferase (LCABL_22910; AccD) involved in fatty acid biosynthesis, and an arginyl-tRNA synthetase (LCABL_19480) were also induced. In particular, the Clp proteolytic subunit and HtrA were strongly induced (Table 2): Clp level was ten fold higher in the presence of *p*-coumaric acid whereas HtrA was under the detection level in the control conditions.

DISCUSSION

Phenolic compounds caused a significant inhibition of growth of *Lact. casei* BL23 being *p*-coumaric acid the most active against this bacterium. The MIC of *p*-coumaric acid for *Lact. casei* BL23 was similar to that previously reported for *Lact. plantarum* (Landete *et al.* 2007) whereas *Lact. casei* was more sensitive to the other three compounds. *Lact. plantarum* is able to decarboxylate *p*-coumaric, ferulic acid, caffeic acid to less toxic derivatives by the action of *p*-coumaric acid decarboxylase (PAD) (Rodríguez *et al.* 2008a). PAD is present in other LAB such as *Lactobacillus brevis* (Landete *et al.* 2010b) or *Pediococcus pentosaceus* (Barthelmebs *et al*. 2000). However, the analysis of the available genomic sequences of *Lact. casei* strains do not reveal the presence of this gene. Methyl gallate is metabolized by *Lact. plantarum* strains to gallic acid and subsequently to pyrogallol via tannase and gallate decarboxylase activities (Rodríguez *et al.* 2008b). The gene encoding tannase in *Lact. plantarum* has been identified (Iwamoto *et al.* 2008) and homologous genes are absent in the *Lact. casei* sequenced strains. As far as we know, the gene encoding gallate decarboxylase has not been identified. In summary, there is no evidence indicating that *Lact. casei* can metabolize phenolic compounds which may partly explain the higher sensitivity of this bacterium to these substances.

286 Phenolic acids are weak organic acids that can enter the cell by passive diffusion in their
287 undissociated form where they can acidify the cytoplasm and denature proteins.
288 Furthermore, they can insert and destabilize the cell membrane due to their amphipathic
289 nature. Campos et al. (Campos *et al.* 2009) observed that phenolic acids induced ion
290 leakage and proton influx on LAB isolated from wine thus indicating an increase in cell
291 membrane permeability. A previous study had shown that several TCS encoded by
292 *Lact. casei* BL23 were involved in the response against cell envelope damages caused
293 by different agents (Alcántara *et al*. 2011). However, the assay of the response of a
294 series of derivative strains defective in each TCS encoded by *Lact. casei* BL23 did not
295 reveal any significant differences with the parental strain in their response to phenolic
296 compounds thus indicating that TCS are not involved in the response against these
297 substances under our experimental conditions. There are very few studies dealing with
298 the regulation of the response of LAB to phenolic compounds. Gury *et al.* (2004)
299 observed that the *padR* gene encodes the negative transcriptional regulator of PAD
300 (*pad*A) in *Lact. plantarum.* The *padA* gene is cotranscribed with a downstream gene,
301 *usp1*, which encodes a putative universal stress protein. The *usp1* gene is overexpressed
302 in presence of phenolic acids and the role to inactivate PadR indicates that it could serve
303 as an important mediator in phenolic acids response. *Lact. casei* BL23 harbours a gene
304 (LCABL_27180; 179 amino acids) significantly similar to the *Lact. plantarum* (34%
305 identical residues in a 130 positions alignment with gene *padR* of *Lact. plantarum*
306 WCFS1) and some *Bacillus padR* genes (for example, 43% identical residues in a 163
307 positions alignment with gene BCE_1910 of *Bacillus cereus* ATCC 10987). However,
308 there is no evidence about the function of this gene.
309 The results obtained with 2D-SDS PAGE are consistent with damages in the cell
310 membrane and cytoplasmic proteins caused by *p*-coumaric acid since the exposition to

311 this substance led to a strong induction of ClpP and HtrA, two proteases involved in
312 protein turnover (Table 2). The ATP-dependent Clp proteases consist of separately
313 encoded ATPase and peptidase subunits. Clp proteases have been the subject of
314 extensive research that have evidenced that ClpP-containing proteolytic complexes play
315 indispensable roles in cellular protein quality control systems by refolding or degrading
316 damaged proteins and that the chaperone activity of Clp ATPases are important for
317 controlling stability and activity of central transcriptional regulators in low-GC Gram
318 positive bacteria (Frees *et al.* 2007). A number of studies have shown the induction of
319 ClpP expression under varied stress conditions in lactobacilli (Fernández et al. 2008;
320 Hörmann et al. 2006; Weiss and Jespersen 2010). ClpP is under control of the stress
321 response regulator CtsR in *Lact. plantarum* (Fiocco *et al.* 2010). Interestingly, a search
322 of the genome sequence of *Lact. casei* BL23 with the CtsR binding site consensus
323 sequence (Derré et al. 1999) reveals a putative CtsR binding site upstream *clpP* (Fig. 3).
324 Similar sites can be found upstream *clpB* and *clpE* (data not shown). Although it is
325 tempting to speculate that the CtsR regulon is involved in the response against *p*-
326 coumaric acid, additional evidence is required to prove this point.
327 There is scarce information concerning the role of HtrA in lactobacilli. HtrA expression
328 is induced in *Lactobacillus helveticus* after exposure to 4% (w/v) NaCl and to a lesser
329 extent after exposure to puromycin, ethanol, or heat although a mutant defective in HtrA
330 only showed heat sensitivity (Smeds *et al.* 1998). On the basis of the amino-acid
331 sequence, the location of HtrA was predicted at the cell membrane (Smeds *et al.* 1998).
332 HtrA degrades abnormal exported proteins in the closely related bacterium *Lactococcus*
333 *lactis* (Poquet *et al.* 2000). HtrA may play a similar role in lactobacilli. The induction of
334 the expression of these two proteins suggests that *p*-coumaric acid disturbed protein
335 structure both at the cell envelope and the cytoplasm under our experimental conditions.

Our results also suggest that *p*-coumaric acid affected the membrane. Acetyl-CoA carboxyl transferase catalizes the first step in the synthesis of fatty acids the conversion of acetyl-CoA to malonyl-CoA. Rozès and Peres (Rozès and Peres 1998) observed that increasing amounts of caffeic and ferulic acids induced a gradual increase in the amounts of myristic, palmitoleic, stearic and methylenehexadecanoic acid with a concomitant decrease of lactobacillic acid in *Lact. plantarum*. The overexpression of the subunit beta of acetyl-coA carboxyl transferase (AccD) observed in this work might indicate an increase in fatty acid biosynthesis for modification of the lipid composition of the membrane. We did not detect the alpha subunit in our analysis although this may be due to limitations of the technique.

Gene *argS* is the only one encoding an arginyl-tRNA synthetase in *Lact. casei* BL23 and possibly is essential for normal protein synthesis. The induction of an aminoacyl-tRNA synthetase might suggest an increase in the metabolism of proteins in response to the presence of *p*-coumaric acid although no other aminoacyl-tRNA synthetase was detected as significantly more abundant. Whether this is due to the limitations of the 2D-SDS PAGE technique remains to be determined. Clearly, further research is required to ascertain this point.

Interestingly, exposure to *p*-coumaric acid led to the downregulation of two genes, *purL* and *purN*, whose products catalyze two consecutive steps in the purine biosynthetic pathway. Gene *purN* encodes phosphoribosylglycinamide formyltransferase (EC:2.1.2.2). This enzyme converts 5'-phosphoribosylglycinamide into 5'-phosphoribosyl-N-formylglycinamide which in turn is the substrate of the phosphoribosylformylglycinamidine synthase (EC:6.3.5.3) encoded by *purL*. PurL synthesizes 2-(formamido)-N1-(5-phospho-D-ribosyl)acetamidine. Downregulation of these enzymes suggests a decrease in nucleic acid synthesis thereby reducing the

361 requirement of nucleotides. This effect would agree with previous observations of
362 inhibition of DNA replication by phenolic compounds (Smith and Dou 2001). Navarro-
363 Perán *et al*. (Navarro-Perán et al. 2007) observed that epigallocatechin-3-gallate
364 decreased the cellular production of nucleotides in a human colon carcinoma cell line
365 and disrupted the purine metabolism.
366 In summary, our results indicate that *p*-coumaric acid disturbs protein structure and may
367 also affects the properties of the cell membrane of *Lact. casei* BL23. In addition, the
368 inhibition of genes involved in purine biosynthesis suggests that *p*-coumaric acid may
369 interfere with DNA replication as previously observed for other phenolic compounds.
370 These results open new lines of research to understand the effects of phenolic
371 compounds in LAB.

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474 **Table 1** *Lactobacillus. casei* strains used in this work

Strains	Characteristics	Reference
BL23	wild type	B. Chassy (Univ. Illinois)
TC01	LCABL_02080 insertional mutant (RR01); Ery^r	(Alcántara et al. 2011)
TC02	LCABL_05260 insertional mutant (RR02); Ery^r	(Alcántara et al. 2011)
TC03	LCABL_07760 insertional mutant (RR03); Ery^r	(Alcántara et al. 2011)
TC04	LCABL_10480 insertional mutant (RR04); Ery^r	(Alcántara et al. 2011)
TC05	LCABL_10640 insertional mutant (RR05); Ery^r	(Alcántara et al. 2011)
TC06	LCABL_12050 insertional mutant (RR06); Ery^r	(Alcántara et al. 2011)
TC07	LCABL_14280 insertional mutant (RR07); Ery^r	(Alcántara et al. 2011)
TC08	LCABL_14430 insertional mutant (RR08); Ery^r	(Alcántara et al. 2011)
TC09	LCABL_16430 insertional mutant (RR09); Ery^r	(Alcántara et al. 2011)
TC10	LCABL_18830 insertional mutant (RR10); Ery^r	(Alcántara et al. 2011)
TC11	LCABL_18980 insertional mutant (RR11); Ery^r	(Alcántara et al. 2011)
TC12	LCABL_19600 insertional mutant (RR12); Ery^r	(Alcántara et al. 2011)
TC13	LCABL_25620 insertional mutant (RR13); Ery^r	(Alcántara et al. 2011)
TC14	LCABL_27650 insertional mutant (RR14); Ery^r	(Alcántara et al. 2011)
TC15	LCABL_28720 insertional mutant (RR15); Ery^r	(Alcántara et al. 2011)
TC16	LCABL_30130 insertional mutant (RR16); Ery^r	(Alcántara et al. 2011)
TC17	BL23 Δ*maeR* (RR17)	(Landete et al. 2010a)

475

476 **Table 2**. Citoplasmic protein spots differentially expressed in the presence of *p*-coumaric acid in *Lact. casei* BL23

Spot	Functional category*	Putative function	Mr†	pI‡	Locus_tag/gene	Spot volume ×10^3 §	N**	Spot volume ×10^3	N	Fold ratio††	Matched peptides	C
						Control		**p-coumaric acid**				
413	Unknown					15.2 ± 4.1	5	1.0	1	0.1		
1804	Nucleotide transport and metabolismo	Phosphoribosylformylglycinamidine synthase II	78924	4.77	LCABL_19700 *purL*	26.0 ± 9.7	6	2.7 ± 0.8	4	0.1	8	1
2107	Posttranslational modification, protein turnover, chaperones	ATP-dependent Clp protease proteolytic subunit	21511	5.09	LCABL_10770 *clpP*	4.2 ± 2.4	4	40.4 ± 12.7	5	10.0	5	2
2418	Posttranslational modification, protein turnover, chaperones	Trypsin-like serine protease	44924	5.99	LCABL_30080 *htrA*	-	-	47.6 ± 22.1	3	+§§	2	7
2808	Unknown					25.1 ± 9.8	6	14.5 ± 4.7	4	0.6		
4610	Unknown					5.6 ± 1.7	4	19.0 ± 1.2	4	3.5		
6101	Nucleotide transport and metabolism	Folate-dependent phosphoribosylglycinamide formyltransferase	20282	5.67	LCABL_19670 *purN*	42.2 ± 1.5	4	3.6 ± 2.0	5	0.1	5	3

6304	Fatty acid and phospholipid metabolism	Acetyl-CoA carboxylase beta subunit	29416	6.07	LCABL_22910 *accD*	-	-	4.9 ± 0.3	3	+	5	2
6704	Transcripcional regulators	Arginyl-tRNA synthetase	62265	5.79	LCABL_19480 *argS*	5.55 ± 1.4	5	14.6 ± 1.8	5	2.6	6	1
7206	Unknown					55.1 ± 16.9	6	13.8 ± 6.5	5	0.3		
7705	Unknown					6.5 ± 3.4	6	13.4 ± 4.3	5	2.0		

477 * Functional categories assigned according to the cluster of orthologous groups of proteins (COGs; www.ncbi.nlm.nih.gov/COG/) .

478 † Theoretical molecular mass.

479 ‡ Theoretical isoelectric point.

480 § Means ± standard deviations.

481 ** Number of gels analyzed.

482 †† Proteins with values over 1 are up-regulated in response to p-coumaric acid, proteins with values below 1 are down-regulated.

483 ‡‡ Percentage of amino acid coverage (peptides observed/theoretical value from sequence data).

484 §§ Not detected in the control.

485

FIGURE LEGENDS

Figure 1. Growth of *Lact. casei* BL23 after addition of increasing amounts of *p*-coumaric acid. The arrow indicates the time of addition of *p*-coumaric acid to the final concentrations indicated: 0 mmol l^{-1}; 10 mmol l^{-1}; 15 mmol l^{-1}; 20 mmol l^{-1}; 25 mmol l^{-1}. Error bars indicate standard deviation.

Figure 2. Silver-stained two-dimensional electrophoresis gels of total soluble cytoplasmic proteins extracted from *Lact. casei* BL23 cells untreated (A) and treated with *p*-coumaric acid 15 mM (B). The figure shows one representative gel of each sample. Spot numbers indicate differentially expressed proteins. Identified differentially expressed proteins are also indicated.

Figure 3. *Lact. casei* BL23 DNA sequence upstream gene *clpP*. The putative CtsR binding site, ribosomal binding site (RBS) and the *clpP* start-codon are shown. The CtsR consensus sequence (Fiocco et al. 2010) is indicated below its corresponding sequence. The numbers indicate the coordinates in the *Lact. casei* BL23 genome sequence (Acc. Nº FM177140).

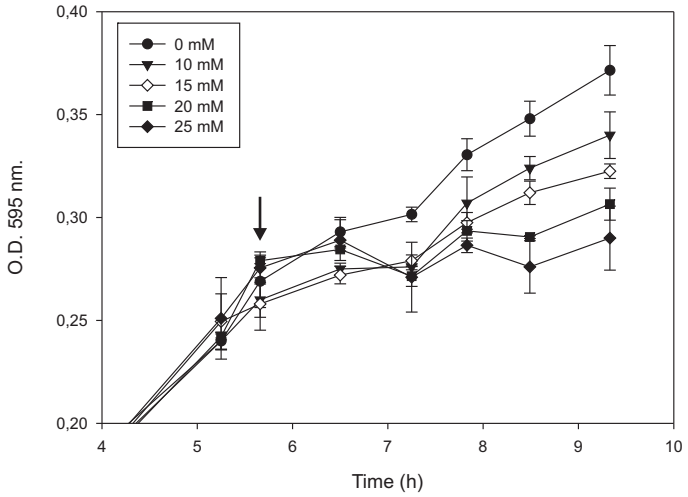
0,40
0,35
0,30
0,25
0,20
O.D. 595 nm.
4
5
6
7
8
9
10
Time (h)
0 mM
10 mM
15 mM
20 mM
25 mM

Fig. 1

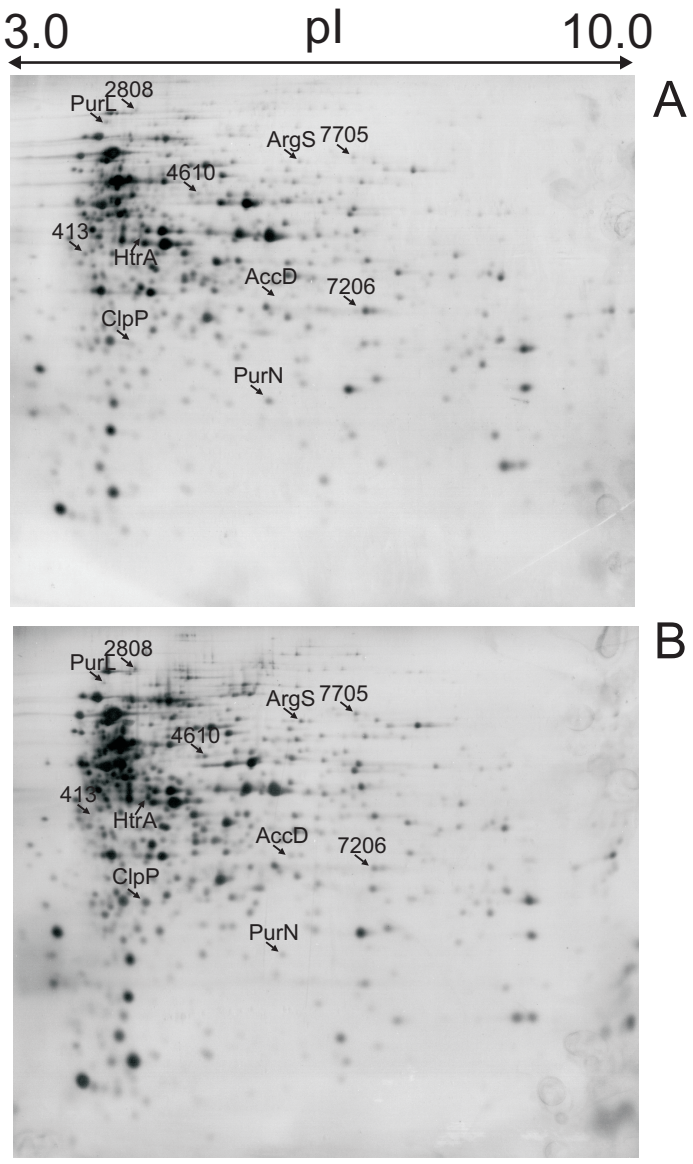
3.0
pI
10.0
A
PurL
2808
ArgS
7705
4610
413
HtrA
AccD
7206
ClpP
PurN
B
PurL
2808
ArgS
7705
4610
413
HtrA
AccD
7206
ClpP
PurN

Fig. 2

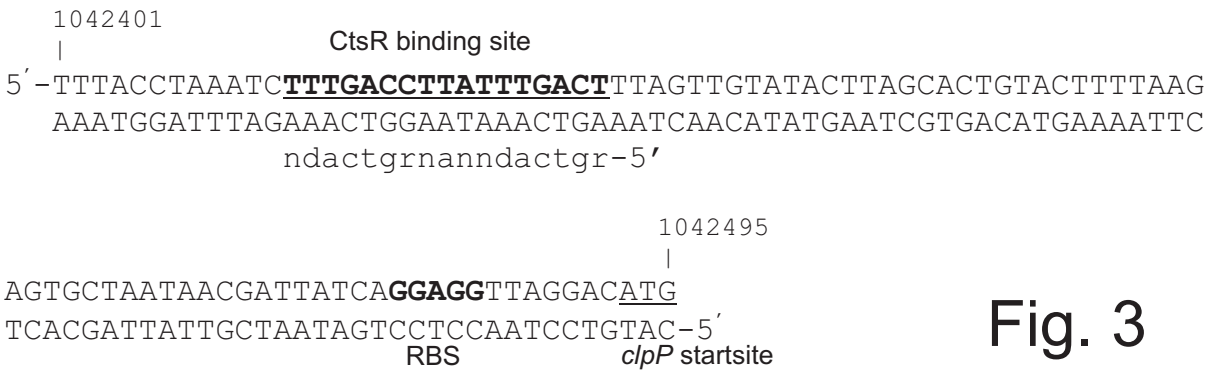
1042401
CtsR binding site
5′-TTTACCTAAATCTTTGACCTTATTTGACTTTAGTTGTATACTTAGCACTGTACTTTTAAG
AAATGGATTTAGAAACTGGAATAAACTGAAATCAACATATGAATCGTGACATGAAAATTC
ndactgrnanndactgr-5′
1042495
AGTGCTAATAACGATTATCAGGAGGTTAGGACATG
TCACGATTATTGCTAATAGTCCTCCAATCCTGTAC-5′
RBS
clpP startsite

Fig. 3

Table S1 Increment in optical density (Δ OD) values after 24 h growth in RPMmod10 and pairwise two way ANOVA in the presence of different phenolic compounds

Strain	Control	Ferulic acid 20 mM		Methyl gallate 20 mM		Caffeic acid 20 mM		*p*-coumaric acid 20 mM	
	Δ OD*	Δ OD	P†	Δ OD	P	Δ OD	P	Δ OD	P
BL23	0.39 ± 0.03	0.16 ± 0.04		0.14 ± 0.05		0.13 ± 0.04		0.23 ± 0.02	
TC01	0.39 ± 0.04	0.12 ± 0.02	0.21	0.16 ± 0.04	0.64	0.14 ± 0.04	1.00	0.22 ± 0.02	0.57
TC02	0.40 ± 0.03	0.20 ± 0.10	0.54	0.15 ± 0.05	0.90	0.14 ± 0.04	0.98	0.23 ± 0.02	0.71
TC03	0.41 ± 0.04	0.14 ± 0.03	0.41	0.16 ± 0.02	0.94	0.13 ± 0.04	0.58	0.22 ± 0.01	0.53
TC04	0.36 ± 0.03	0.14 ± 0.03	0.70	0.18 ± 0.04	0.13	0.16 ± 0.02	0.11	0.18 ± 0.01	0.44
TC05	0.41 ± 0.01	0.15 ± 0.04	0.47	0.19 ± 0.06	0.55	0.13 ± 0.02	0.39	0.23 ± 0.02	0.29
TC06	0.30 ± 0.10	0.11 ± 0.02	0.31	0.10 ± 0.07	0.35	0.08 ± 0.01	0.40	0.12 ± 0.01	0.92
TC07	0.40 ± 0.02	0.14 ± 0.01	0.46	0.19 ± 0.08	0.39	0.16 ± 0.05	0.58	0.23 ± 0.04	0.66
TC08	0.39 ± 0.02	0.15 ± 0.03	0.66	0.17 ± 0.05	0.47	0.15 ± 0.03	0.55	0.25 ± 0.02	0.58
TC09	0.41 ± 0.01	0.15 ± 0.02	0.38	0.18 ± 0.04	0.56	0.13 ± 0.02	0.44	0.23 ± 0.02	0.44
TC10	0.36 ± 0.02	0.15 ± 0.05	0.62	0.17 ± 0.04	0.14	0.12 ± 0.03	0.53	0.23 ± 0.01	0.30
TC11	0.40 ± 0.02	0.15 ± 0.03	0.57	0.14 ± 0.05	0.76	0.13 ± 0.03	0.58	0.20 ± 0.01	0.09
TC12	0.31 ± 0.04	0.09 ± 0.01	0.68	0.10 ± 0.03	0.31	0.08 ± 0.01	0.51	0.17 ± 0.01	0.74
TC13	0.40 ± 0.03	0.16 ± 0.02	0.84	0.19 ± 0.05	0.27	0.16 ± 0.03	0.39	0.23 ± 0.03	0.92
TC14	0.38 ± 0.04	0.16 ± 0.02	0.71	0.16 ± 0.04	0.39	0.13 ± 0.01	0.65	0.23 ± 0.03	0.63
TC15	0.40 ± 0.02	0.17 ± 0.04	0.79	0.17 ± 0.07	0.57	0.17 ± 0.07	0.54	0.24 ± 0.02	0.98
TC16	0.39 ± 0.02	0.14 ± 0.02	0.56	0.16 ± 0.03	0.55	0.13 ± 0.02	0.95	0.22 ± 0.01	0.76
TC17	0.39 ± 0.02	0.13 ± 0.03	0.41	0.15 ± 0.04	0.89	0.14 ± 0.03	0.99	0.20 ± 0.01	0.86

***** Arithmetic means and standard deviation values.

† Only the interaction p values are shown.