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

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RUNNING HEADLINE: Response 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 \textit{Lactobacillus casei}. \textit{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 \textit{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 \textit{Lact. casei} BL23 (Mazé \textit{et al.} 2010). Hence, this study aims to determine the antimicrobial activity of some phenolic compounds against \textit{Lact. casei} BL23, to analyze the response of \textit{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 \textit{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 \textit{et al.} 2011).

\textbf{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 °C 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 \textit{Lact. casei} BL23 and TCS-defective mutants by phenolic compounds

The effect on the growth of \textit{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 \(\mu\)g 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 \(\mu\)g 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 \textit{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 °C 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 °C 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
samples were then loaded on IPG-strips providing a non-linear 3-10 pH gradient (Bio-
rad) and allowed to rehydrate overnight. Isoelectric focusing was carried out by using an
Ettan IPGPhor II (GE Healthcare) system. The isoelectric focusing was performed using
the following program: voltage was increased from 0 to 500 V during the first 5 h,
maintained at 500 V for 5 h, increased from 500 V to 8000 V in 9 h, and fixed at 8000
V for 5 h. After isoelectrofocusing, the strips were first equilibrated for 20 min in a
buffer containing 6 mol l⁻¹ urea, 30% (w/v) glycerol, 2% (w/v) SDS, 0.05 mol l⁻¹ Tris-
HCl pH 8.8, and 1% (w/v) DTT (reduction step), and subsequently for 20 min in a
buffer containing 6 mol l⁻¹ urea, 30%(w/v) glycerol, 2% (w/v) SDS, 0.05 mol l⁻¹ Tris-
HCl pH 8.8, and 2.5% (v/v) iodoacetamide (alkylation step). The strips were then
deposited onto a 12.5 % (w/v) polyacrylamide-SDS gels and run at 1 W/gel for 16 h in
an Ettan DALtsix Large Vertical System (GE Healthcare). Silver staining was
performed as described by Blomberg (Blomberg 2002).
Six biological replicates were obtained for each growth condition. Gels were scanned
using a HP ScanJet 5100C (300 dpi, 12-bit image) and the images analysed with the
PDQuest 8.0 software (Bio-Rad). Spot detection was performed using the PDQuest
automated spot detection algorithm. Normalization was performed automatically using
the local regression model implemented in the software to compensate image
differences caused by variations in experimental conditions (e.g. protein loading or
staining). A master gel image was automatically generated by the software and spots in
the master gel were then matched across all gels. Matching was then visually inspected
and corrected when required. Subsequently, gels were divided into three different
groups: untreated samples, ethanol-supplemented samples and p-coumaric-
supplemented samples. Matching features of the software were used to relate and
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 (NCBI 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/](http://www.ncbi.nlm.nih.gov/COG/)). KEGG database ([http://www.genome.jp/kegg/](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
growth of *Lact. casei*. The MIC values determined were 25 mmol l⁻¹ for *p*-coumaric acid and 40 mmol l⁻¹ for the other phenolic compounds assayed.

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

Next, we tested whether TCS play a role in the response to phenolic compounds since these systems had been previously shown to be involved in the tolerance to a number of stress conditions in *Lact. casei* (Alcántara et al. 2011). To this end, 17 TCS-defective mutants and the parental strain *Lact. casei* BL23 were grown in presence of *p*-coumaric acid, ferulic acid, caffeic acid and methyl gallate. No significant loss of erythromycin resistance in the insertional mutants was observed under the experimental conditions used (data not shown) thus indicating that reversion to the wild-type phenotype was negligible. The results obtained are shown in Table S1 in Supplemental Information. Since some mutants showed significant differences with the parental strain in RPMmod10 (TC06 and TC12; determined by the Student’s *t* test after assessing equality of variances by the Levene’s test), pairwise two-way ANOVA analyses, considering treatment (control *versus* phenolic compound) and strain (BL23 *versus* each mutant strain) as variables, were performed in order to determine whether the differences observed were due to the phenolic compound (in this case a significant interaction should be detected). Addition of phenolic compounds resulted in significant inhibition of all strains (Table S1), however in no case significant differences were observed between the parental strain and any of the mutants (data not shown) thus indicating that TCS do not play a significant role in the response against phenolic compounds of *Lact. casei* BL23. However, involvement of TCS cannot be ruled out under the experimental different growth conditions used here.
2D-PAGE analysis of the response of *Lact. casei* BL23 to *p*-coumaric acid

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

Fig. 2 shows representative gels resulting from the analysis of the total proteins. No significant differences were detected between the control samples and the samples supplemented with ethanol (data not shown). When the samples supplemented with *p*-coumaric acid were compared with the control samples, eleven spots showed statistical differences according to the criteria established (see Materials and Methods). Table 2 summarizes the quantitative data of the spots identified as differentially expressed. In response to *p*-coumaric acid, six proteins were upregulated and five downregulated in *Lact. casei* BL23. Six spots could be identified by mass spectrometry, the other five did not render reliable mass spectra. Among the six identified proteins, two of them were significantly less abundant in the presence of *p*-coumaric acid (Table 2): a putative phosphoribosylformylglycinamidine synthase II and a folate-dependent phosphoribosylglycinamidie formyltransferase (LCABL_19700 and LCABL_19670, respectively). Both proteins catalyze consecutive steps in the purine biosynthetic pathway. Among the upregulated proteins, two of them are involved in protein turnover: the Clp protease proteolytic subunit and the trypsin-like serine protease HtrA (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 \textit{Lact. casei} BL23
being \( p \)-coumaric acid the most active against this bacterium. The MIC of \( p \)-coumaric
acid for \textit{Lact. casei} BL23 was similar to that previously reported for \textit{Lact. plantarum}
(Landete et al. 2007) whereas \textit{Lact. casei} was more sensitive to the other three
compounds. \textit{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 \textit{Lactobacillus brevis}
(Landete et al. 2010b) or \textit{Pediococcus pentosaceus} (Barthelmebs et al. 2000). However,
the analysis of the available genomic sequences of \textit{Lact. casei} strains do not reveal the
presence of this gene. Methyl gallate is metabolized by \textit{Lact. plantarum} strains to gallic
acid and subsequently to pyrogallol via tannase and gallate decarboxylase activities
(Rodriguez et al. 2008b). The gene encoding tannase in \textit{Lact. plantarum} has been
identified (Iwamoto et al. 2008) and homologous genes are absent in the \textit{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 \textit{Lact. casei} can
metabolize phenolic compounds which \textit{may} partly explain the higher sensitivity of this
bacterium to these substances.
Phenolic acids are weak organic acids that can enter the cell by passive diffusion in their undissociated form where they can acidify the cytoplasm and denature proteins. Furthermore, they can insert and destabilize the cell membrane due to their amphipathic nature. Campos et al. (Campos et al. 2009) observed that phenolic acids induced ion leakage and proton influx on LAB isolated from wine thus indicating an increase in cell membrane permeability. A previous study had shown that several TCS encoded by *Lact. casei* BL23 were involved in the response against cell envelope damages caused by different agents (Alcántara et al. 2011). However, the assay of the response of a series of derivative strains defective in each TCS encoded by *Lact. casei* BL23 did not reveal any significant differences with the parental strain in their response to phenolic compounds thus indicating that TCS are not involved in the response against these substances under our experimental conditions. There are very few studies dealing with the regulation of the response of LAB to phenolic compounds. Gury et al. (2004) observed that the *padR* gene encodes the negative transcriptional regulator of PAD (*padA*) in *Lact. plantarum*. The *padA* gene is cotranscribed with a downstream gene, *usp1*, which encodes a putative universal stress protein. The *usp1* gene is overexpressed in presence of phenolic acids and the role to inactivate PadR indicates that it could serve as an important mediator in phenolic acids response. *Lact. casei* BL23 harbours a gene (LCABL_27180; 179 amino acids) significantly similar to the *Lact. plantarum* (34% identical residues in a 130 positions alignment with gene *padR* of *Lact. plantarum* WCFS1) and some *Bacillus padR* genes (for example, 43% identical residues in a 163 positions alignment with gene BCE_1910 of *Bacillus cereus* ATCC 10987). However, there is no evidence about the function of this gene. The results obtained with 2D-SDS PAGE are consistent with damages in the cell membrane and cytoplasmic proteins caused by *p*-coumaric acid since the exposition to
this substance led to a strong induction of ClpP and HtrA, two proteases involved in
protein turnover (Table 2). The ATP-dependent Clp proteases consist of separately
encoded ATPase and peptidase subunits. Clp proteases have been the subject of
extensive research that have evidenced that ClpP-containing proteolytic complexes play
indispensable roles in cellular protein quality control systems by refolding or degrading
damaged proteins and that the chaperone activity of Clp ATPases are important for
controlling stability and activity of central transcriptional regulators in low-GC Gram
positive bacteria (Frees et al. 2007). A number of studies have shown the induction of
ClpP expression under varied stress conditions in lactobacilli (Fernández et al. 2008;
Hörmann et al. 2006; Weiss and Jespersen 2010). ClpP is under control of the stress
response regulator CtsR in Lact. plantarum (Fiocco et al. 2010). Interestingly, a search
of the genome sequence of Lact. casei BL23 with the CtsR binding site consensus
sequence (Derré et al. 1999) reveals a putative CtsR binding site upstream clpP (Fig. 3).
Similar sites can be found upstream clpB and clpE (data not shown). Although it is
tempting to speculate that the CtsR regulon is involved in the response against p-
coumaric acid, additional evidence is required to prove this point.
There is scarce information concerning the role of HtrA in lactobacilli. HtrA expression
is induced in Lactobacillus helveticus after exposure to 4% (w/v) NaCl and to a lesser
extent after exposure to puromycin, ethanol, or heat although a mutant defective in HtrA
only showed heat sensitivity (Smeds et al. 1998). On the basis of the amino-acid
sequence, the location of HtrA was predicted at the cell membrane (Smeds et al. 1998).
HtrA degrades abnormal exported proteins in the closely related bacterium Lactococcus
lactis (Poquet et al. 2000). HtrA may play a similar role in lactobacilli. The induction of
the expression of these two proteins suggests that p-coumaric acid disturbed protein
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 catalyzes 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
requirement of nucleotides. This effect would agree with previous observations of inhibition of DNA replication by phenolic compounds (Smith and Dou 2001). Navarro-Perán et al. (Navarro-Perán et al. 2007) observed that epigallocatechin-3-gallate decreased the cellular production of nucleotides in a human colon carcinoma cell line and disrupted the purine metabolism.

In summary, our results indicate that p-coumaric acid disturbs protein structure and may also affect the properties of the cell membrane of *Lact. casei* BL23. In addition, the inhibition of genes involved in purine biosynthesis suggests that p-coumaric acid may interfere with DNA replication as previously observed for other phenolic compounds. These results open new lines of research to understand the effects of phenolic compounds in LAB.
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Table 1 *Lactobacillus* *casei* strains used in this work

<table>
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<tr>
<th>Strains</th>
<th>Characteristics</th>
<th>Reference</th>
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<td>BL23</td>
<td>wild type</td>
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<td>TC17</td>
<td>BL23 ΔmaeR (RR17)</td>
<td>(Landete et al. 2010a)</td>
</tr>
</tbody>
</table>
Table 2. Citoplasmic protein spots differentially expressed in the presence of *p*-coumaric acid in *Lact. casei* BL23

<table>
<thead>
<tr>
<th>Spot</th>
<th>Functional category*</th>
<th>Putative function</th>
<th>Mr†</th>
<th>pI‡</th>
<th>Locus_tag/gene</th>
<th>Spot volume ×10³ §</th>
<th>N**</th>
<th>Spot volume ×10³</th>
<th>N</th>
<th>Fold ratio††</th>
<th>Matched peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>413</td>
<td>Unknown</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15.2 ± 4.1</td>
<td>5</td>
<td>1.0</td>
<td>1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>1804</td>
<td>Nucleotide transport and metabolism</td>
<td>Phosphoribosylformylglycinamidine synthase II</td>
<td>78924</td>
<td>4.77</td>
<td>LCABL_19700 purL</td>
<td>26.0 ± 9.7</td>
<td>6</td>
<td>2.7 ± 0.8</td>
<td>4</td>
<td>0.1</td>
<td>8</td>
</tr>
<tr>
<td>2107</td>
<td>Posttranslational modification, protein turnover, chaperones</td>
<td>ATP-dependent Clp protease proteolytic subunit</td>
<td>21511</td>
<td>5.09</td>
<td>LCABL_10770 clpP</td>
<td>4.2 ± 2.4</td>
<td>4</td>
<td>40.4 ± 12.7</td>
<td>5</td>
<td>10.0</td>
<td>5</td>
</tr>
<tr>
<td>2418</td>
<td>Posttranslational modification, protein turnover, chaperones</td>
<td>Trypsin-like serine protease</td>
<td>44924</td>
<td>5.99</td>
<td>LCABL_30080 htrA</td>
<td>-</td>
<td>4</td>
<td>47.6 ± 22.1</td>
<td>3</td>
<td>+§§</td>
<td>2</td>
</tr>
<tr>
<td>2808</td>
<td>Unknown</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25.1 ± 9.8</td>
<td>6</td>
<td>14.5 ± 4.7</td>
<td>4</td>
<td>0.6</td>
<td></td>
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<tr>
<td>4610</td>
<td>Unknown</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.6 ± 1.7</td>
<td>4</td>
<td>19.0 ± 1.2</td>
<td>4</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>6101</td>
<td>Nucleotide transport and metabolism</td>
<td>Folate-dependent phosphoribosylglycinamidase formyltransferase</td>
<td>20282</td>
<td>5.67</td>
<td>LCABL_19670 purN</td>
<td>42.2 ± 1.5</td>
<td>4</td>
<td>3.6 ± 2.0</td>
<td>5</td>
<td>0.1</td>
<td>5</td>
</tr>
</tbody>
</table>

*Mr* and *pI* are molecular weight and isoelectric point, respectively. *N* is the number of matched peptides.
| Fatty acid and phospholipid metabolism | Acetyl-CoA carboxylase beta subunit | 29416 | 6.07 | LCABL_22910 accD | 4.9 ± 0.3 | 3 | 5 | 2 |
| 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 |
| Unknown | Unknown | 7206 | 55.1 ± 16.9 | 6 | 13.8 ± 6.5 | 5 | 0.3 |
| Unknown | Unknown | 7705 | 6.5 ± 3.4 | 6 | 13.4 ± 4.3 | 5 | 2.0 |

* Functional categories assigned according to the cluster of orthologous groups of proteins (COGs; www.ncbi.nlm.nih.gov/COG/).
† Theoretical molecular mass.
‡ Theoretical isoelectric point.
§ Means ± standard deviations.
** Number of gels analyzed.
†† Proteins with values over 1 are up-regulated in response to p-coumaric acid, proteins with values below 1 are down-regulated.
‡‡ Percentage of amino acid coverage (peptides observed/theoretical value from sequence data).
§§ Not detected in the control.
**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).
Fig. 1
CtsR binding site

5′-TTTACCTAAATC**TTTGACCTTATTTGACT**TTAGTTGATATACTTAGCACTGTACTTTTAAG
AAATGGATTTAGAAACTGGAATAAACTGAAATCAACATATGAATCGTGACATGAAAATTC
ndactgrnanndactgr-5′

AGTGCTAATAACGATTATCA**GGAGGTTAGGACATG**
TCACGATTATGCTAAATAGTCTCTCCAATCCTGTAC-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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control</th>
<th>Ferulic acid 20 mM</th>
<th>Methyl gallate 20 mM</th>
<th>Caffeic acid 20 mM</th>
<th>p-coumaric acid 20 mM</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Δ OD</td>
<td>Δ OD</td>
<td>P†</td>
<td>Δ OD</td>
<td>Δ OD</td>
</tr>
<tr>
<td>BL23</td>
<td>0.39 ± 0.03</td>
<td>0.16 ± 0.04</td>
<td>0.14 ± 0.05</td>
<td>0.13 ± 0.04</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>TC01</td>
<td>0.39 ± 0.04</td>
<td>0.12 ± 0.02</td>
<td>0.21</td>
<td>0.16 ± 0.04</td>
<td>0.64</td>
</tr>
<tr>
<td>TC02</td>
<td>0.40 ± 0.03</td>
<td>0.20 ± 0.10</td>
<td>0.54</td>
<td>0.15 ± 0.05</td>
<td>0.90</td>
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<tr>
<td>TC03</td>
<td>0.41 ± 0.04</td>
<td>0.14 ± 0.03</td>
<td>0.41</td>
<td>0.16 ± 0.02</td>
<td>0.94</td>
</tr>
<tr>
<td>TC04</td>
<td>0.36 ± 0.03</td>
<td>0.14 ± 0.03</td>
<td>0.70</td>
<td>0.18 ± 0.04</td>
<td>0.13</td>
</tr>
<tr>
<td>TC05</td>
<td>0.41 ± 0.01</td>
<td>0.15 ± 0.04</td>
<td>0.47</td>
<td>0.19 ± 0.06</td>
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<tr>
<td>TC06</td>
<td>0.30 ± 0.10</td>
<td>0.11 ± 0.02</td>
<td>0.31</td>
<td>0.10 ± 0.07</td>
<td>0.35</td>
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<tr>
<td>TC07</td>
<td>0.40 ± 0.02</td>
<td>0.14 ± 0.01</td>
<td>0.46</td>
<td>0.19 ± 0.08</td>
<td>0.39</td>
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<tr>
<td>TC08</td>
<td>0.39 ± 0.02</td>
<td>0.15 ± 0.03</td>
<td>0.66</td>
<td>0.17 ± 0.05</td>
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<tr>
<td>TC09</td>
<td>0.41 ± 0.01</td>
<td>0.15 ± 0.02</td>
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<td>0.18 ± 0.04</td>
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<tr>
<td>TC10</td>
<td>0.36 ± 0.02</td>
<td>0.15 ± 0.05</td>
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<td>0.17 ± 0.04</td>
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<tr>
<td>TC11</td>
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<td>0.14 ± 0.05</td>
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<tr>
<td>TC12</td>
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<td>0.09 ± 0.01</td>
<td>0.68</td>
<td>0.10 ± 0.03</td>
<td>0.31</td>
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<tr>
<td>TC13</td>
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<td>0.16 ± 0.02</td>
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<td>0.19 ± 0.05</td>
<td>0.27</td>
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<tr>
<td>TC14</td>
<td>0.38 ± 0.04</td>
<td>0.16 ± 0.02</td>
<td>0.71</td>
<td>0.16 ± 0.04</td>
<td>0.39</td>
</tr>
<tr>
<td>TC15</td>
<td>0.40 ± 0.02</td>
<td>0.17 ± 0.04</td>
<td>0.79</td>
<td>0.17 ± 0.07</td>
<td>0.57</td>
</tr>
<tr>
<td>TC16</td>
<td>0.39 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>0.56</td>
<td>0.16 ± 0.03</td>
<td>0.55</td>
</tr>
<tr>
<td>TC17</td>
<td>0.39 ± 0.02</td>
<td>0.13 ± 0.03</td>
<td>0.41</td>
<td>0.15 ± 0.04</td>
<td>0.89</td>
</tr>
</tbody>
</table>

* Arithmetic means and standard deviation values.
† Only the interaction p values are shown.