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RESEARCH ARTICLE

Response of a *Lactobacillus plantarum* human isolate to tannic acid challenge assessed by proteomic analyses

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Scope: To gain insight on the mechanisms used by intestinal bacteria to adapt and resist the antimicrobial action of dietary tannins and identify targets for tannic acid in *Lactobacillus plantarum*.

Methods and results: A proteomic analysis of an *L. plantarum* human isolate exposed to the tannic acid challenge was undertaken. Tannic acid targeted proteins involved in outstanding processes for bacterial stress resistance including cyclopropanation of membrane lipids, stress response at population scale and maintenance of cell shape. To respond to this aggression, tannic acid-misfit cells of *L. plantarum* challenged with tannic acid reorganized their metabolic capacity to economize energy and express proteins involved in oxidative stress defense and cell wall biogenesis, indicating that the injury incurred by tannic acid was based on oxidative damage and disruption of the cell envelope. The induction of 3-octaprenyl-4-hydroxybenzoate carboxy-lyase, which is sensitive to changes in redox conditions and involved in ubiquinone biosynthesis in other bacteria, suggests for a tannic acid-induced redox imbalance.

Conclusion: The results reveal the adaptation of a gastrointestinal isolate of *L. plantarum* to tannic acid and identify antibacterial targets for this dietary compound. This provides the basis for the selection of tannin-resistant microorganisms and their use to obtain health benefits from tannin-containing diets.

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1 Introduction

Tannins are polyphenolic compounds found in nearly all plant foods and feed and therefore part of animal and human diets. These dietary metabolites are mainly classified as condensed tannins (proanthocyanidins) and hydrolysable tannins. Based on several epidemiological studies and in vitro assays that evidenced antioxidant, radical scavenging or antimutagenic properties, the main interest of tannin intake for human health is its possible implication in chronic disease prevention [1]. To understand the biological effects of tannins the insight on the metabolic fate and bioavailability of these metabolites in the human body is crucial. This knowledge is currently scarce but it is known that most of the ingested tannins escape the upper gastrointestinal tract (GI tract) digestion and reach the large intestine where they meet with the indigenous colonic microbiota. The interaction of tannins with gut microbiota will determine in great extent its absorption at different sites in the host GI tract and the direct physiological effects of these metabolites.

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Abbreviations: AMC, activated methyl cycle; **CFA,** cyclopropane fatty acyl; **GI,** gastrointestinal; **GluRS,** glutamyl-tRNA synthetase; **LAB,** lactic acid bacteria; **NO,** nitric oxide; **NOS,** nitric oxide synthase; **NPR,** NADH-peroxidase; **Pgm,** phosphoglycerate mutase; **ROS,** reactive oxygen species; **SAM,** *S*-adenosylmethionine; **TyrS,** tyrosine-tRNA synthetase

Besides direct effects on the host, other mechanisms by which tannins may benefit human health could involve effects on microbial population [2]. In fact, some experimental data obtained in animal models show that dietary tannins are involved in defining the distribution and abundance of microbial populations in the digestive tract. Thus, polyphenol-fed rats shifted the predominant fecal microbiota from *Bacteroides*, *Clostridium* and *Propionibacterium* spp. toward *Bacteroides* and the regarded as safe *Lactobacillus* and *Bifidobacterium* spp. [3]. Regarding animal nutrition, tannin-rich diets are considered antinutritional as they reduce intake, protein and carbohydrate digestibility and prevent weight gain. Some reports have evidenced that tannin-resistant rumen microbial populations prevent the adverse effects of tannins [4] and may be able to confer protection to animals not adapted to tannin-containing diets [5]. These microorganisms improve digestion, prevent bloat and improve animal production by increasing the body weight [6]. In humans, the sole tannin-degrading bacteria of human origin found in a previous search for tannin-degrading bacteria from human feces and fermented foods was *Lactobacillus plantarum* [7] which, interestingly, also exerts different positive health effects on human volunteers and animals [8]. Furthermore, judging by previous studies [9] *L. plantarum* might colonize the human GI tract considerably better than other tested lactobacilli.

L. plantarum displays a striking capacity to adapt to several ecological niches. This bacterium is successfully adapted to plant habitats and part of the human colonic microbiota [10, 11] and GI tract from animals [8]. Data set of specific expression profiles describing adaptation of *L. plantarum* to plant-based media [12], the passage through the GI tract of mice [13] or bile stress [14] have been reported and reflect the versatility of this bacterium.

Regarding adaptation and resistance to tannins, a recent study has reported on the protein expression profile of stationary phase cells of an *L. plantarum* wine strain that resumed growth after adaptation to tannic acid [15]. Starved cells already adapted to tannic acid and submitted to medium containing tannic acid displayed a prolonged viability during stationary phase. The upregulated proteins were mainly related to the energy metabolism (glycolysis) and protein-synthesizing capacity (ribosomal proteins). Since (i) the protein profile of *L. plantarum* has been investigated on stationary cells already adapted to tannic acid and (ii) the protein profile in this bacterium changes during growth [16], we suspect that many proteins and therefore mechanisms involved in adaptation and resistance to tannins have been not yet identified.

In view of the contribution of host tannin-resistant bacteria in both, benefiting the host from tannin-containing diets and in determining the fate of these metabolites, knowledge on the mechanisms that bacteria use to withstand the inhibitory effects of tannins is necessary for a better understanding of their biological effects. To provide additional insight on the mechanisms involved in adaptation and resistance to tannins in intestinal bacteria, tannic acid-misfit log phase cells of an *L. plantarum* human isolate were challenged with tannic acid and the response comparatively evaluated with non-treated cells by 2-D-PAGE analysis.

2 Material and methods

2.1 Bacterial strain, culture conditions and chemicals

L. plantarum WCFS1 used through this study was kindly provided by Dr Michiel Kleerebezem (NIZO Food Research, The Netherlands). This strain is a colony isolate of *L. plantarum* NCIMB 8826, which was isolated from human saliva. It survives the passage through the human stomach [17], persists in the digestive tract of mice and humans better than other *Lactobacillus* spp. isolated from the human intestine [18] and reduces the total translocation of endogenous microbiota of mice suffering from colitis [18]. Complex MRS [19] or semi-synthetic RPM media [20] were used to grow *L. plantarum* WCFS1 at 30°C without shaking in microaerobic conditions. The composition of RPM is the following: glucose (2 g/L), trisodium citrate dehydrate (0.5 g/L), D-, L-malic acid (5 g/L), casamino acids (Difco, Detroit, MI, USA) (1 g/L), yeast nitrogen base lacking amino acids (Difco) (6.7 g/L) and the pH adjusted to 5.5.

Based on preliminary assays where MRS proteins precipitated in the presence of 1 mM tannic acid (not shown), RPM was used as the medium of choice to test the effects of tannic acid on the protein expression of *L. plantarum* WCFS1. A single colony from MRS agar plate was inoculated into RPM medium. After 18-h incubation at 30°C in microaerobic conditions, this culture was used to inoculate 250 mL of fresh RPM medium.

Tannic acid (T0125) was obtained from Sigma (Madrid, Spain). A 100-mM tannic acid stock solution was prepared in 5% v/v acetone. Appropriate dilutions were prepared to adjust the tannic acid to 1 mM final concentration in RPM.

2.2 Extraction of cell-free proteins

L. plantarum cultures (250 mL) were grown in RPM lacking tannic acid to mid-exponential phase ($OD_{600} \approx 0.6$) and then supplied with tannic acid to bring their final concentration to 1 mM. The tannic acid-induced cultures of *L. plantarum* and their corresponding control cultures devoid of tannic acid were further incubated for 2 h at 30°C. Cultures were then centrifuged ($8000 \times g$ for 10 min at 4°C) and washed thrice with 0.1 M Tris-HCl buffer, pH 7.5, for 15 min. Bacterial pellets were suspended in 5 mL of 0.1 M Tris-HCl buffer, pH 7.5, and broken by a single passage through a cell disrupter (Basic Z, Constant Systems, Daventry, UK) set at 2.5×10^5 Pa. Unbroken cells and cell debris were removed

by centrifugation at 4500 × *g* for 15 min at 4°C. Membrane vesicles were discarded from the solution by ultracentrifugation at 50 000 × *g* for 30 min at 4°C. The protein concentration was estimated using the Bradford method according to the manufacturer's instructions (Coomassie Protein Assay Reagent; Pierce Biotechnology, Rockford, IL, USA).

2.3 2-D electrophoresis conditions

Sample preparation of *L. plantarum* proteins was performed according to [21]. For 2-D electrophoresis, isoelectrofocusing strips of pH 4.0–7.0 (BIORAD) were rehydrated for 12 h at 50 V using the Protean IsoElectric Focusing Cell II (BIORAD) and then focused at 10 000 V/h. Focused immobilized pharmalyte gradient strips were equilibrated sequentially for 15 min in 1 M Tris-HCl buffer, pH 6.8, containing 6 M urea, 30% vol/vol glycerol (Merck KGaA) and 1% wt/vol sodium dodecyl sulfate (Merck KGaA) supplemented with 0.83% wt/vol dithiothreitol in the first equilibration step and with 7.5% wt/vol iodoacetamide in the second one. The second dimension was performed by SDS-PAGE on gels containing 12.5% polyacrylamide and carried out with a Protean II xi cell (BIORAD). Proteins were resolved overnight at 4°C by using a 20-mA/gel constant current, visualized following staining with Bio-safe colloidal Coomassie blue (BIORAD) according to the manufacturer's instructions and scanned with an ImageScanner (Amersham Biosciences, Piscataway, NJ, USA). Spot detection and volume quantitation were carried out with the SameSpot software (Non Linear Dynamics). At least three independent experiments (cultures) for each growth condition and three gels per experiment were performed. Effects of tannic acid on the expression of proteins were considered if the mean normalized spot volume varied at least twofold with respect to the control as confirmed by variance analysis at a significance level of $p \leq 0.05$.

2.4 Identification of proteins by peptide mass fingerprinting

Individual spots displaying significant and reproducible intensities that differed under the presence of tannic acid were excised from the gels and submitted to tryptic digestion. Then, mass spectrometry analyses were performed on a Voyager DESTR instrument (Applied Biosystems) as previously described [22]. MS-Fit (University of California San Francisco Mass Spectrometry Facility; http://prospector.ucsf.edu) and Mascot (Matrix Science, Boston, MA, USA; http://www.matrixscience.com/search_form_select.html), installed locally, were used to identify proteins from peptide mass fingerprints. All searches were performed against the database for *L. plantarum* WCFS1 (http://www.ncbi.nlm.nih.gov/genomes/).

3 Results

3.1 Alterations in the protein expression profile of *L. plantarum* WCFS1 in response to tannic acid stress

To identify the proteins involved in the adaptation and response of *L. plantarum* WCFS1 to tannic acid, we investigated the proteomic profile of exponentially growing tannic acid-misfit cells after 2 h of exposure to 1 mM of tannic acid. Semi-synthetic RPM instead complex MRS medium was used to avoid precipitation of proteins from MRS medium caused by the addition of tannic acid. Protein expression was considered significantly up or downregulated when a difference in the spot intensities $\geq$2-fold was observed with the SameSpot software (Non Linear Dynamics) among the tested growth conditions. We observed that the synthesis profile of 22 out of approximately 350 spots detected on the 2-D-PAGE gels was significantly altered ($p<0.05$) in response to 1 mM tannic acid exposure (Supporting Information Fig. 1). Thirteen spots were upregulated and nine were downregulated after tannic acid exposure. The identified proteins mainly fell into three groups corresponding to cell wall biogenesis, oxidative stress and transcription–translation processes (Table 1). Altered expression of a general stress protein, a protein involved in carbon metabolism, two putative oxidoreductases and three unclassified proteins were also observed.

3.1.1 Cell wall and membrane biogenesis

(i) LdhD: D-lactate dehydrogenase, encoded by *ldhD* (lp_2057), is upregulated under tannic acid stress compared with the control conditions (Table 1, Fig. 1). LdhD catalyzes the conversion of pyruvate into D-lactic acid and concomitantly recycles NAD^+. D-lactate, the product of LdhD, is incorporated as the last residue in the pentapeptide assembled to the *N*-acetylmuramic acid, to give rise to the muramyl-pentapeptide, which is a basic component, together with *N*-acetylglucosamine, of the peptidoglycan thread.

(ii) DapF: Diaminopimelate epimerase (DapF), encoded by *dapF* (lp_2185), is the last enzyme in the biosynthetic pathway of diaminopimelic acid and catalyzes the epimerization of L, L-diaminopimelate to form *meso* D, L-diaminopimelate. This amino acid is one of the amino acids that successively incorporate into the pentapeptide attached to *N*-acetylmuramic acid. DapF was also more abundant under tannic acid stress.

(iii) TagE6: TagE6, the product of *tagE6* gene (lp_2844), is a putative poly (glycerol-phosphate) α-glucosyltransferase, which is induced after exposure of *L. plantarum* WCFS1 to tannic acid stress (Table 1, Fig. 1).

(iv) Cfa2: The amounts of cyclopropane-fatty-acyl-phospholipid synthase, encoded by the annotated *cfa2* gene

Table 1. Proteins differentially expressed in misfit cells of *L. plantarum* WCFS1 challenged with 1 mM tannic acid

Group and spot	Protein	Function	lp no. (locus)	Classification	MOWSE[a)]	Fold [b)]
Cell wall/membrane biogenesis						
1	LdhD	D-Lactate dehydrogenase	lp_2057	Glycolysis	9.8 E+03	4.5
2	DapF	Diaminopimelate epimerase	lp_2185	Cell envelope biogenesis	1.2 E+04	3.2
3	TagE6	Putative poly(glycerol-phosphate) α-glucosyltransferase	lp_2844	Cell envelope biogenesis	1.78 E+01	3.4
4	Cfa2	Cyclopropane-fatty-acyl-phospholipid synthase	lp_3174	Lipid metabolism	2.9 E+01	−3.4
Cellular processes						
5	LuxS	Autoinducer-2 synthesizing protein	lp_0774	Quorum sensing	5.5 E+02	−2.6
6	MetE	Cobalamine-independent methionine synthase	lp_1375	Aminoacid metabolism	2.06 E+01	−4.5
Oxidative stress						
7	Npr2	NADH-peroxidase	lp_2544	Detoxification	1.4 E+08	2.8
8	UbiD	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	lp_2945	Ubiquinone biosynthesis	1.7 E+07	7.6
9	RecA	Recombinase A	lp_2301	DNA repair and recombination	4.53 E+01	8.3
Miscellaneous						
10	LP_3045	Short-chain dehydrogenase/oxidoreductase	lp_3045	Miscellaneous	2.7 E+02	2.3
11	LP_0244	Putative oxidoreductase	lp_0244	Miscellaneous	1.9 E+03	7
Glycolysis						
12	Pgm8	Phosphoglycerate mutase	lp_0901	Glycolysis	4.0 E+02	3.2
Stress						
13	Asp1	Alkaline shock protein	lp_0929	General stress	2.1 E+02	2.8
Transcriptional-translational factors						
14	PyrG	CTP synthase	lp_0481	Pyrimidine biosynthesis	1.65 E+01	−2.6
15	GreA	GreA2 transcription elongation factor	lp_1563	Transcription	4.8 E+02	2.2
16	GltX (GluRS)	Glutamyl-tRNA synthetase	lp_0609	Translation	3.3 E+05	−17
17	TyrS	Tyrosine-tRNA synthetase	lp_2807	Translation	2.25 E+01	−3.6
18	S30EA	30S ribosomal interface protein S30EA	lp_0737	Translation	1.6 E+03	−3.1
19	LP_1153	Putative transcription regulator	lp_1153	Transcription	4.4 E+04	−2.7
Unclassified[c)]						
20	LP_0052	Putatitve 6-pyruvoyl-tetrahydropterin synthase	lp_0052	Cofactor metabolism	4.3 E+01	−2.2
21	LP_3397	Putative nucleoside deoxyribosyltransferase	lp_3397	Pyrimidine metabolism	1.2 E+02	4.8
22	LP_2877	Putative UspA family nucleotide-binding protein	lp_2877	General stress	1.35 E+02	2.0

a) Molecular weight search score.
b) Variation in protein expression in tannic acid versus control conditions. Negative values show decreased protein expression.
c) The putative function for these proteins is suggested on the basis of BLASTP analysis. See text for details.

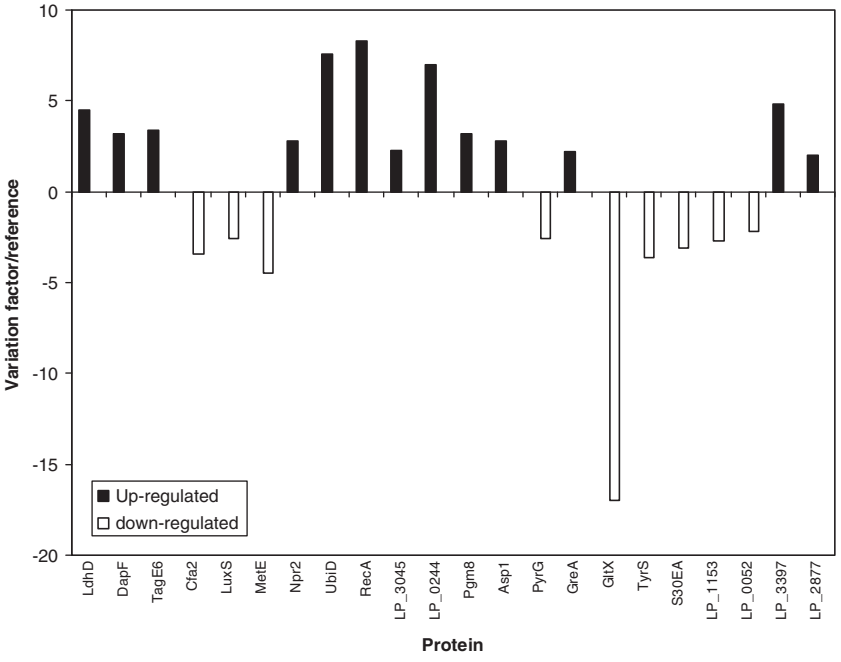

Figure 1. Variation factors of proteins differentially expressed in the presence or absence of tannic acid. The variation factor was calculated by dividing each spot volume of the corresponding protein observed under tannic acid stress by the spot volume of the same protein observed under control conditions.

(lp_3174), were clearly downregulated in the presence of tannic acid (Table 1, Fig. 1). Cyclopropane fatty acyl synthases (CFA synthases) catalyze the addition of a methylene group from *S*-adenosylmethionine (SAM) across *cis* double bonds of monounsaturated fatty acyl.

3.1.2 Activated methyl cycle (AMC)

(i) LuxS: The amounts of LuxS from *L. plantarum* WCFS1, encoded by the *luxS* gene (lp_0774), were diminished in presence of 1 mM tannic acid (Table 1, Fig. 1). LuxS is the enzyme required for producing AI-2 which is an autoinducer used by bacteria as signalling molecule for cell–cell communication and organize their behavior at the population scale via the regulation of gene expression of a variety of vital functions necessary for survival in specific niches [23].

Besides its role in cell–cell communication, the reaction catalyzed by LuxS is part of an important metabolic cycle termed ''activated methyl cycle'' (AMC) [24]. The AMC fulfils several important functions in the cell, the main of which is to generate methyl groups with a high transfer potential. Homocysteine is released during the LuxS-catalyzed reaction being subsequently recycled to methionine and SAM, which is the main methyl donor in the cell.

(ii) MetE: The amount of MetE (cobalamine-independent methionine synthase) encoded by lp_1375, was increased in response to tannic acid (Table 1, Fig. 1). MetE is part of the AMC cycle and involved in SAM recycling. The function of MetE is to enter the methyl groups into AMC through the conversion of homocysteine into methionine.

3.1.3 Oxidative stress

(i) Npr2: NADH-peroxidase (NPR) encoded by the *npr2* gene (lp_2544), was upregulated in the presence of tannic acid compared with control conditions (Table 1, Fig. 1). NPR catalyzes the detoxification of H_2O_2 to H_2O with the concomitant oxidation of NADH.

(ii) UbiD: The amounts of 3-octaprenyl-4-hydroxybenzoate carboxy-lyase (UbiD) encoded by the *ubiD* gene (lp_2945), were increased under tannic acid exposure (Table 1, Fig. 1). UbiD is involved in the biosynthesis of ubiquinones, a lipid-soluble compound that serves as electron carrier.

(iii) RecA: The RecA protein encoded by the *recA* gene (lp_2301) was strongly induced by tannic acid stress (Table 1, Fig. 1). This protein is known to provide protection against oxidative damage, in particular against the DNA damage caused by reactive oxygen species (ROS) species [25]. RecA also cross-protects against other environmental stresses such as the heat stress [26].

3.1.4 Miscellaneous

(iv) lp_3045, lp_0244: These two genes encode respectively a putative short-chain dehydrogenase/oxidoreductase and a putative oxidoreductase.

3.1.5 Carbon metabolism

(i) Pgm8: The phosphoglycerate mutase (Pgm), encoded by *pgm8* (lp_0929), catalyzes the conversion of 3-phosphogly-

cerate to 2-phosphoglycerate and was found to be upregulated after tannic acid exposure (Table 1, Fig. 1).

3.1.6 General stress proteins

(i) Asp1: Increased amounts of the alkaline shock protein (Asp1) encoded by lp_0929 were observed under tannic acid stress (Table 1, Fig. 1). This is a general stress protein that is expressed in response to a variety of environmental stress conditions.

3.1.7 Translational and transcriptional factors

(i) PyrG: The amounts of CTP synthase, encoded by the *pyrG* gene (lp_0481), were clearly diminished under tannic acid (Table 1, Fig. 1). This protein catalyzes the conversion of UTP into CTP and all four ribonucleotides are involved in the CTP synthase reaction: UTP and CTP are substrate and product in the reaction, respectively, while ATP and GTP act as the energy source and allosteric activator, respectively. CTP synthase plays a key role in pyrimidine metabolism and its regulation is independent of PyrR, which regulates the pyrimidine biosynthetic genes.

(ii) GltX: Glutamyl-tRNA synthetase (GluRS), encoded by the *gltX* gene (lp_0609), was strongly downregulated under tannic acid stress (Table 1, Fig. 1). This protein forms Glu-tRNA by the esterification of glutamate to the 3′ end of tRNA. The *gltX* annotated in the *L. plantarum* WCFS1 genome is a non-discriminating synthetase, which recognizes both $tRNA^{Gln}$ and $tRNA^{Glu}$. In addition to their crucial role in aminoacyl-tRNA formation during protein synthesis, aminoacyl-tRNA synthetases have acquired additional functions including regulation of gene expression, RNA splicing, or cytokine activity [27].

(iii) TyrS: Exposure of *L. plantarum* WCFS1 to tannic acid diminished the amounts of tyrosine-tRNA synthetase (TyrS), encoded by lp_2807, which catalyzes the formation of Tyr-tRNA by the esterification of tyrosine to the 3′ end of tRNA (Table 1, Fig. 1).

(iv) Ribosomal protein S30EA: S30EA protein, encoded by lp_0737, was downregulated upon tannic acid stress (Table 1, Fig. 1). Ribosomal proteins, besides their main function in protein synthesis, may also sense environmental changes.

(v) GreA: GreA (LP_1563) is the only differentially expressed protein involved in translational and transcriptional processes that is upregulated under tannic acid stress (Table 1, Fig. 1). This protein allows RNA polymerase to overcome obstacles encountered during elongation and to increase transcription fidelity.

3.1.8 Hypothetical proteins LP_0052, LP_3387 and LP_2877

LP_0052 is a hypothetical protein that was downregulated upon tannic acid stress (Table 1). A BLASTP analysis shows that excluding hits to *L. plantarum*, the three best BLASTP hits of this protein were found with the 6-pyruvoyl-tetrahydropterin synthase of other lactic acid bacteria (LAB) (identity $\geq$42%). LP_3397 and LP_2877 were upregulated in the presence of tannic acid (Table 1, Fig. 1). LP_2877 displays good homology with nucleotide-binding proteins of the universal stress protein family (UspA) present in other LAB (three best BLASTP hits with identity $\geq$58%). LP_3397 is homologous to nucleoside deoxyribosyltransferases (NDT) from other LAB (three best BLASTP hits with identity $\geq$51%).

4 Discussion

We have observed a different proteomic response of tannic acid-misfit cells submitted to tannic acid challenge when compared with the previously reported response of tannic acid-adapted cells placed under tannic acid stress [15]. The differences observed in both studies are probably the consequence of the different experimental strategies employed for the pursued goals. Growth of *L. plantarum* on tannic acid requires an extended lag phase for adaptation [15, 20]. Since the proteome of *L. plantarum* WCFS1 changes during growth [16] it could be that the proteins involved in adaptation to tannic acid are differently or not expressed after the extended lag phase. Then, to detect the proteins involved in the adaptation to tannic acid the cells were grown under standard conditions before being challenged with tannic acid. This strategy allowed unravelling the response of *L. plantarum* to this dietary compound, shedding light on the mechanisms that bacteria use to overcome the inhibitory effects of tannins as well as on the targets for tannic acid antimicrobial action.

4.1 Tannic acid affects the expression of proteins involved in the cell wall/membrane synthesis

The increased synthesis of three *L. plantarum* proteins involved in the biogenesis of cell wall precursors suggests that tannic acid affects the integrity of the cell envelope, which has been reported for *L. plantarum* [28] and other bacteria [29]. Two of these proteins, LdhD and DapF, play a key role in the peptidoglycan biosynthesis.

Previous construction and analysis of mutants affecting D-lactate production demonstrated that LhdD is essential for cell wall biosynthesis in *L. plantarum* [30]. Strikingly, the incorporation of a D-lactate residue into the pentapeptide is the basis of the natural resistance of *L. plantarum* to high concentrations of vancomycin [31]. Similarly, the construction and analysis of a *dapF* mutant of *Escherichia coli* has shown that this enzyme is essential for peptidoglycan integrity, maintenance of cell shape and cell viability [32]. These reports on LdhD or DapF mutants highlight the crucial role of these enzymes for peptidoglycan integrity and we can hypothesize that the upregulation of LdhD and

DapF is necessary to alleviate the damage caused by tannic acid in *L. plantarum* and maintain the integrity of the cell envelope.

TagE6, another protein induced by tannic acid, is also putatively involved in the biosynthesis of cell wall precursors. The glycosylation of the main chain of teichoic or lipoteichoic acids contributes greatly to the functionality of the cell wall polymer [33]. Most Gram-positive bacteria possess at least two types of cell wall glycopolymers, one attached to peptidoglycan and the other to lipids [34]. One of the suggested roles ascribed to glycopolymers that decorate the teichoic or lipoteichoic acids is to impede the passage of host defense molecules such as bacteriocins, antibiotics or surfactants by clogging the pores and cavities between the peptidoglycan threads or modifying the physicochemical properties of the cell wall [34]. From the six *tagE* paralogs present in the genome of *L. plantarum* WCFS1, *tagE1- tagE2 - tagE3* were suggested to be involved in the glycosylation of a surface-associated protein encoded by the adjacent *sdr* gene to give rise to mucin-like structures [35, 36]. Besides, protein glycosylation TagE has been reported as a glycosyltransferase involved in glycosylation of poly(glycerol phosphate). Therefore, we suggest that TagE6 could be involved in the biosynthesis of a cell wall glycopolymer that decorates teichoic or lipoteichoic acids to protect against the impact caused by tannic acid on the cell envelope.

The increased expression of LdhD, DapF and TagE6, which are involved in the biogenesis of precursors for cell wall synthesis, suggests that *L. plantarum* drives part of its metabolic capacity to repair the damage caused by tannic acid on the cell wall and maintain its integrity. However, Cfa2, a membrane protein involved in the cyclopropanation of membrane lipids, which protect bacteria from adverse conditions such as acidity [37, 38], freeze-drying [39] or dessication [40], was downregulated. We also observed a downregulation of MetE and LuxS by tannic acid, which are part of the AMC cycle. The coincidental decrease under tannic acid stress of LuxS and MetE, which are involved in SAM recycling, and Cfa2 which uses SAM as methyl donor, leads us to suggest that cyclopropanation of membrane phospholipids by Cfa2 is an important target for the antimicrobial mechanism of tannic acid and probably other polyphenolic compounds. In agreement with this hypothesis, quantitative real-time PCR experiments have recently shown that cranberry concentrates containing anthocyanin and non-anthocyanin polyphenolic compounds markedly downregulated the *cfa* gene from *E. coli* 0157:07 to exert its antimicrobial effects [41].

Beside its role in the AMC cycle, LuxS is also involved in stress resistance by setting up the specific behavior of the bacterial population via biofilm formation [42–46]. Biofilms shelter bacteria from penetration by antibiotics [47] and tannic acid possesses well-documented antimicrobial properties [48, 49] so it could be that downregulation of LuxS by tannic acid deactivates a possible response at the population scale.

4.2 Response to the oxidative stress generated by tannic acid

Growing evidence shows that regardless of the diversity of their action, antimicrobials share a common mechanism to kill bacteria: through overproduction of ROS [50, 51]. Autooxidation of tannins generates H_2O_2 and hence promotes oxidative stress [52]. The response of *L. plantarum* to tannic acid supports this hypothesis as the overproduction of proteins known to be involved in the oxidative stress response was observed. We observed the upregulation of Npr2 in response to tannic acid. This result is consistent with the need to detoxify the H_2O_2 arising from the autooxidation of this phenolic compound. It is also in agreement with the previously reported resistance to condensed tannins of an *E. coli* mutant that displayed a tenfold increased level of hydroperoxidase [52]. The overproduction of Npr2 in *L. plantarum* would be part of the first defense against oxygen damage, which consists of the enzymatic inactivation of toxic oxygen radicals.

Our results show that, in addition to Npr2, other tannic acid-induced proteins constitute a second line of defense to repair the damage of oxygen radicals on DNA or proteins. This second line of defense involves RecA, which was strongly overexpressed under tannic acid stress (8.3-fold induction) (Table 1, Fig. 1). This protein repairs DNA and is important by both its recombination properties and capacity to induce a set of DNA repair genes known as SOS response. This was corroborated by the concomitant induction of the general stress protein UspA. Indeed, UspA is among the known set of proteins induced by RecA and its overproduction lead to a continuous growth-arrest state [53].

We observed that the expression of a protein implicated in the biosynthesis of ubiquinones, UbiD, was augmented under tannic acid exposure. Ubiquinones, beside their role as electron carriers in the respiratory chain activity, have been shown to be effective in limiting the accumulation of H_2O_2 and protect lipids against oxidative stress [54]. However, the increased production of UbiD by *L. plantarum* in response to tannic acid stress is unlikely related to ubiquinone production since (i) this bacterium does not bear a complete gene set for ubiquinone biosynthesis (Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/)), (ii) this species does not produce mena or ubiquinones and it needs the exogenous addition of at least menaquinones for heme-assisted respiration [55] and (iii) plants, the natural habitat of *L. plantarum*, can provide ubiquinones. In *E. coli*, *ubiD* expression responds to redox signals and is increased in response to the reducing agent DTT [56]. In our conditions, the upregulation of UbiD probably responds to tannic acid-induced redox imbalance. Curiously in *Lactococcus lactis*, MenD and MenB, two among the whole set of proteins involved in the formation of menaquinones, were also claimed to play a role in protection against oxidative stress since they were upregulated in a thioredoxin reductase

mutant displaying a diminished response to oxidative stress [57]. However, the possibility that UbiD from *L. plantarum* is under redox control requires further investigation. Our findings show that other two oxidoreductases (LP_3045) (LP_0244) were overproduced on tannic acid. Although we do not rule out the possibility that these two proteins are involved in metabolic functions, their overproduction in addition to UbiD seems to further suggest that a redox imbalance is induced by tannic acid. Curiously, the induction of four genes encoding for four oxidoreductases was also observed in cells of *L. plantarum* WCFS1 grown in the presence of bile salts which, like the tannic acid, also damage the membrane [14].

As mentioned above, the hypothetical protein LP_0052 was downregulated under tannic acid stress and displayed identity to the 6-pyruvoyl-tetrahydropterin synthase (PTPS) of various LAB. 6-Pyruvoyl-tetrahydropterin synthase is one of the three enzymes necessary for de novo biosynthesis of tetrahydrobiopterin (BH_4), which is an essential cofactor for nitric oxide synthase (NOS), an enzyme that protects bacteria against oxidative stress. Nitric oxide (NO), the product of NOS, has been recently shown to protect bacteria against the oxidative stress imposed by a broad spectrum of antibiotics [58]. Thus, we suggest that tannic acid would diminish NO production, via LP_0052 downregulation, to deactivate a first defense against oxidative damage. Nevertheless, tetrahydrobiopterin production, the presence of NOS or a NOS-like protein and NO production remain to be elucidated in *L. plantarum*.

4.3 Effects on translational and transcriptional factors

Another group of proteins significantly affected by tannic acid corresponds to translational and transcriptional factors. CTP synthase (pyrG) was shown to have full control of the CTP concentration and is absolutely required for the biosynthesis of ribo and deoxiribonucleotides [59]. Its function in cellular metabolism is therefore crucial as RNA requires ribonucleotides and DNA replication requires deoxyribonucleotides. In the absence of cytidine, CTP synthase is essential for growth. The downregulation of CTP-synthase under tannic acid could indicate good accessibility to nucleotides and hence made unnecessary the pyrimidine rescue. However, we observed the overexpression of LP_3397, a protein highly homologous to nucleoside deoxyribosyltransferase of other LAB, which play a key role in nucleotide salvage. At first, the concomitant downregulation of PyrG and the overexpression of LP_3397 may appear contradictory since both enzymatic functions aim at nucleotide rescue. However, it has been recently reported that PyrG posses not only enzymatic but also structural properties as it forms cytoskeletal filaments [60]. These filaments have been proposed to play a role in maintaining cell shape and probably in secondary yet unknown structural properties. We hypothesize that PyrG could be a target for tannic acid due to its structural properties while the need for nucleotide rescue necessary for DNA repair imposed by tannic acid stress could be fulfilled by LP_3397. The increased amounts of GreA would increase transcription fidelity to alleviate the stress incurred by tannic acid.

We observed diminished amounts of GluRS (17-fold repression; Table 1, Fig. 1) and TyrS in the presence of tannic acid. Recently, the activity of a GluRS from *Acidithiobacillus ferroxidans* that was oxidatively damaged by heme could be rescued by the general antioxidant NAPDH. This led to hypothesize that the damage exerted by heme was caused by a redox process [61]. In addition to GluRS, only TyrS, among five other aminoacyl-tRNA synthetases, was significantly damaged by oxidative stress [61]. These results support the hypothesis that certain aminoacyl-tRNA transferases are sensitive to cellular oxidative stress. Accordingly, the downregulation of GluRS and TyrS observed here could be the consequence of oxidative/redox inhibition imposed by tannic acid. The diminished amounts of the ribosomal S30EA protein suggests a protein synthesis modulation in response to tannic acid stress, which is not surprising as the adjustment of protein synthesis is a common mechanism of bacterial adaptation to stress conditions. In particular, the amounts of the S30EA protein have been also shown to diminish in *Lactobacillus sakei* after adaptation to the GI tract of axenic mice [62] or in *Bifidobacterium animalis* in the presence of bile [63].

4.4 Carbon metabolism and energy saving

The synthesis of one of the different Pgm paralogs of *L. plantarum* WCFS1 was increased under tannic acid stress. Variations in the expression of this protein have been also observed in other LAB submitted to several types of stress conditions such as heme-dependent respiration [64], exposure to glutathione [65] or hop [66], suggesting that this protein plays a role in adjusting metabolism to cope with stress. The increased amounts of Pgm, which is an efficient catalyst, could result in the accumulation of 2-phosphoglycerate, which was previously claimed to serve as an endogenous energy source in starved cells of *L. lactis* [67]. Thus, the expression patterns of Pgm8, UspA, GluRS, TyrS and S30A upon tannic acid suggest that metabolic adaptations into an energy-saving mode occur; since growth-arrest state promoted by overexpression of UspA is characteristic of stress conditions that account for energy economy, 2-phosphoglycerate (product of Pgm) is considered as an endogenous energy source and diminished expression of GluRS, TyrS and S30A would prevent luxurious protein de novo synthesis.

4.5 Stress response

L. plantarum WCFS1 overproduced two general stress proteins under tannic acid stress, Asp1 and UspA. Regard-

ing Asp1, the physiological role of alkaline shock proteins is not yet clear but it is known to be involved not only in tolerance to alkaline pH but also in other non-specific stress conditions such as that imposed by oxidative stress. UspA is a general stress protein that cooperates in the defense against DNA damage and the transcription of the *uspA* gene is activated by RecA [68]. UspA is synthesized in response to growth inhibition caused by starvation for carbon, nitrogen, sulfate or phosphate, by osmotic shock, high pH, heat or by heavy metals, oxidants, acids and antibiotics [69]. As mentioned above, the overproduction of UspA leads to a continuous growth-arrest state.

In summary, the results show that tannic acid targeted proteins involved in key processes for bacterial stress resistance including the cyclopropanation of membrane lipids, organization at the population scale and maintenance of cell structure. The results also suggest that tannic acid targets nitric oxide production, which could highlight the role of nitric oxide as part of defense against oxidative damage. Among the mechanisms used by the *L. plantarum* human isolate to overcome the toxic effects of tannic acid stand out those involved in first and second line of defense against oxidative damage caused by ROS, which support previous hypothesis involving the overproduction of destructive ROS as a common mechanism of bactericidal antimicrobials to kill bacteria [50]. According to this hypothesis [50] the observed response of *L. plantarum* WCFS1 to tannic acid would be expected as the hydroxyl radicals generated following the application of antimicrobials (in this case tannic acid) are extremely toxic and will readily damage proteins, membrane lipids and DNA. The related bacterium *Lactobacillus hilgardii* displayed a different proteomic response to tannic acid, which was characterized by a diminished expression of specific metabolic enzymes [70]. It was hypothesized that in certain environments hydrogen peroxide arising from tannin auto-oxidation would provide the oxidative conditions to favor the formation of covalent bonds between tannic acid and these specific metabolic proteins. According to the response observed in *L. plantarum* WCFS1, it seems that tannic acid acted as an antimicrobial rather than as a compound whose auto-oxidation favor the complexation of tannic acid with specific metabolic proteins. These apparent discrepancies could reflect the different responses to tannic acid by distinct microorganisms or the different conditions applied in both studies. Additional and complementary experimental approaches (e.g. global transcriptomic assays) could provide key information on the response to tannic acid in these bacteria. Strategies for maintaining the integrity of the cell wall and for energy saving are also among the tools used by *L. plantarum* to overcome the inhibitory effects of dietary tannins. It should be noted that among the proteins involved in the resistance and adaptation to tannic acid, there could be proteins constitutively expressed that escape the tannic acid effect. It seems to be the case of the tannase protein (encoded by the lp_2956 gene), which was not induced in this study. The lack of induction of tannase protein is consistent with previous proteomic analysis performed in other *L. plantarum* strains [15] and the related bacterium *L. hilgardii* [70] when grown on tannic acid.

Supported by the findings of this study, the selection of tannin-resistant microorganisms may now be facilitated. The ability of these microorganisms to overcome the toxic and antinutritional effects of tannins is essential not only for improving animal production on tannin-containing feeds but also for inoculation among animals with the goal of improving the digestibility of tannin-rich diets. Regarding human nutrition, it has been hypothesized that some of the health benefits of tannins in humans are due to their effect on the microbial population of the GI tract [2]. The presence of tannin-resistant microorganisms in the human GI tract may protect themselves and also the sensitive microbial population, such as the cellulolytic bacteria, from the antimicrobial effect of tannins. The protective effect of tannin-resistant bacteria would also prevent sudden population shifts that could lead to the establishment of pathogenic bacteria or a reduction of nutrient digestibility.

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