Food Research International 157 (2022) 111464

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Proteomics reveal the protective effects of chlorogenic acid on *Enterococcus faecium* Q233 in a simulated pro-oxidant colonic environment

P. Padilla [a,b], M. Estévez [a,*], M.J. Andrade [b], F.J. Peña [c], J. Delgado [b]

[a] Food Technology and Quality (TECAL), Institute of Meat and Meat Products (IPROCAR), University of Extremadura, Cáceres, Spain
[b] Food Hygiene and Safety (HISEALI), Institute of Meat and Meat Products (IPROCAR), University of Extremadura, Cáceres, Spain
[c] Spermatology Laboratory, University of Extremadura, Cáceres, Spain

ARTICLE INFO

Keywords:
E. faecium
Chlorogenic acid
Oxidative stress
Proteomics
Flow cytometry

ABSTRACT

Certain phytochemicals have been found to promote the beneficial effects of probiotic bacteria although the molecular mechanisms of such interactions are poorly understood. The objective of the present study was to evaluate the impact of the exposure to 0.5 mM chlorogenic acid (CA) on the redox status and proteome of *Enterococcus faecium* isolated from cheese and challenged with 2.5 mM hydrogen peroxide (H_2O_2). The bacterium was incubated in anaerobic conditions for 48 h at 37 °C. CA exposure led to a more intense oxidative stress and accretion of bacterial protein carbonyls than those induced by H_2O_2. The oxidative damage to bacterial proteins was even more severe in the bacterium treated with both CA and H_2O_2, yet, such combination led to a strengthening of the antioxidant defenses, namely, a catalase-like activity. The proteomic study indicated that H_2O_2 caused a decrease in energy supply and the bacterium responded by reinforcing the membrane and wall structures and counteracting the redox and pH imbalance. CA stimulated the accretion of proteins related to translation and transcription regulators, and hydrolases. This phytochemical was able to counteract certain proteomic changes induced by H_2O_2 (i.e. increase of ATP binding cassete (ABC) transporter complex) and cause the increase of Rex, a redox-sensitive protein implicated in controlling metabolism and responses to oxidative stress. Although this protection should be confirmed under *in vivo* conditions, such effects point to benefits in animals or humans affected by disorders in which oxidative stress plays a major role.

1. Introduction

Oxidative stress is involved in a variety of pathological conditions including cardiometabolic and neurological disorders, diabetes, and chronic inflammatory diseases, among others (Dos Santos et al., 2019). Scientific evidence supports the potential benefits of dietary interventions in alleviating the oxidative stress occurred in the lumen and tissues of the gastrointestinal tract (GIT) (Ballini et al., 2019; Sugihara et al., 2019). Among these antioxidant strategies, the intake and colonization of probiotic bacteria (Ballini et al., 2019) and certain dietary phytochemicals (Mastrogiovanni et al., 2019) have been identified as effective in protecting against some of the above mentioned intestinal health disorders.

The beneficial impact of probiotic bacteria from *Lactobacillus* and *Bifidobacterium* genera on gut health is profusely documented (Jakubczyk et al., 2020). Other bacteria such as certain species and strains from *Enterococcus* have been much less studied for their potential health benefits. Strains of *Enterococcus faecium*, isolated from fermented food products such as cheeses, have been recently characterized for their biosafety and probiotic potential (Oruc et al., 2021). Several works have recently demonstrated *in vivo* beneficial effects of *E. faecium* when administrated alone or in combination with other probiotic bacteria to mice (Choeisoongnern et al., 2021; Divyashri et al., 2015; Huang et al., 2021). In these studies, the authors emphasized the necessity of pinpointing the molecular mechanisms behind these probiotic effects.

Phytochemicals with assorted bioactivities such as antioxidant, antimicrobial, anti-inflammatory, antidiabetic and antiproliferative, among others, have also attracted considerable attention for their potential health benefits (Shahidi and Ambigaipalan, 2015). Chlorogenic acid (CA), a bioactive component of assorted plant materials and foods such as coffee and apples, has been studied for its antioxidant, anti-inflammatory (Farah & Lima, 2019), antihypertensive (Loader et al., 2017) and antiglycemic effects (Wang et al., 2008). CA may contribute to protecting against cardiovascular, metabolic, cancerogenic and

* Corresponding author at: Institute of Meat and Meat Products (IPROCAR), University of Extremadura, Avd. Universidad, sn. 10003, Cáceres, Spain.
E-mail address: mariovet@unex.es (M. Estévez).

https://doi.org/10.1016/j.foodres.2022.111464
Received 28 March 2022; Received in revised form 29 May 2022; Accepted 1 June 2022
Available online 6 June 2022

neurological disorders (Tajik et al., 2017). Interestingly, CA has been described to act as prebiotic and hence, enable the occurrence of functional properties exerted by probiotic bacteria. In the study carried out by Palócz et al. (2016), the combination of CA with *Lactobacillus plantarum* 2142 alleviated intestinal inflammation and oxidative stress in IPEC-J2 cells. Certain species of bifidobacteria and other gut microbiota are known to biotransform CA into bioactive compounds that are speculated to exert some of the aforementioned health benefits (Raimondi et al., 2015; Tomás-Barberán et al., 2014). Yet, the underlying molecular mechanisms of the interactions between CA and probiotic bacteria are not well understood.

The present study aims to decipher the molecular basis of the interaction between CA and *E. faecium* isolated from matured cheese, in a simulated anaerobic and pro-oxidant colonic environment. To achieve this objective, the bacteria were exposed to dietary concentrations of CA (0.5 mM) for 48 h and subsequently analyzed by means of high-resolution mass spectrometry-based proteomics using a Nanoliquid Chromatography-Orbitrap MS/MS. Supportive flow cytometry studies and protein oxidation measurements were applied to assess the impact of the pro-oxidant environment and CA on the bacteria.

2. Material and methods

2.1. 1. Chemicals and raw material

All chemicals and reagents used in this study were of ACS analytical grade and purchased from Sigma Chemicals (Sigma-Aldrich, USA), Scharlab S.L. (Spain), Pronadisa (Conda Laboratory, Spain), Applied Biosystems (USA), Epicentre (USA) and Acros Organics (Spain). *E. faecium* Q233 was isolated from matured soft cheese and identified by 16S rRNA gene sequencing (Ordiales et al., 2013). This identification was further confirmed by the proteomic results.

2.2. Experimental settings

Stock cultures of *E. faecium* were stored at −80 °C in Man, Rogosa and Sharpe (MRS) broth / glycerol to a final concentration of 20% (v/v). Before experimental use, *E. faecium* was subcultured twice under anaerobic conditions at 37 °C for 24 h in MRS broth supplemented with 0.5% L-Cysteine (m/v). Four experimental groups were considered depending on the chemical challenges applied to the bacteria: CONTROL (*E. faecium* in MRS), H_2O_2 (*E. faecium* in MRS + 2.5 mM H_2O_2), CA (*E. faecium* in MRS + 0.5 mM CA) and H_2O_2 + CA (*E. faecium* in MRS + 2.5 mM H_2O_2 + 0.5 mM CA). Before its incorporation in the corresponding cultures, CA was dissolved in dimethyl sulfoxide (DMSO) to facilitate solubility. The volume of DMSO used to dissolve and deliver the CA to treated cultures (CA and H_2O_2 + CA) was also added to the other groups (CONTROL and H_2O_2). Three replicates were carried out for each treatment. Experimental groups were incubated at 37 °C for up to 48 h in anaerobic conditions. Samples of the cultures were collected in four times (0, 12, 24 and 48 h) from the inoculation. For further analyses, culture medium was removed by washing with a phosphate buffered saline solution (PBS, pH 7.4) twice. For counting of viable cells, 100 μL of *E. faecium* were inoculated on MRS agar at the same sampling time and conditions as already reported for the experimental groups.

2.3. Analytical procedures

2.3.1. Reactive oxygen species (ROS) generation by flow-cytometry analyses

Flow cytometry detection of ROS in *E. faecium* was carried out according to the previous described protocol (Padilla et al., 2021). Briefly, *E. faecium* ($1x10^6$ cfu/mL) sampled at 48 h was suspended in 1 mL of PBS and stained with Hoechst 33,342 (0.5 μM) (Sigma, Steinheim, Germany) to discriminate bacterium from debris and CellRox Deep Red (5 μM) (ThermoFisher, CA, USA) for the detection of intracellular ROS. Excitation and emission wavelengths were set at 345 nm and 488 nm, respectively, for the Hoechst 33342, and at 644 and 645 nm, respectively, for the CellRox Deep Red. Prior to analyses, the samples were filtered through MACS® SmartStrainer 30 μm filters and immediately run on a Cytoflex® flow cytometer (Beckman Coulter, CA, USA) equipped with violet, blue and red lasers. The instrument was daily calibrated using specific calibration beads provided by the manufacturer. A compensation overlap was performed before each experiment. However due to emission and excitation characteristics of the combination of probes used, spectral overlap was negligible. Files were exported as FCS files and analyzed using FlowjoV 10.5.3 Software for Mac OS (Ashland, OR, USA). Unstained, single-stained and Fluorescence Minus One (FMO) controls were used to determine compensations and positive and negative events, as well as to set regions of interest.

2.3.2. Quantification of hydrogen peroxide (H_2O_2)

H_2O_2 was quantified in *E. faecium* by using the procedure described by Jiang et al. (1990) with some modifications. Briefly, *E. faecium* ($1x10^6$ cfu/mL) sampled at 0, 12, 24 and 48 h were diluted in hexane/isopropanol (3:1, v/v). The mixture was vigorously vortexed and subsequently dispensed in quartz cuvettes to measure absorbance at 240 nm in an 1800 Shimadzu spectrophotometer (SHIMADZU EUROPA GmbH, Duisburg, Germany). Standard curves were prepared with standard H_2O_2 for quantification purposes using the extinction coefficient of 43.6 $M^{-1} * cm^{-1}$ at 240 nm. Data is expressed as pmol of H_2O_2/L.

2.3.3. Assessment of catalase-like activity

The ability of *E. faecium* to decompose H_2O_2 was assessed by the procedure reported by Li and Schellhorn (2007) with some modifications. Briefly, *E. faecium* ($1x10^6$ cfu/mL) sampled at 48 h was exposed to a 20 mM H_2O_2 solution and allowed to stand at room temperature (22 °C) for 180 s. Each 30 s, the depletion of H_2O_2 was assessed by measuring absorbance at 240 nm in an 1800 Shimadzu spectrophotometer (SHIMADZU EUROPA GmbH, Duisburg, Germany). Standard curves were prepared with standard H_2O_2 for quantification purposes using the extinction coefficient of 43.6 $M^{-1} * cm^{-1}$. Data was expressed as pmol of depleted H_2O_2/min*mL.

2.3.4. Quantification of bacterial protein carbonyls

Total protein carbonyls were determined in *E. faecium* sampled at 48 h by means of the dinitrophenylhydrazine (DNPH) method as described by Estévez et al. (2019) with minor modifications. Briefly, proteins from *E. faecium* ($1x10^6$ cfu/mL) were precipitated by the addition of 1 mL of cold 10% trichloroacetic acid (TCA), followed by centrifugation at 4 °C at 600 g for 5 min. The supernatants were discarded and protein pellets were treated with 1 mL of a 2 M HCl solution with 0.2% DNPH and incubated at room temperature for 1 h. Proteins were subsequently precipitated with 1 mL of cold 10% TCA, followed by centrifugation at 4 °C, 1200 g for 10 min and washed twice with 1 mL of ethanol:ethyl acetate (1: 1 v/v). The pellets were dissolved in 1.5 mL of 20 mM Na_3PO_4 buffer pH 6.5 added with guanidine hydrochloride to reach 6 M. The amount of carbonyls was expressed in nmoles of protein hydrazones per mg of protein using a molar extinction coefficient of hydrazones of 21.0 nM^{-1} cm^{-1} at 370 nm.

2.3.5. Quantification of bacterial TBARS

Malondialdehyde (MDA) and other TBARS were quantified in each *E. faecium* culture, sampled at 0, 12, 24 and 48 h by adding 200 μL of the bacterial suspension to 500 μL thiobarbituric acid (0.02 M) and 500 μL trichloroacetic acid (10%). Samples were incubated during 20 min at 90 °C. After cooling, a 5 min centrifugation at 600 g was made and the supernatant was measured at 532 nm. Results are expressed as mg TBARS per L of sample.

2.3.6. Sample preparation for LC-MS/MS based proteomics

Sample preparation for proteomics was carried out with 200 mL of

culture of each experimental group sampled at 48 h with three biological replicates per treatment. Cells were collected by centrifugation at 4 °C, 600 g for 10 min, supernatant was discarded, and the pellet was resuspended in 4 mL lysis buffer pH 7,5 (100 mM Tris-HCl, 50 mM NaCl, 10% glycerol, 20 mM EDTA pH 8,5), 1 mM PMSF (Phenylmethansulfonylfluorid) and 1 μg/mL Pepstatin A. The collected cells were subjected to rupture by pressure difference using a French press with approximately 68 atm. The resulting lysate was placed at 4 °C for 1 h and then centrifuged at 4 °C, 1200 g, for 15 min to remove cell debris and residual intact cells. The clarified supernatants containing the majority of proteome of *E. faecium* were precipitated with trichloroacetic acid/acetone (Carpentier et al., 2005).

The protein precipitated lysates were resuspended in 8 M urea. Protein concentration was measured with a Coomasie Protein Assay Reagent Ready to Use employing a Nanodrop 2000c Spectophotometer and a Nanodrop 2000 software (Thermo Scientific, Waltham, MA, USA) to ensure homogeneous protein quantities in the different samples. Five aliquots per treatment, three biological replicates plus two technical replicates, containing 50 μg of proteins were treated as previously described by Delgado, Acosta, et al., 2015; Delgado, Owens, Doyle, Asensio, & Nuñez, 2015 and Owens et al. (2015). Briefly, samples were incubated with 0.5 M DTT (Dithiothreitol) in 50 mM ammonium bicarbonate for 20 min at 56 °C for protein reduction. The resulting free thiol (-SH) groups were alkylated by incubating the samples with 0.55 M iodoacetamide in 50 mM ammonium bicarbonate for 15 min at room temperature in the dark. Sequencing-grade trypsin (Promega, Madison, WI, USA) and ProteaseMAX surfactant (Promega, Madison, WI, USA) were added and incubated at 50 °C for 1 h. Finally, 1 μL of 100% formic acid was added to stop the proteolysis reaction. Supernatant was removed and placed into new screw-capped Eppendorf tubes for drying in a vacuum concentrator. Digested samples were then desalted, prior to spectrometric analysis, using Pierce™ Peptide Desalting Spin Columns (Thermo Scientific, Waltham, MA, USA).

Before analyzing the samples on the Orbitrap LC-MS/MS, digested samples were resuspended in loading buffer (98% milli-Q water, 2% acetonitrile, 0.05% trifluoroacetic acid), and sonicated in a water bath for 5 min and centrifuged at 1200 g for 15 min at room temperature and put them into vials for LC-MS/MS Orbitrap.

2.3.7. Label-free quantitative (LFQ) proteomic analyses

A Q-Exactive Plus mass spectrometer coupled to a Dionex Ultimate 3000 RSLCnano chromatogragh (Thermo Scientific, Waltham, MA, USA) analyzed around 2 μg from each digest. Data was collected using a Top15 method for MS/MS scans (Delgado et al., 2019). Comparative proteome abundance and data analysis were carried out using MaxQuant software (v. 1.6.0.15.0; https://www.maxquant.org/download_asset/maxquant/latest) and Perseus (v 1.6.14.0) to organize the data and perform statistical analysis. Carbamidomethylation of cysteines was set as a fixed modification; oxidation of methionines and acetylation of N-terminals were set as variable modifications. Database searching was performed against *E. faecium* protein database (https://www.uniprot.org). The maximum peptide/protein false discovery rates (FDR) were set to 1% based on comparison to a reverse database. The LFQ algorithm was used to generate normalized spectral intensities and infer relative protein abundance. Proteins were identified with at least two peptides, and those proteins that matched to a contaminant database or the reverse database were removed, and proteins were only retained in final analysis if they were detected in at least two replicates from at least one treatment. Quantitative analysis was performed using a *t*-test to compare treatments with the CONTROL. The qualitative analysis was also performed to detect proteins that were found in at least three replicates of a given treated group but were undetectable in the comparison CONTROL group. All proteins satisfying one of the two aforementioned criteria are identified as "discriminating proteins".

2.4. Statistical analysis

All experiments were performed five times (3 biological replicates + 2 technical replicates) and each individual sample was analyzed twice for flow cytometry. Data was analyzed for normality and homoscedasticity. The effect of the exposure to H_2O_2 and AC was assessed by Analysis of Variance (ANOVA). The Tukey's test was used for multiple comparisons of the means. The effect of the incubation time on the same measurements was assessed by Student's *t*-test. The significance level was set at $p < 0.05$. SPSS (v. 15.0) was used for statistical analysis of the data.

3. Results & DISCUSSION

3.1. Onset of oxidative stress in E. Faecium exposed to H_2O_2 and CA

After the incubation of *E. faecium* under the tested experimental conditions, the counts for the four experimental groups were similar, reaching the stationary phase in 12 h (Fig. 1). While H_2O_2 did not compromise the survival of *E. faecium* at the applied concentrations, the achievement of the stationary phase in bacteria treated with H_2O_2 (H_2O_2 and H_2O_2 + CA), was retarded (Fig. 1). This is in agreement with previous studies that tested the effect of H_2O_2 on *Lactobacillus reuteri* and other probiotic bacteria and reported a growth delay along with oxidative damage in bacterial proteins and lipids via ROS formation (Arcanjo et al., 2019; Sanders et al., 2004). On the other hand, the presence of CA alleviated the negative effect that H_2O_2 had on the growth of the bacteria (Fig. 1). These results agree with a previous work where the presence of resveratrol acted as a protector of the bacteria against oxidative stress (Arcanjo et al., 2019). Other authors have also encountered a positive effect of selected phenolic compounds on the survival, growth and REDOX status of probiotic bacteria at the expense of pathogenic microorganisms, since phenolic compounds have the ability to inhibit oxidative stress by acting as metal chelators and radicals scavengers (Cardona et al., 2013; Estévez and Heinonen, 2010).

Among the techniques to measure oxidative stress in bacteria, flow cytometry was used to analyze the population of CelRox + bacteria (%) (Fig. 2A) and the total fluorescence emitted by ROS in *E. faecium* (Fig. 2B) at the end of the incubation period (48 h).

This technique showed that the bacterial populations are distributed into several groups depending on the amount of CelRox probe bound to the ROS in these cells: CelRox + bacteria with a significant amount of ROS and CelRox- bacteria that may have an indeterminate amount of ROS, but it is not enough to be detected. The results displayed significant differences between treatments, which prove that the presence of both H_2O_2 and CA, and their combination, affected the generation of free radicals and their concentration in *E. faecium*. As expected, H_2O_2 stimulated the formation of free radicals in bacteria (H_2O_2 *vs* CONTROL). The decomposition of H_2O_2 into the hydroxyl radical through the Fenton reaction with a transition metal is well described in the literature and is represented below:

$$M^n + H_2O_2 \rightarrow M^{n+1} + HO^{\bullet} + HO^-$$

Where M is a transition metal, HO − is the hydroxyl ion and HO • is the hydroxyl radical, the most abundant and dangerous free radical in biological systems (Davies, 2005). Despite a transition metal was not included, the medium has many of them that could have catalyzed the decomposition of H_2O_2. Fluorescence emitted by the probe specifically binds to ROS present in the cells and its intensity is directly related to their amount of ROS. Therefore, the incubation with H_2O_2 not only increased the number of bacteria with significant ROS concentration (Fig. 2A), but the total amount of ROS in these samples was also higher than those of the CONTROL (Fig. 2B).

CA stimulated the formation of ROS in *E. faecium* in such way that the population of CelRox + was significantly higher in CA-treated bacteria

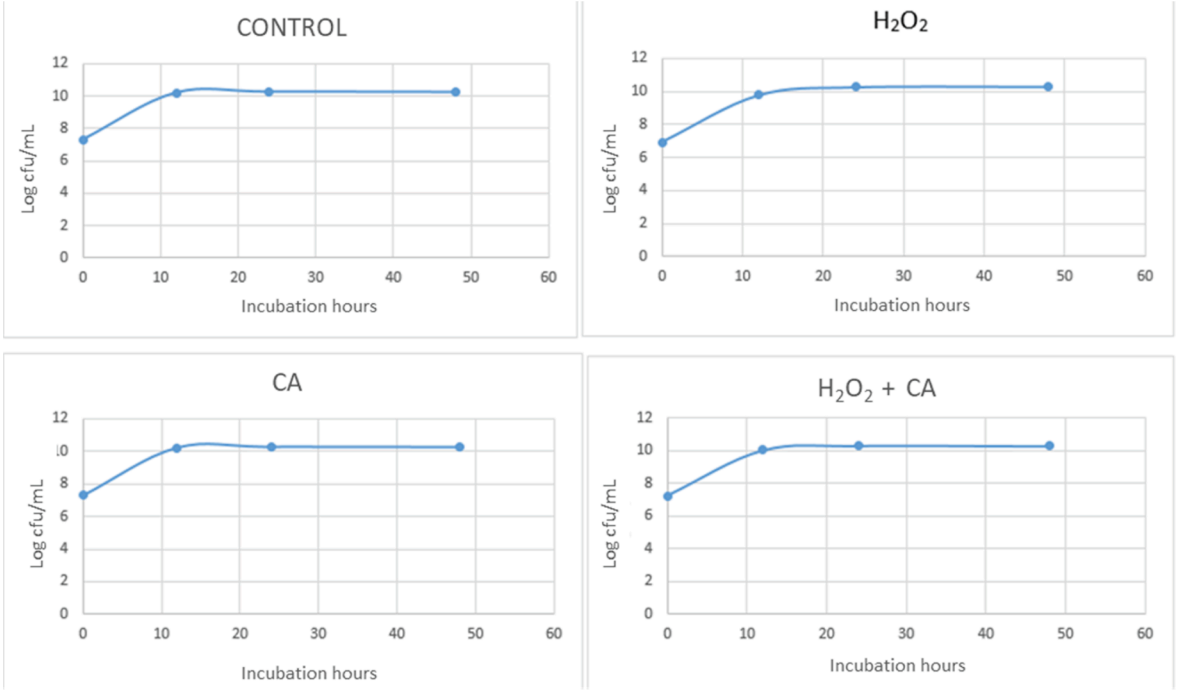

Fig. 1. Evolution of the growth of *Enterococcus faecium* Q233 during its incubation in the presence of H_2O_2, chlorogenic acid (CA) and the combination of both (H_2O_2 + CA) compared to CONTROL group. CONTROL (*E. faecium* in MRS), H_2O_2 (*E. faecium* in MRS + 2.5 mM H_2O_2), CA (*E. faecium* in MRS + 0.5 mM CA) and H_2O_2 + CA (*E. faecium* in MRS + 2.5 mM H_2O_2 + 0.5 mM CA).

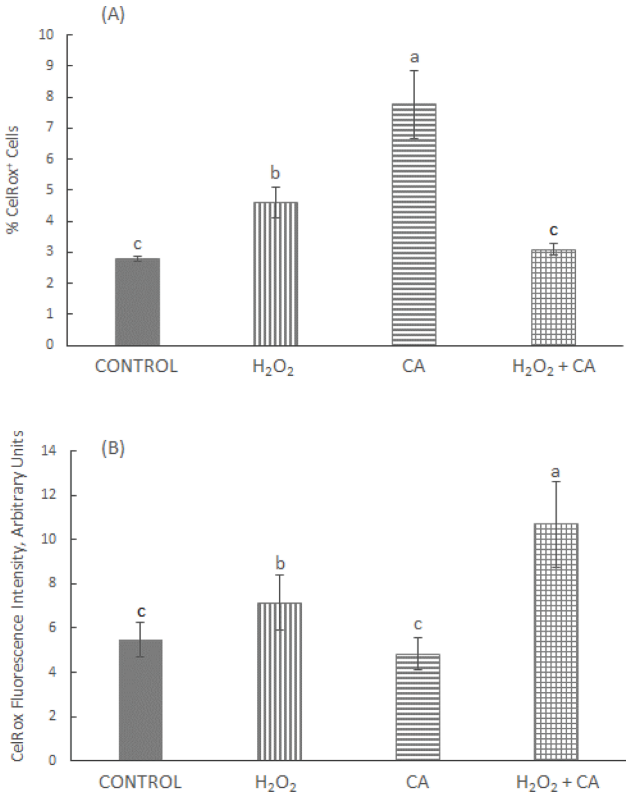

Fig. 2. Subpopulation of *Enterococcus faecium* Q233 (%) phenotyped by Reactive oxygen species (ROS) occurrence (Cell Rox +; A), and total fluorescence emitted by ROS in *E. faecium* (B). Results expressed as means ± standard deviations. Different letters on top of bars denote significant differences ($p < 0.05$) between group of samples. CONTROL (*E. faecium* in MRS), H_2O_2 (*E. faecium* in MRS + 2.5 mM H_2O_2), CA (*E. faecium* in MRS + 0.5 mM CA) and H_2O_2 + CA (*E. faecium* in MRS + 2.5 mM H_2O_2 + 0.5 mM CA).

than in those incubated with the pro-oxidant H_2O_2. These results could be considered contradictory because of the antioxidant effect attributed to this phenolic compound (Sato et al., 2011). Nevertheless, it is also well documented the ability of CA to act as a pro-oxidant and to stimulate the formation of ROS in biological systems (Hou et al., 2017). Specifically, the increase in ROS formation by CA has been observed in human cancer cells (CACO-2) and non-transformed epithelial cells cultured *in vitro* (Yan et al., 2015). Furthermore, a recent study performed with flow cytometry in *Bacillus subtilis* and *Escherichia coli* showed that their incubation with CA induced high levels of ROS, which resulted in oxidative damage to the bacteria (Wang et al., 2020). However, previous research examined the formation of ROS and the survival of *E. coli* incubated with CA, and reported a reduction in ROS content (Lee and Lee, 2018), which is opposite to the results obtained in the present study. Nevertheless, the mechanisms involved in the generation of ROS by CA are still to be thoroughly defined and may be affected by many factors such as the concentrations of CA and H_2O_2, among others (Utrera and Estévez, 2012). CA increased the population of CelRox+ (Fig. 2A) though the total fluorescence levels were lower than those found in cells incubated with H_2O_2 and similar to those of the CONTROL group (Fig. 2B). These results reflect that the amount of ROS in the population of CelRox + bacterium and, in general, in the groups treated with CA, is much lower than those treated with H_2O_2. In other words, CA was able to induce a mild but significant oxidative stress in a higher number of bacteria than H_2O_2. The mechanisms of ROS generation by CA, still unknown, seem to be different from those of H_2O_2 and may respond to more complex cellular mechanisms than the simple decomposition of a reactive molecule, as occurs with H_2O_2.

An opposite result was observed when the bacterium was exposed to the combination of H_2O_2 and CA. The population of CelRox + bacteria treated with both substances was similar to the CONTROL group (Fig. 2A). Nevertheless, total fluorescence levels (Fig. 2B) were significantly higher than the other groups under study. Therefore, cells exposed to H_2O_2 and CA had, on average, the highest levels of radicals, but they were concentrated in a small population of the bacterium (similar to the CONTROL group). Compared to the effect of H_2O_2, alone,

the combination with CA decreases the number of CelRox + bacteria, which proves a protective effect of the phenolic compound against the propagation of oxidative stress.

3.2. Oxidative damage to lipids and proteins from E. Faecium exposed to H_2O_2 and CA

Fig. 3 shows the oxidative damage to lipids (Fig. 3A) and proteins (Fig. 3B) from *E. faecium* after 48 h of incubation. The concentration of MDA was consistent with the generation of free radicals. Incubation with H_2O_2 caused an increase in ROS content that led, in turn, to oxidative damage to cell lipids. The addition of CA, led to a significant reduction in MDA levels irrespective to the presence of H_2O_2. The protective effect of this phenolic compound against lipid oxidation could have been caused via the induction of slight oxidative stress (observed in the flow cytometry analysis), that would, in turn, have caused a reinforcement of the cell antioxidant defences, (as observed in the catalase-like activity analysis, discussed in the following section). Protection against lipid oxidation is essential since lipids of biological membranes constitute (along with the cell wall) the first and most important barrier of the cell and enables its interaction with the environment. The results found in this work are consistent with previous works in which the addition of a phenolic compound increases the oxidative stability of the lipids of probiotic bacteria incubated in a free radical-generating system (Arcanjo et al., 2019).

The results of protein oxidation were different than those obtained from lipid oxidation, which highlights that the oxidation of lipids and proteins respond to different biological mechanisms. Unlike lipid oxidation, protein oxidation has been described as a mechanism of cell regulation and signalling in bacteria and eukaryotic cells (Arcanjo et al., 2019; Ezraty et al., 2017). The addition of CA, alone or in combination with H_2O_2, caused a remarkable increase of protein carbonyls. The pro-oxidant effect of CA on bacterial proteins could respond to the well-known pro-oxidant mechanism of its oxidized quinone form (Utrera and Estévez, 2012). As already proposed by Ezraty et al. (2017) and Arcanjo et al. (2019), protein carbonylation could be a cell signalling mechanism that trigger the strengthening of endogen antioxidant defence systems. This hypothesis would explain the increase in protein carbonylation and the protection against cell lipids. In the present study, such strengthening effect on the antioxidant defences would include, among others, an increased ability of the bacterium to degrade H_2O_2 (catalase-like activity, discussed in the following section). In agreement with the present results, Arcanjo et al. (2019) observed how the stimulation of carbonyl formation in *L. reuteri* by resveratrol occurred along with an upregulation of genes encoding for enzymes with antioxidant activity which, ultimately, protected bacterial lipids against oxidation. These authors also proposed that the protection of resveratrol against oxidative stress in *L. reuteri* would have made via strengthening the antioxidant defences of the cell at the expense of stimulating protein oxidation. This latter oxidative damage may act as a sign of a pro-oxidative threat and the triggering event for the strengthening of the antioxidant defences via gene expression.

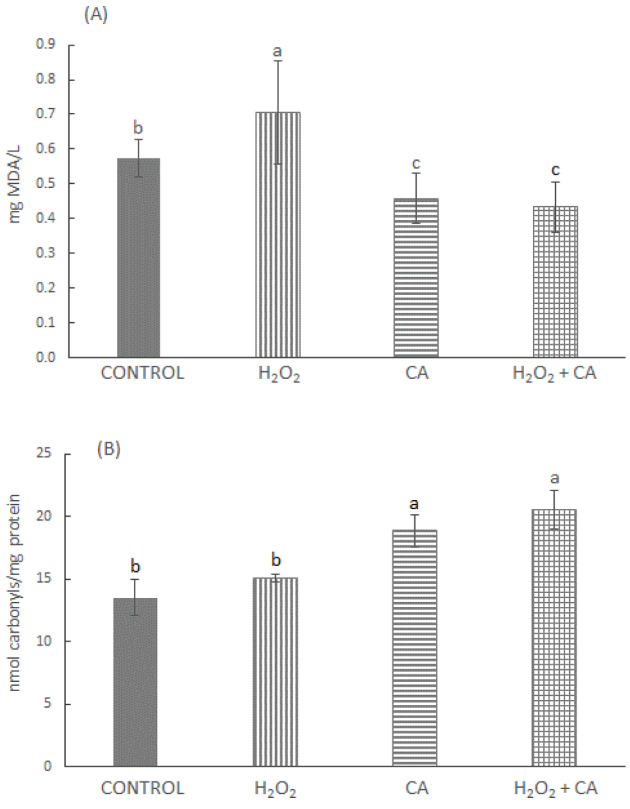

Fig. 3. Oxidative damage to lipids (A) and proteins (B) from *Enterococcus faecium* Q233 as analyzed by the concentration of thiobarbituric reactive substances (TBARS) and concentration of protein carbonyls, respectively. Results expressed as means ± standard deviations. Different letters on top of bars denote significant differences ($p < 0.05$) between group of samples. CONTROL (*E. faecium* in MRS), H_2O_2 (*E. faecium* in MRS + 2.5 mM H_2O_2), CA (*E. faecium* in MRS + 0.5 mM CA) and H_2O_2 + CA (*E. faecium* in MRS + 2.5 mM H_2O_2 + 0.5 mM CA).

3.3. Catalase-like activity in E. Faecium exposed to H_2O_2 and CA

The ability of *E. faecium* to detoxify H_2O_2 after 48 h of incubation is shown in Fig. 4. This probiotic bacterium is Gram positive and catalase negative, with this condition making it highly susceptible to oxidative stress in the presence of H_2O_2. Catalase decomposes two molecules of this reactive species into molecules of water and molecular oxygen according to the following reaction:

$$H_2O_2 \rightarrow 2\ H_2O + O_2$$

However, it is also known that some lactic acid bacteria possess an activity called catalase-like, which usually depends on an NADH-dependent oxidoreductase enzyme (Tanaka et al., 2018). This characteristic could respond not only to an antioxidant protection mechanism and REDOX balance control, but also to a protection mechanism against other bacteria and energy metabolism regulation. In a previous work, the *dhaT* gene, which encodes for an NADH-dependent oxidoreductase enzyme, was observed to be overexpressed in bacteria *L. reuteri* exposed to oxidative stress induced by H_2O_2 (Arcanjo et al., 2019). According to the authors, the enzyme would be able to detoxify H_2O_2. Kang et al.

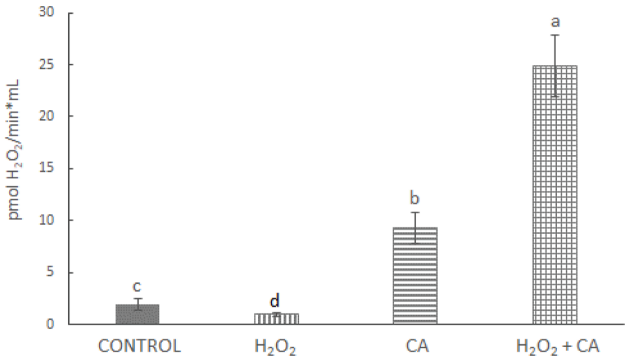

Fig. 4. Catalase-like activity of *Enterococcus faecium* Q233 as analyzed by its ability to decompose H_2O_2. Results expressed as means ± standard deviations. Different letters on top of bars denote significant differences ($p < 0.05$) between group of samples. CONTROL (*E. faecium* in MRS), H_2O_2 (*E. faecium* in MRS + 2.5 mM H_2O_2), CA (*E. faecium* in MRS + 0.5 mM CA) and H_2O_2 + CA (*E. faecium* in MRS + 2.5 mM H_2O_2 + 0.5 mM CA).

(2013) have also reported a similar enzyme in *Lactobacillus panis*.

In this work, *E. faecium* showed a catalase-like activity, which differed between experimental units (Fig. 4). The catalase-like activity of the CONTROL group was significantly reduced in bacteria exposed with H_2O_2. However, incubation with CA increased the catalase-like activity by 5-fold. The pathway by which the phenolic compound causes an increase in this antioxidant activity, still unknown, is considerably enhanced by the co-exposure with H_2O_2. This result contributes to explaining the protective effect of CA against lipid oxidation in *E. faecium* caused by H_2O_2 (described above) and reinforces the hypothesis that the mechanism may involve the oxidized form of CA. In this regard, it seems reasonable to hypothesize that CA (or more likely its oxidized quinone form) causes a mild oxidative stress in cells (measured by flow cytometry and confirmed by protein carbonylation) that triggers the catalase-like activity described above. Thus, H_2O_2, which itself causes oxidative stress without activating the antioxidant defences, would contribute to oxidize CA, which in turn would cause a considerable increase in catalase-like activity. This hypothesis is in accordance with the findings from Arcanjo et al., (2019) in a test carried out *in vitro* where *L. reuteri* was incubated in the presence of H_2O_2 and resveratrol. These authors demonstrated that the gene *dhaT*, which encodes the enzyme 1,3-propanediol oxidoreductase (1,3-PDO), is overexpressed in a situation of oxidative stress and that resveratrol has regulatory effect on such expression. The authors proposed that it would be the oxidized form of resveratrol which could be behind the signalling mechanism that would trigger the expression of the gene and the activation of this antioxidant defence. In the present research, the increase in catalase-like activity observed could respond to the presence of a similar enzyme, such as the one described by Tanaka et al. (2018).

3.4. Proteome response of E. Faecium to the exposure to H_2O_2 and CA

The LFQ proteomic analysis carried out revealed a total of 1041 proteins in at least three out of the five replicas in at least one of the four treatments (CONTROL, CA, H_2O_2 and CA + H_2O_2). Each one of these proteins was identified by at least two peptides and its FDR was lower than 1%. (Supplementary Tables 1, 2 and 3).

3.4.1. E. Faecium proteome response to H_2O_2 exposure.

Among the proteins significantly modified in quantity ($p < 0.05$) in *E. faecium* because of the exposure to H_2O_2, 77 proteins were found in lower quantity and 211 were in higher quantity as compared to the CONTROL. All these proteins were identified as discriminating proteins as their relative concentration was significantly affected by the exposure to H_2O_2 (Supplementary Table 1).

According to ClueGO classification, the proteins found in lower quantity belonged to several metabolic routes, namely glycolytic processes (53.70%), carboxylic acid metabolic processes (18.52%) and exopeptidase activities (16.67%). Non-mentioned pathways accounted for less than the 10% (Supplementary Fig. 1A). While this is, to our knowledge, the first time that bacterial proteome is studied in relation to the impact of H_2O_2, our results confirm those from some other previous studies. For instance, the lower relative abundance of proteins involved in glycolytic processes matches with results from Ranjbar et al. (2020) who indicated the disruption of the glycolytic pathway in *E. coli* in a pro-oxidative environment (such as that made by H_2O_2) that could explain, in turn, a reduced bacterial growth (Sanman et al., 2016). Likewise, Zhang et al. (2021) found a down-regulation of nucleotide biosynthesis in *Pediococcus pentosaceus* R1 exposed to H_2O_2, which is consistent with the lower abundance of proteins involved in carboxylic acid metabolic process and exopeptidase activity, found in the present study (Table 1). Unlike the previous studies, the present work indicates how the exposure to H_2O_2 affected the concentration of specific proteins playing a relevant role in essential bacterial processes such as energy supply and biomolecules synthesis (Table 1), providing, like this, a completer and more accurate picture of the impairment of biological processes in the bacterium affected by a pro-oxidative challenge. Additionally, proteins involved in the glutathione (GSH) pathway were assessed in the proteomic data. Unexpectedly, a GSH peroxidase protein coded by *gpxA* was reduced in quantity (Fold change 0.77) in H_2O_2 group, whilst two glutathione-disulfide reductase proteins coded by *gor* and *gor2* were found both in lower and higher relative quantity after the treatment with this prooxidant (Fold change 0.87 and 1.24, respectively). Globally, these findings indicate an impairment of the antioxidant defense exerted by GSH under oxidative stress conditions. This result, along with those from the assessment of the catalase-like activity, shows the inability of *E. faecium* to activate effective antioxidant defenses against the pro-oxidative threat of H_2O_2. Further, other proteins involved in this defense, such as superoxide dismutase, were found in the proteomic analysis but no differences were detected between the H_2O_2 and control groups. Thus, seemingly this enzyme may not be key in the antioxidant response of *E. faecium* to H_2O_2.

On the other hand, the increased routes were related to organic substance biosynthetic processes (48%), anion binding (30%) and peptidoglycan biosynthesis (10%). Non-mentioned pathways accounted for less than the 10% (Supplementary Fig. 1B). Therefore, the exposure to H_2O_2 led to an increase of proteins involved in DNA and proteins synthesis and transcription regulators, ATP binding cassete (ABC) transporters, oxidoreductases, DNA repair proteins and peptidoglycan biosynthesis (Table 1). These results are coherent with a previous study in which lactic acid bacteria adaptation responses to stress were studied (Lebeer et al., 2011). According to the authors, proteins related to nutritional adaptation (hydrolases, ABC transporters and phosphotransferase systems [PTS]), and stress mechanisms, (membrane modification), were found to be activated under stress conditions (Lebeer et al., 2011). According to our results, *E. faecium* would be activating routes related to protect biologically relevant molecules (proteins, DNA and peptidoglycan) and recover the physiological impairments caused by H_2O_2 in terms of energy supply, redox status and pH. The possible function of ATPases found in anion binding route could be to ensure the pH homeostasis, as seen by Arena et al. (2006). Since *E. faecium* was incubated in anaerobiosis, fermentation of glucose leads to a pH decline, which would activate the synthesis of proteins found in acid environment stress, such as ATPases. This situation may be worsened in the presence of H_2O_2 owing to the connection between oxidative stress, altered redox status and impaired pH (Tsai et al., 1997). This relation has been described in eukaryotic cells (Tsai et al., 1997), human tissues (Loh et al., 2002) and bacteria (Oktyabrskii and Smirnova, 2012). These findings support the hypothesis, previously formulated by Arena et al., (2006), among others, that bacterial adaptation to oxidative stress may involve the contribution of different physiological mechanisms.

The exposure of *E. faecium* to H_2O_2 also caused an increase in two histidine kinases that are part of the two-component system (Table 1). The two-component systems are bacterial mechanisms of adaptation to environmental changes. They are composed by a sensor histidine kinase that autophosphorylate and subsequently transfer the phosphate group to their cognate response regulators thus modulating their activity, usually as transcriptional regulators (Monedero et al., 2017). In line with the present results, Zhou et al. (2010) found an upregulation of one gene encoding for histidine kinases in *Desulfovibrio vulgaris* Hildenborough incubated with H_2O_2. These proteins seem to be involved in the detection and response to oxidative stress as regulators.

Serine/Threonine kinases were also found in higher quantity in *E. faecium* treated with H_2O_2 (Table 1). These enzymes regulate protein activity through its association with phosphatases, to quench signalling cascades (Pereira et al., 2011) and it is well known that these proteins are involved in stress responses (Sasková et al., 2007).

The *cgtA* gene, also named *obg*, encodes for an essential GTPase that plays a role in adjusting the cellular energy status, control DNA replication, ribosome biogenesis and stress adaptation pathways (stringent response, sporulation, general stress response) (Kint et al., 2014) (Table 1). Nevertheless, these proteins seem to exert its effect

Table 1
Discriminating proteins from *Enterococcus faecium* as affected by the exposure to 2.5 mM H_2O_2 for 48 h at 37 °C and anaerobiosis.

H_2O_2 < CONTROL					
PROTEIN NAME	GENE NAME	*p*-value	Fold-change[1]	BIOLOGICAL FUNCTION	FASTA accession number
Biomolecule synthesis proteins					
Serine hydroxymethyltransferase	*glyA*	0.00452454	0.86	Amino-acid biosynthesis	I3U4H4
Leucine–tRNA ligase	*leuS*	0.03758580	0.94	Protein biosynthesis	Q3Y3J5
Pyrroline-5-carboxylate reductase	*proC*	0.00355311	0.86	Amino-acid biosynthesis,	Q3XYJ7
Serine–tRNA ligase	*serS*	0.04602920	0.92	Protein biosynthesis	I3U1P9
Cysteine synthase	*cysK2*	0.01176790	0.84	Amino-acid biosynthesis	I3TZB4
4-hydroxy-tetrahydrodipicolinate synthase	*dapA*	0.00355311	0.86	Amino-acid biosynthesis	Q3Y1B7
Pyrroline-5-carboxylate reductase	*proC*	0.00452454	0.86	Amino-acid biosynthesis	I3U4H4
Glycolytic Process					
Enolase	*eno*	0.0268079	0.86	glycolytic process	Q3Y0T4
Fructose-bisphosphate aldolase	*fba2*	0.00616979	0.78	glycolytic process	I3U007
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	*gpmA*	0.00266733	0.86	Gluconeogenesis, Glycolysis	Q3XXS6
Triosephosphate isomerase	*tpiA*	0.00005370	0.84	Gluconeogenesis, glycolysis	Q3XX07
L-lactate dehydrogenase	*ldh*	0.00554119	0.84	glycolytic process	Q3XWM4
Glucose-6-phosphate isomerase	*pgi*	0.00944010	0.82	Gluconeogenesis, glycolysis	Q3XZ32
Pyruvate oxidase	*poxB*	0.02742560	0.90	pyruvate oxidase activity	Q3XZD0
Other pathways					
Glutathione peroxidase	*gpxA*	0.01341360	0.77	Response to oxidative stress	Q3XX43
Glutathione-disulfide reductase	*gor*	0.04558070	0.87	Cell redox homeostasis	Q3XWK1
H_2O_2 > CONTROL					
Peptidoglycan Biosynthesis					
Lipid II isoglutaminyl synthase (glutamine-hydrolyzing) subunit GatD	*cobQ*	0.02700000	1.17	Peptidoglycan synthesis	Q3Y1G9
UDP-N-acetylglucosamine 1-carboxyvinyltransferase	*murAB*	0.02075460	1.23	Peptidoglycan synthesis	I3U008
UDP-N-acetylmuramoyl-L-alanyl-D-glutamate–L-lysine ligase	*murE*	0.01949580	1.27	Peptidoglycan synthesis	Q3XXY2
UDP-N-acetylglucosamine–N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	*murG*	0.00009240	1.24	Peptidoglycan synthesis	Q3Y2H8
Endolytic murein transglycosylase	*pabC*	0.01949580	1.27	Cell wall biogenesis/degradation	Q3XXY2
Acyl carrier protein	*acpP2*	0.01869130	1.41	Fatty acid biosynthesis	Q3Y0S6
3-oxoacyl-[acyl-carrier-protein] synthase 3	*fabH*	0.01146540	1.35	Fatty acid biosynthesis	Q3Y0S7
Transcription					
DNA-directed RNA polymerase subunit alpha	*ntd*	0.04760460	1.53	transcription	Q3Y0A5
Histidine kinase	*vicK*	0.00153634	1.25	Two-component regulatory system	Q3XYG9
Histidine kinase	*HMPREF0351_12688*	0.00159151	1.22	Two-component regulatory system	Q3XYJ6
Chaperone protein ClpB	*clpB*	0.00005960	1.57	Stress response	Q3XZF2
Protein RecA	*recA*	0.00000009	1.79	DNA damage, DNA recombination, DNA repair, SOS response	Q3Y1B9
DNA replication and repair protein RecF	*recF*	0.04744940	1.35	DNA damage, DNA replication, DNA repair, SOS response	Q3XZE3
DNA gyrase subunit A	*gyrA*	0.01182610	1.09	DNA-dependent DNA replication, DNA topological change	Q3XZE1
Transcriptional regulator	*HMPREF0351_10139*	0.01222660	2.63		I3TYC5
LysR family transcriptional regulator	*HMPREF0351_10567*	0.00470219	1.24		I3TZK3
Response regulator	*HMPREF0351_12687*	0.02887400	1.12	Transcription, Transcription regulation	I3U5M3
DeoR family transcriptional regulator	*fruR*	0.01297940	1.40	Transcription, Transcription regulation	I3U2J9
Heat-inducible transcription repressor HrcA	*hrcA*	0.00002960	1.57	Stress response, transcription, transcription regulation	Q3XWX7
Lactose PTS family porter repressor	*lacR*	0.00277818	1.28	Transcription, Transcription regulation	Q3XYZ6
Transcription-repair-coupling factor	*mfd*	0.00343941	1.14	DNA damage, DNA repair	Q3Y182
DNA-binding response regulator	*vicR*	0.00164261	1.16	Transcription, Transcription regulation	I3U4P8
Chromosomal replication initiator protein DnaA	*dnaA*	0.01143460	1.10	DNA replication	Q3XZE6
Endonuclease MutS2	*mutS*	0.02171570	1.21	mismatch repair, negative regulation of DNA recombination	Q3XXL7
Endonuclease MutS2	*mutS3*	0.00391418	1.22	mismatch repair, negative regulation of DNA recombination	Q3Y1S0
ATPases					
GTPase Obg	*cgtA*	0.00710349	1.15	ribosome biogenesis	Q3XWL6
ATP-binding subunit of chaperone	*HMPREF0351_10960*	0.00015300	1.43	ATPase activity, ATP binding	Q3Y0T6
p-ATPase superfamily P-type ATPase heavy metal transporter	*HMPREF0351_10990*	0.00419889	1.82	ATP binding, hydrolase activity	Q3Y0Q6
p-ATPase superfamily cation transporter	*HMPREF0351_11086*	0.00040997	1.29	ATPase activity, ATP binding	I3U122
p-ATPase superfamily P-type ATPase copper (Cu) transporter	*copB*	0.01729900	1.33	ATP binding	I3U3B6
DNA repair protein RadA	*radA*	0.00655028	1.28	DNA damage, DNA repair, stress response	I3U5E2
Teichoic acid ABC superfamily ATP binding cassette transporter, ABC protein	*tagH*	0.01394620	1.24	ATPase-coupled transmembrane transporter activity	Q3XY30
p-ATPase superfamily cation transporter	*yoaB*	0.00018857	1.21	ATPase activity, ATP binding	I3U105
ABC transporter					

(*continued on next page*)

Table 1 (*continued*)

H_2O_2 < CONTROL					
PROTEIN NAME	GENE NAME	*p*-value	Fold-change[1]	BIOLOGICAL FUNCTION	FASTA accession number
ABC superfamily ATP binding cassette transporter, ABC protein	*HMPREF0351_10042*	0.00127376	1.19	ATPase-coupled transmembrane transporter activity, ATP binding, hydrolase activity	Q3XXS0
ABC superfamily ATP binding cassette transporter, ABC protein	*HMPREF0351_10435*	0.00054375	1.32	ATPase-coupled transmembrane transporter activity, ATP binding, hydrolase activity	Q3Y2U7
ABC superfamily ATP binding cassette transporter, ABC protein	*HMPREF0351_10455*	0.04460830	1.20	ATPase-coupled transmembrane transporter activity, ATP binding, hydrolase activity	Q3Y2W7
ABC superfamily ATP binding cassette transporter, ABC protein	*HMPREF0351_10571*	0.00345350	1.38	ATPase-coupled transmembrane transporter activity, ATP binding, hydrolase activity	I3TZK7
ABC superfamily ATP binding cassette transporter, ABC protein	*HMPREF0351_11163*	0.01365780	1.26	ATPase-coupled transmembrane transporter activity, ATP binding	Q3XWK0
ABC superfamily ATP binding cassette transporter, membrane protein	*HMPREF0351_12246*	0.04519430	1.21	ATPase-coupled transmembrane transporter activity, ATP binding	I3U4D2
ABC superfamily ATP binding cassette transporter, membrane protein	*HMPREF0351_12247*	0.00014756	1.32	ABC-type amino acid transporter activity, ATPase-coupled transmembrane transporter activity. ATP binding, hydrolase activity	Q3XWY8
Glutamate ABC superfamily ATP binding cassette transporter, ABC protein	*glnQ*	0.04555700	1.20	ABC-type amino acid transporter activity, ATPase-coupled transmembrane transporter activity. ATP binding, hydrolase activity	Q3XZW4
Phosphotransferase System					
HPr kinase/phosphorylase	*hprK*	0.03843200	1.18	Carbohydrate metabolism	Q3XWV5
Phosphoenolpyruvate-protein phosphotransferase	*proS*	0.00368226	1.08	Phosphotransferase system, sugar transport	Q3XZ69
Serine/Threonine kinases					
Non-specific serine/threonine protein kinase	*HMPREF0351_12565*	0.00797501	1.15	Kinase, Serine/threonine-protein kinase, Transferase	Q3Y195
HPr kinase/phosphorylase	*hprK*	0.03843200	1.18	Carbohydrate metabolism	Q3XWV5
Other pathways					
Glutathione-disulfide reductase	*gor2*	0.00581890	1.24	Cell redox homeostasis	Q3XY74
General stress protein Gls20	*gls20*	0.00054684	1.47	Stress response	Q3Y329

[1] C: only found in CONTROL samples. T: only found in treated samples. Fold change displayed as the result of the ratio H_2O_2 /C.

specifically through the regulator RecA, also found in *E. faecium*, which is involved in regulation of homologous recombination on top of its function in promoting the auto-proteolysis of LexA, repressor of SOS regulon (Cox, 2007). This means that *cgtA* and RecA are entailed in stress response. Interestingly, we have identified the clpB protein which has been recently described in *E. faecium* as part of a stress induced multi-chaperone system, in cooperation with dnaK, dnaJ and GrpE. This system is involved in the recovery of the cell from heat stress and in the correct folding of newly formed proteins (Alam et al., 2021). Yet no significant differences were found by H_2O_2 treatment.

The enzyme alkyl hydroperoxide reductase (ahpC), which is part of AhpF-AhpC complex, provides *Streptococcus mutans* with peroxidase activity (Higuchi et al., 2000), catalyzing the reduction of peroxide into water or alcohol. AhpC is also involved in a chaperone function, as found in *Helicobacter pylori,* stabilizing the protein folding in oxidative stress conditions (Chuang et al., 2006). Although no significative differences were found among treatments for AphC, the relative abundance of this proteins in the CONTROL group was the lowest, indicating that chemical challenges would have stimulated the synthesis of this protective protein.

Peptidoglycan biosynthesis has been considered as a vital strategy for lactic acid bacteria to cope with various stress conditions (Yang et al., 2021) (Table 1). The exposure to H_2O_2 stimulated the occurrence of proteins related to cell wall and membrane synthesis, encoded in *E. faecium* by genes such as *acp* (Acyl carrier protein) and *fab* (3-oxoacyl-[acyl-carrier-protein] synthase 3). This mechanism was also found in *L. plantarum* ZDY2013 in response to an acid challenge (Huang et al., 2016). These higher quantity in cell wall proteins may contribute to maintaining cell functions of the bacteria under stress condition (Yang et al., 2021).

Finally, Gls20 (general stress protein) was found in 1.47 and 1.17-fold higher quantities in H_2O_2 and CA groups, respectively (Table 1 and Table 2). Both groups showed higher ROS levels than the CONTROL treatment (Section 3.1). This protein has been described to be essential for multiple stress adaptation in *E. faecalis* (Giard et al., 2001; Teng et al., 2005) and for oxidative stress in *S. thermophilus* (Arena et al., 2006).

3.4.2. *E. Faecium proteome response to CA exposure.*

Among the proteins significantly modified in quantity ($p < 0.05$) in *E. faecium* as a result of the exposure to CA, 98 were in lower quantity and 119 were in higher quantity, as compared to the CONTROL. In the qualitative analysis just four proteins were found only in the CA treatment (Supplementary Table 2).

Based on the ClueGO classification, the proteins found in lower quantity belonged to several metabolic routes, namely to carboxylic acid metabolic processes (30.3%), to anion binding processes (30.3%) and to organonitrogen compound metabolic processes (24.2%) (Supplementary Fig. 2A). Basically, the proteins found in these routes are related to biomolecules synthesis, including subpathways to the biosynthesis of nitrogen-containing compounds, lipids, and fatty acids (Table 2). This finding is coherent with our previous results, as CA appeared to cause oxidative stress to bacterium and in this situation, the bacterial response is manifested in a slower growth. To similar conclusions came Arena et al. (2006) who found that exposure of *S. thermophilus* to oxidative stress led to inhibition of bacterial growth. Yet the proteome shift caused by CA is notably different to that induced by H_2O_2 which could be indicative that the underlying mechanisms of the stress caused by each substance is different and leads to different bacterial responses. While H_2O_2 induced a remarkable decline in the cellular mechanisms related to glycolysis and energy supply, CA seems to affect to a higher extent the biosynthesis of assorted bacterial biomolecules, namely organonitrogen compounds and lipids.

Among the biomolecules that were likely affected by the impairment of the proteome, some of them, such as the GSH, is, as aforementioned, related to the antioxidant defence against oxidative stress. A glutathione pathway involved protein, the superoxide dismutase, coded by *sodA*, was found in lower relative quantity in *E. faecium* when treated with CA (Fold change 0.71). These results are remarkable as superoxide dismutase-like activity has been reported in previous studies as a

Table 2
Discriminating proteins from *E. faecium* as affected by the exposure to 0.5 mM chlorogenic acid (CA) for 48 h at 37 °C and anaerobiosis.

PROTEIN NAME	GENE NAME	*p*-value	Fold-change[1]	BIOLOGICAL FUNCTION	FASTA accession number
CA < CONTROL					
Biomolecule Synthesis					
Arginine–tRNA ligase	*argS*	0.01116870	0.84	Protein biosynthesis	Q3XZA3
Asparagine–tRNA ligase	*asnS*	0.00141641	0.89	Protein biosynthesis	Q3XYR6
Aminotransferase	*aspB*	0.01942280	0.77	biosynthetic process	Q3XYR7
Aspartate aminotransferase	*aspC2*	0.03357750	0.91	biosynthetic process	I3U1B3
Aspartate–tRNA ligase	*aspS*	0.04581640	0.91	Protein biosynthesis	Q3XY17
Glycine–tRNA ligase alpha subunit	*glyQ*	0.00078655	0.78	Protein biosynthesis	I3U3K9
Glycerol-3-phosphate dehydrogenase [NAD(P) +]	*gpsA*	0.01454940	0.69	Lipid biosynthesis	Q3XWV3
GMP synthase [glutamine-hydrolyzing]	*guaA*	0.00426934	0.90	Purine biosynthesis	Q3XZ01
Elongation factor 4	*lepA*	0.00289396	0.85	Protein biosynthesis	I3U2C9
Leucine–tRNA ligase	*leuS*	0.00839025	0.92	Protein biosynthesis	Q3Y3J5
Enoyl-[acyl-carrier-protein] reductase [NADH]	*fabI*	0.00066351	0.83	Fatty acid biosynthesis, Fatty acid metabolism, Lipid biosynthesis, Lipid metabolism	I3U1N1
5′-methylthioadenosine/S-adenosylhomocysteine nucleosidase	*mtnN*	0.04147730	0.79	Amino-acid biosynthesis, Methionine biosynthesis	Q3Y2D9
Other pathways					
Superoxide dismutase	*sodA*	0.02996350	0.71	Cell redox homeostasis	I3U0N1
Glutathione-disulfide reductase	*gor2*	0.02685480	0.82	Cell redox homeostasis	Q3XY74
6-phosphogluconate dehydrogenase	*gnd*	0.00048277	0.84	Gluconate utilization, pentose shunt	Q3XYQ5
Glucose-6-phosphate 1-dehydrogenase	*zwf*	0.00202413	0.89	Carbohydrate metabolism, Glucose metabolism	Q3Y2I8
CA > CONTROL					
Transcription Regulator					
Protein RecA	*recA*	0.01405560	1.09	DNA damage, DNA recombination, DNA repair, SOS response	Q3Y1B9
Global transcriptional regulator Spx	*spxA2*	0.01181280	1.12	Transcription, Transcription regulation	Q3XYC5
RNA-binding heat shock protein	*HMPREF0351_12550*	0.03768560	1.50	Stress response	Q3Y180
Bifunctional ligase/repressor BirA	*birA*	0.03732550	1.13	Transcription, Transcription regulation	I3U5K7
Chromosomal replication initiator protein DnaA	*dnaA*	0.00037060	1.08	DNA replication	Q3XZE6
Replicative DNA helicase	*dnaB*	0.00831094	1.16	DNA replication	Q3XZD5
ATP synthase subunit beta	*atpD*	0.03054790	1.13	ATP synthesis, Hydrogen ion transport, Ion transport, Transport	Q3XY89
GTPase Obg	*cgtA*	0.00796315	1.19	ribosome biogenesis	Q3XWL6
Endonuclease MutS2	*mutS3*	0.01121180	1.18	mismatch repair, negative regulation of DNA recombination	Q3Y1S0
Bifunctional ligase/repressor BirA	*lplA*	0.00003430	1.15	Transcription regulation	Q3XZT0
Crp family transcriptional regulator	*crp*	0.04832190	1.22	Transcription regulation	Q3XZ99
DeoR family transcriptional regulator	*fruR*	0.00515452	1.44	Transcription regulation	I3U2J9
Transcription termination/antitermination protein NusA	*nusA*	0.03089720	1.18	Transcription regulation	Q3XZ66
Biomolecule Synthesis					
Cysteine–tRNA ligase	*cysS*	0.02557540	1.14	Protein biosynthesis	Q3Y163
Glutamate–tRNA ligase	*gltX*	0.00431243	1.15	Protein biosynthesis	Q3Y161
Histidine–tRNA ligase	*hisS*	0.04707060	1.07	Protein biosynthesis	Q3XY18
Serine–tRNA ligase	*serS*	0.00258078	1.15	Protein biosynthesis	Q3XYJ7
50S ribosomal subunit assembly factor BipA	*tufA*	0.00047291	1.15	Protein biosynthesis, Ribosome biogenesis	I3TY41
Elongation factor Tu	*tufA2*	0.00214930	1.16	Protein biosynthesis	Q3XX23
Valine–tRNA ligase	*valS*	0.02503110	1.09	Protein biosynthesis	Q3Y009
Chorismate synthase	*aroC*	0.00805999	1.24	Amino-acid biosynthesis, Aromatic amino acid biosynthesis	Q3XXZ3
Glutamate–tRNA ligase	*gltX*	0.00431243	1.15	Protein biosynthesis	Q3Y161
Translation initiation factor IF-3	*infC*	0.03253310	1.15	Protein biosynthesis	I3TYL6
Ribosomal Proteins					
50S ribosomal protein L1	*rplA*	0.00082174	1.25	Translation regulation	I3U4S7
Ribosomal protein L2	*rplB2*	0.02491510	1.14	translation	Q3XYY6
50S ribosomal protein L3	*rplC*	0.00135762	1.23	translation	I3TY43
50S ribosomal protein L6	*rplF*	0.00429825	1.30	translation	Q3XYX4
50S ribosomal protein L13	*rplM*	0.02673010	1.14	translation	Q3Y121
50S ribosomal protein L15	*rplO*	0.04525910	1.15	translation	Q3XYX0
50S ribosomal protein L17	*rplQ*	0.03345080	1.13	translation	Q3XYW2
50S ribosomal protein L18	*rplR*	0.00618316	1.33	translation	Q3XYX3
50S ribosomal protein L22	*rplV*	0.03667110	1.20	translation	Q3XYY4
50S ribosomal protein L30	*rpmD*	0.01396360	1.36	translation	Q3XYX1
50S ribosomal protein L33	*rpmG*	0.03880200	1.47	translation	I3U2V2
50S ribosomal protein L35	*rpmI*	0.00013040	1.26	translation	Q3XXD4
30S ribosomal protein S3	*rpsC*	0.00111061	1.30	translation	Q3XYY3
30S ribosomal protein S4	*rpsD*	0.00626310	1.19	translation	I3TYB5
30S ribosomal protein S5	*rpsE*	0.01739870	1.13	translation	Q3XYX2
30S ribosomal protein S10	*rpsJ*	0.01263070	1.13	translation	I3TY42
30S ribosomal protein S11	*rpsK*	0.03949810	1.28	translation	Q3XYW4
30S ribosomal protein S12	*rpsL*	0.00264341	1.41	translation	I3TY38

(*continued on next page*)

Table 2 (*continued*)

CA < CONTROL					
PROTEIN NAME	GENE NAME	*p*-value	Fold-change[1]	BIOLOGICAL FUNCTION	FASTA accession number
30S ribosomal protein S14	*rpsN2*	0.00637147	1.26	translation	Q3XWD4
30S ribosomal protein S17	*rpsQ*	0.00335901	1.20	translation	Q3XYY0
30S ribosomal protein S19	*rpsS*	0.02018250	1.14	translation	Q3XYY5
30S ribosomal protein S21	*rpsU*	0.01578630	1.19	translation	Q3Y1C1
Oxidoreductase					
GMP reductase	*guaC*	0.00129811	1.15	purine nucleotide metabolic process	Q3XYY6
Glyceraldehyde-3-phosphate dehydrogenase	*gap*	0.03239580	1.15	glucose metabolic process	I3TY43
Other pathways					
General stress protein Gls20	*Gls20*	0.04784370	1.17	Stress response	Q3Y329
Universal stress protein	*uspA4*	0.00550011	1.15	Stress response	I3U4Y2
Universal stress protein	*uspA2*	0.03765640	1.22	Stress response	I3U0Z8
Universal stress protein	*uspA*	0.02312300	1.15	Stress response	Q3Y093

[1] C: only found in CONTROL samples. T: only found in treated samples. Fold change displayed as the result of the ratio CA/C.

Table 3
Discriminating proteins from *E. faecium* as affected by the exposure to 2.5 mM H_2O_2, and 0.5 mM chlorogenic acid (CA) for 48 h at 37 °C and anaerobiosis.

H_2O_2 + CA < H_2O_2 PROTEIN NAME	GENE NAME	*p*-value	Fold-change[1]	BIOLOGICAL FUNCTION	FASTA accession number
ABC transporter					
Amino acid ABC superfamily ATP binding cassette transporter, membrane protein	*HMPREF0351_10153*	0.01382750	0.86	amino acid transport	I3TYD9
Amino acid ABC superfamily ATP binding cassette transporter, membrane protein	*HMPREF0351_12278*		H	Amino-acid transport, Transport	I3U4G4
Glycine betaine/carnitine/choline ABC superfamily ATP binding cassette transporter, membrane/binding protein	*proWX*		H	Amino-acid transport, Transport	Q3Y0D9
H_2O_2 + CA > H_2O_2					
ribosomal protein					
50S ribosomal protein L6	*rplF*	0.03095780	1.28	translation	Q3XYX4
50S ribosomal protein L9	*rplI*	0.02268260	1.12	translation	Q3XZD6
50S ribosomal protein L13	*rplM*	0.03842160	1.10	translation	Q3Y121
50S ribosomal protein L19	rplS	0.00981996	1.23	translation	I3U367
50S ribosomal protein L33	rpmG	0.00154458	1.84	translation	I3U2V2
50S ribosomal protein L35	rpmI	0.00567683	1.19	translation	Q3XXD4
30S ribosomal protein S7	rpsG	0.02470560	1.12	translation	I3TY39
30S ribosomal protein S10	rpsJ	0.04101320	1.14	translation	I3TY42
30S ribosomal protein S12	rpsL	0.00367784	1.44	translation	I3TY38
30S ribosomal protein S17	rpsQ	0.00029301	1.16	translation	Q3XYY0
Transcription regulation					
RNA polymerase sigma factor SigA	sigA	0.02820870	1.23	Transcription, Transcription regulation	Q3XWZ6
Global transcriptional regulator Spx	spxA2	0.03816490	1.09	Transcription, Transcription regulation	Q3XYC5
Bifunctional ligase/repressor BirA	lplA	0.03079810	1.09	Transcription, Transcription regulation	Q3XZT0
GTP-sensing transcriptional pleiotropic repressor CodY	codY	0.04498310	1.13	Transcription, Transcription regulation	I3U178
Redox-sensing transcriptional repressor Rex	*rex*	0.03838950	1.09	Transcription, transcription regulation	Q3Y0H7
Amidase activity					
C40 family peptidase	*HMPREF0351_10449*	0.00039152	2.19	Cell wall organization	Q3Y2W1
Peptidoglycan hydrolase	*HMPREF0351_12531*	0.00018541	2.46	Cell cycle, Cell division, Cell wall biogenesis/degradation, septation	Q3Y3J6
Peptidoglycan hydrolase	*amiC*	0.00328786	2.46	Cell cycle, Cell division, Cell wall biogenesis/degradation, septation	Q3XWX2

[1] H: only found in H_2O_2 samples. M: only found in H_2O_2 + CA samples. Fold change displayed as the result of the ratio H_2O_2 + CA /H_2O_2.

relevant biological mechanisms for probiotic bacteria such as *Lactobacillus* strains to protect against oxidative stress and intestinal inflammation (Tomusiak-Plebanek et al., 2018). This does not seem to be the case of *E. faecium*.

On the other hand, the increased routes were principally related to transcription, translation and synthesis of proteins including several interesting oxidoreductases (Table 2). Pathways accounting for less than the 10% of total metabolic routes affected by CA, were not enumerated (Supplementary Fig. 2B).

Among the translation proteins, an important number of ribosomal proteins were found to be upregulated in *E. faecium* exposed to CA with these proteins having the ability to build the ribosomal small and large units. Furthermore, they display extra-ribosomal functions, as being a part of an operon, which activity could modulate, in final steps, even stress responses (Singh et al., 2009). Ribosomal proteins (RplJ, RpsF) were found to be increased in *L. sakei* as a response to stress caused by high hydrostatic pressure (Jofré et al., 2007). These family of proteins are plausibly related to the response of *E. faecium* towards the induced stress caused by CA.

The analysis of flow cytometry and oxidation markers indicated that CA stimulates the generation of ROS and the carbonylation of bacterial proteins in *E. faecium*. On the other hand, the exposure to CA stimulated the catalase-like activity which was reflected in antioxidant protection against lipid oxidation. The promotion of the biological processes implicated in proteins biosynthesis as revealed by the analysis of the proteome, would support the hypothesis of CA being able to trigger signalling cascades aimed to activate the antioxidant defences of the cell. According to Lushchak (2011), the activation of the antioxidant

responses follows several steps that includes sensing reactive species and passing the signal through regulators to transcription and translation machineries. As a major transcriptional regulator described in the response to oxidative stress (Kajfasz et al., 2012), we identified SpxA2 (Table 2) that was found in 1.12-fold higher quantities in the bacteria exposed to CA, than in CONTROL bacteria. The mechanism of action of Spx protein involves the interaction with the C-terminal domain of the RNA polymerase α-subunit as demonstrated *in vitro* and is thus, a modulator of the transcriptional regulator – RNA polymerase interaction (Zuber, 2004). In the *Streptococcaceae* family, SpxA2 maintain activation of oxidative stress genes and in addition is involved in cell envelope homeostasis (Nilsson et al., 2019). Other oxidoreductases with potential implication in the redox status of the cell were, as well, upregulated in bacteria exposed to CA. Among these, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), encoded by *gap* gene (Table 2), is worthmentioning as it has been recently emphasized as a multifunctional protein with implications in the protection against oxidative stress in eukaryotic cells (Muronetz et al., 2020; Nakajima et al., 2017). While in bacteria, the non-glycolytic functions of GAPDH are much less understood, previous works already proposed the role of this enzyme in protecting bacteria against stress and oxidative damage of biomolecules (Ferreira et al., 2015; Hillion et al., 2017). Interestingly, dietary CA was reported to enhance gut health in piglets through the promotion of beneficial microbiota and their peroxidase activity, which would suggest the prebiotic effect of this phytochemical in certain probiotic bacteria (Zhang et al., 2018).

Finally, in the anion binding route, we discovered several hydrolases that could be involved in the degradation pathway of the CA, because one of the steps of the transformation of this compound is the hydrolysis (Tomas-Barberan et al., 2014). In *Bifidobacterium animalis*, a feruloyl esterase activity capable of hydrolysing CA was identified (Raimondi et al., 2015) and although this enzyme was not found in *E. faecium*, one of these hydrolases could have this function.

3.4.3. *E. Faecium proteome response to H_2O_2 + CA exposure.*

Considering the initial hypothesis that CA may counteract the potential negative effects of H_2O_2 in *E. faecium*, the effect of H_2O_2 + CA exposure on the proteome is compared to that exerted by just H_2O_2. Among the proteins significantly modified in quantity ($p < 0.05$) in *E. faecium* because of the exposure to H_2O_2 + CA, 47 were in lower quantity and 65 were in higher quantity, as compared to the bacteria exposed to H_2O_2. In the qualitative analysis, five proteins were found only in the H_2O_2 treatment (Supplementary Table 3).

Based on the ClueGO classification, the simultaneous addition of CA + H_2O_2 to bacteria, led to a decrease in quantity of proteins involved in just one metabolic route, namely to the ABC transporter complex (100%) (Supplementary Fig. 3A). It is worth recalling that this group of proteins was increased in bacteria treated with H_2O_2 as compared to the CONTROL bacteria. Since processes governed by the ABC transporter complex plays a crucial role in bacterial survival under energy-limited conditions or in environments in which toxic compounds are present (Konings et al., 1997), the downregulation of this route by CA may be a reflection of the protective effect of the phytochemical against the toxic effects of H_2O_2.

On the other hand, the increased routes were related to organonitrogen compound biosynthetic process (92%) and to other processes that, despite of being quantitatively less marked, would play a relevant role in the response of the bacteria to the combination of H_2O_2 + CA, such as amidase and oxidoreductase activities (Supplementary Fig. 3B).

Bacterial amidases have been recently described to be involved in the protection and maintenance of the bacterial cell wall against outer membrane stress situations (Gurnani Serrano et al., 2021). Hence, CA seems to activate mechanisms of cell wall protection in the presence of H_2O_2. Since the combination of CA + H_2O_2 increased the ability of the bacteria to protect itself against oxidative stress and lipid oxidation and that occurred with a remarkable increase in a catalase-like activity of the bacteria, changes in the proteome would be indicating a strengthening of the endogenous antioxidant defenses of *E. faecium*. Among proteins found in greater quantity, and that could play a role in this regard, we found ribosomal proteins and transcriptional regulators such as the abovementioned SpxA2. A transcriptional repressor, which is also related to response to oxidative stress, Rex, showed significant differences between treatments. This protein is a regulator that respond to cellular NAD^+/NADH ratio in order to modulate gene expression in central metabolism, oxidative stress response and biofilm formation (Vesić and Kristich, 2013). Vesić and Kristich (2013) reported that Rex factor influenced on the detoxification of H_2O_2 in *E. faecalis* which is coherent with the current hypothesis of CA improving the ability of *E. faecium* to neutralize the pro-oxidative effect of H_2O_2. Along with the exposure to CA, the combination of the phytochemical with H_2O_2, increased, once again, the concentration of oxidoreductases, that would likely play a role in the maintenance of the redox status of the cell.

The proteomic analysis of *E. faecium* indicates that the remarkable increase in the catalase-like activity observed in bacteria exposed to CA cannot be attributed to a particular enzyme or group of enzymes but may actually be the reflection of the cooperative effect of various physiological mechanisms aimed to strengthening the antioxidant defenses of the cell and protect the cell against oxidative stress. Given the timely connection between bacterial protein carbonylation and the increase in the catalase-like activity in *E. faecium*, this post-translational change may be a sensing mechanism that would eventually trigger the antioxidant response as proposed by Ezraty et al. (2017). This extent requires, however, further confirmation.

4. Conclusions

This paper originally shows that CA displays a protecting effect on probiotic *E. faecium* via modulating its proteome and strengthening its endogenous antioxidant defences. These results and the hypotheses proposed here are in line with modern theories proposing that i) the overall protective effect of certain phytochemicals on the redox status of cells may not be attributed to their radical scavenging activities but actually via inducing a mild oxidative stress that stimulates endogenous antioxidant defences and that ii) protein carbonylation likely induced by the oxidized forms of such phytochemicals in a pro-oxidative environment, would play a role as a signalling mechanisms that would trigger the antioxidant strengthening mechanisms. These antioxidant mechanisms do not involve the glutathione pathway but an indefinite number of oxidoreductases such as GAPDH. Yet, this protection may be proved *in vivo* conditions to guarantee that such effects lead to benefits in experimental animals or human volunteers affected by disorders in which oxidative stress plays a major role.

CRediT authorship contribution statement

P. Padilla: Data curation, Methodology, Formal analysis, Writing – original draft. **M. Estévez:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing. **M.J. Andrade:** Data curation, Methodology, Funding acquisition, Supervision, Formal analysis, Validation, Writing – review & editing. **F.J. Peña:** Data curation, Methodology, Funding acquisition, Supervision, Formal analysis, Validation, Writing – review & editing. **J. Delgado:** Data curation, Methodology, Funding acquisition, Supervision, Formal analysis, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The financial support from the Spanish Ministry of Economics and Competitiveness (SMEC) is acknowledged (project number AGL2017-84586-R).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111464.

References

Alam, A., Bröms, J.E., Kumar, R., Sjöstedt, A., (2021). The Role of ClpB in Bacterial Stress Responses and Virulence. Frontiers Molecular Bioscience https://doi.org/10.3389/fmolb.2021.668910.

Arcanjo, N. O., Andrade, M. J., Padilla, P., Rodríguez, A., Madruga, M. S., & Estévez, M. (2019). Resveratrol protects Lactobacillus reuteri against H 2 O 2 - induced oxidative stress and stimulates antioxidant defenses through upregulation of the dhaT gene. *Free Radic. Biol. Med., 135*, 38–45. https://doi.org/10.1016/j.freeradbiomed.2019.02.023

Arena, S., D'Ambrosio, C., Renzone, G., Rullo, R., Ledda, L., Vitale, F., … Scaloni, A. (2006). A study of Streptococcus thermophilus proteome by integrated analytical procedures and differential expression investigations. *Proteomics, 6*, 181–192. https://doi.org/10.1002/pmic.200402109

Ballini, A., Santacroce, L., Cantore, S., Bottalico, L., Dipalma, G., Topi, S., … Inchingolo, F. (2019). Probiotics efficacy on oxidative stress values in inflammatory bowel disease: A randomized double-blinded placebo-controlled pilot study. Endocrine, Metab. Immune Disord. Targets (Formerly Curr. Drug Targets-Immune. *Endocr. Metab. Disord., 19*, 373–381. https://doi.org/10.2174/1871530319666181221150352

Cardona, F., Andrés-Lacueva, C., Tulipani, S., Tinahones, F. J., & Queipo-Ortuño, M. I. (2013). Benefits of polyphenols on gut microbiota and implications in human health. *Journal of Nutritional Biochemistry, 24*, 1415–1422. https://doi.org/10.1016/j.jnutbio.2013.05.001

Carpentier, S. C., Witters, E., Laukens, K., Deckers, P., Swennen, R., & Panis, B. (2005). Preparation of protein extracts from recalcitrant plant tissues: An evaluation of different methods for two-dimensional gel electrophoresis analysis. *Proteomics, 5*, 2497–2507.

Choeisoongnern, T., Sirilun, S., Waditee-Sirisattha, R., Pintha, K., Peerajan, S., & Chaiyasut, C. (2021). Potential Probiotic Enterococcus faecium OV3-6 and Its Bioactive Peptide as Alternative Bio-Preservation. *Foods, 10*, 2264.

Chuang, M. H., Wu, M. S., Lo, W. L., Lin, J. T., Wong, C. H., & Chiou, S. H. (2006). The antioxidant protein alkylhydroperoxide reductase of Helicobacter pylori switches from a peroxide reductase to a molecular chaperone function. *Proc. Natl. Acad. Sci. U. S. A., 103*, 2552–2557. https://doi.org/10.1073/pnas.0510770103

Cox, M. M. (2007). Motoring along with the bacterial RecA protein. *Nature Reviews Molecular Cell Biology, 8*, 127–138. https://doi.org/10.1038/nrm2099

Davies, M. J. (2005). The oxidative environment and protein damage. *Biochim. Biophys. Acta - Proteins Proteomics, 1703*, 93–109. https://doi.org/10.1016/j.bbapap.2004.08.007

Delgado, J., Acosta, R., Rodríguez-Martín, A., Bermúdez, E., Núñez, F., & Asensio, M. A. (2015). Growth inhibition and stability of PgAFP from Penicillium chrysogenum against fungi common on dry-ripened meat products. *International Journal of Food Microbiology, 205*, 23–29. https://doi.org/10.1016/j.ijfoodmicro.2015.03.029

Delgado, J., Núñez, F., Asensio, M. A., & Owens, R. A. (2019). Quantitative proteomic profiling of ochratoxin A repression in Penicillium nordicum by protective cultures. *International Journal of Food Microbiology, 305*, Article 108243. https://doi.org/10.1016/j.ijfoodmicro.2019.108243

Delgado, J., Owens, R. A., Doyle, S., Asensio, M. A., & Nuñez, F. (2015). Impact of the antifungal protein PgAFP from Penicillium chrysogenum on the protein profile in Aspergillus flavus. *Applied Microbiology and Biotechnology, 9*, 8701–8715.

Divyashri, G., Krishna, G., Muralidhara, P., & S.g.,. (2015). Probiotic attributes, antioxidant, anti-inflammatory and neuromodulatory effects of Enterococcus faecium CFR 3003: In vitro and in vivo evidence. *Journal of Medical Microbiology, 64*, 1527–1540. https://doi.org/10.1099/jmm.0.000184

Dos Santos, J. M., Tewari, S., & Mendes, R. H. (2019). The role of oxidative stress in the development of diabetes mellitus and its complications. *J. Diabetes Res., 2019*. https://doi.org/10.1155/2019/4189813

Estévez, M., & Heinonen, M. (2010). Effect of phenolic compounds on the formation of α-Aminoadipic and γ-Glutamic semialdehydes from myofibrillar proteins oxidized by copper, iron, and myoglobin. *Journal of Agriculture and Food Chemistry, 58*, 4448–4455. https://doi.org/10.1021/jf903757h

Estévez, M., Padilla, P., Carvalho, L., Martín, L., Carrapiso, A., & Delgado, J. (2019). Malondialdehyde interferes with the formation and detection of primary carbonyls in oxidized proteins. *Redox Biology, 26*, Article 101277. https://doi.org/10.1016/j.redox.2019.101277

Ezraty, B., Gennaris, A., Barras, F., & Collet, J.-F. (2017). Oxidative stress, protein damage and repair in bacteria. *Nature Reviews Microbiology, 15*, 385.

Farah, A., & Lima, J. (2019). Consumption of Chlorogenic Acids through Coffeeand Health Implications. *Beverages, 5*, 11. https://doi.org/10.3390/beverages5010011

Ferreira, E., Giménez, R., Alexandra Cañas, M., Aguilera, L., Aguilar, J., Badia, J., & Baldomà, L. (2015). Glyceraldehyde-3-phosphate dehydrogenase is required for efficient repair of cytotoxic DNA lesions in Escherichia coli. *International Journal of Biochemistry & Cell Biology, 60*, 202–212. https://doi.org/10.1016/j.biocel.2015.01.008

Giard, J.-C., Laplace, J.-M., Rincé, A., Pichereau, V., Benachour, A., Leboeuf, C., … Hartke, A. (2001). The stress proteome of Enterococcus faecalis. *Electrophoresis, 22*, 2947–2954. https://doi.org/10.1002/1522-2683(200108)22:14<2947::AID-ELPS2947>3.0.CO;2-K

Gurnani Serrano, C. K., Winkle, M., Martorana, A. M., Biboy, J., Morè, N., Moynihan, P., … Polissi, A. (2021). ActS activates peptidoglycan amidases during outer membrane stress in Escherichia coli. *Molecular Microbiology, 116*, 329–342.

Higuchi, M., Yamamoto, Y., & Kamio, Y. (2000). Molecular biology of oxygen tolerance in lactic acid bacteria: Functions of NADH oxidases and Dpr in oxidative stress. *Journal of Bioscience and Bioengineering, 90*, 484–493. https://doi.org/10.1016/S1389-1723(01)80028-1

Hillion, M., Imber, M., Pedre, B., Bernhardt, J., Saleh, M., Loi, V. V., … Antelmann, H. (2017). The glyceraldehyde-3-phosphate dehydrogenase GapDH of Corynebacterium diphtheriae is redox-controlled by protein S-mycothiolation under oxidative stress. *Scientific Reports, 7*, 1–14. https://doi.org/10.1038/s41598-017-05206-2

Hou, N., Liu, N., Han, J., Yan, Y., & Li, J. (2017). Chlorogenic acid induces reactive oxygen species generation and inhibits the viability of human colon cancer cells. *Anti-Cancer Drugs, 28*, 59–65. https://doi.org/10.1097/CAD.0000000000000430

Huang, J., Huang, J., Yin, T., Lv, H., Zhang, P., & Li, H. (2021). Enterococcus faecium R0026 combined with Bacillus subtilis R0179 prevent obesity-associated hyperlipidemia and modulate gut microbiota in C57BL/6 Mice. *Journal of Microbiology and Biotechnology, 31*, 181–188. https://doi.org/10.4014/JMB.2009.09005

Huang, R., Pan, M., Wan, C., Shah, N. P., Tao, X., & Wei, H. (2016). Physiological and transcriptional responses and cross protection of Lactobacillus plantarum ZDY2013 under acid stress. *Journal of Dairy Science, 99*, 1002–1010. https://doi.org/10.3168/jds.2015-9993

Jakubczyk, K., Dec, K., Kałduńska, J., Kawczuga, D., Kochman, J., & Janda, K. (2020). Reactive oxygen species - sources, functions, oxidative damage. *Pol. Merkur. Lek. organ Pol. Tow. Lek., 48*, 124–127.

Jiang, Z. Y., Woollard, A. C. S., & Wolff, S. P. (1990). Hydrogen peroxide production during experimental protein glycation. *FEBS Letters, 268*, 69–71. https://doi.org/10.1016/0014-5793(90)80974-N

Jofré, A., Champomier-Vergès, M., Anglade, P., Baraige, F., Martín, B., Garriga, M., … Aymerich, T. (2007). Protein synthesis in lactic acid and pathogenic bacteria during recovery from a high pressure treatment. *Research in Microbiology, 158*, 512–520. https://doi.org/10.1016/j.resmic.2007.05.005

Kajfasz, J. K., Mendoza, J. E., Gaca, A. O., Miller, J. H., Koselny, K. A., Giambiagi-deMarval, M., … Lemos, J. A. (2012). The Spx regulator modulates stress responses and virulence in Enterococcus faecalis. *Infection and Immunity, 80*, 2265–2275. https://doi.org/10.1128/IAI.00026-12

Kang, T. S., Korber, D. R., & Tanaka, T. (2013). Glycerol and environmental factors: Effects on 1,3-propanediol production and NAD+ regeneration in Lactobacillus panis PM1. *Journal of Applied Microbiology, 115*, 1003–1011. 10.1111/jam.12291.

Kint, C., Verstraeten, N., Hofkens, J., Fauvart, M., & Michiels, J. (2014). Bacterial Obg proteins: GTPases at the nexus of protein and DNA synthesis. *Critical Reviews in Microbiology, 40*, 207–224. https://doi.org/10.3109/1040841X.2013.776510

Lebeer, S., Claes, J.J., Vandenplas, S., Verhoeven, T.L.A., Tytgat, H., Schoofs, G., De Keersmaecker, S., and Vanderleyden, J., 2011. In vivo espression technology reveals new insights in the intestinal adaptation of Lactobacillus rhamnosus GG, in: Abstract Book in 10th Symposium on Lactic Acid Bacteria, Egmond Aan Zee, The Netherlands, August 28–September 1 2011, Poster D002.

Lee, B., & Lee, D. G. (2018). Depletion of reactive oxygen species induced by chlorogenic acid triggers apoptosis-like death in Escherichia coli. *Free Radic. Res., 52*, 605–615. https://doi.org/10.1080/10715762.2018.1456658

Li, Y., & Schellhorn, H. E. (2007). Rapid Kinetic Microassay for Catalase Activity. *J. Biomol. Tech. JBT, 18*, 185.

Loader, T. B., Taylor, C. G., Zahradka, P., & Jones, P. J. H. (2017). Chlorogenic acid from coffee beans: Evaluating the evidence for a blood pressure-regulating health claim. *Nutrition Reviews, 75*, 114–133. https://doi.org/10.1093/nutrit/nuw057

Loh, S. H., Tsai, C. S., Tsai, Y., Chen, W. H., Hong, G. J., Wei, J., … Lin, C. I. (2002). Hydrogen peroxide-induced intracellular acidosis and electromechanical inhibition in the diseased human ventricular myocardium. *European Journal of Pharmacology, 443*, 169–177. https://doi.org/10.1016/S0014-2999(02)01595-9

Lushchak, V. I. (2011). Adaptive response to oxidative stress: Bacteria, fungi, plants and animals. *Comp. Biochem. Physiol. - C Toxicol. Pharmacol., 153*, 175–190. https://doi.org/10.1016/j.cbpc.2010.10.004

Mastrogiovanni, F., Mukhopadhya, A., Lacetera, N., Ryan, M. T., Romani, A., Bernini, R., & Sweeney, T. (2019). Anti-Inflammatory Effects of Pomegranate Peel Extracts on In Vitro Human Intestinal Caco-2 Cells and Ex Vivo Porcine Colonic Tissue Explants. *Nutrients, 11*, 548.

Monedero, V., Revilla-Guarinos, A., & Zúñiga, M. (2017). Physiological Role of Two-Component Signal Transduction Systems in Food-Associated Lactic Acid Bacteria. *Advances in Applied Microbiology, 99*, 1–51. https://doi.org/10.1016/bs.aambs.2016.12.002

Muronetz, V. I., Melnikova, A. K., Saso, L., & Schmalhausen, E. V. (2020). Influence of oxidative stress on catalytic and non-glycolytic functions of glyceraldehyde-3-phosphate dehydrogenase. *Current Medicinal Chemistry, 27*, 2040–2058.

Nakajima, H., Itakura, M., Kubo, T., Kaneshige, A., Harada, N., Izawa, T., … Takeuchi, T. (2017). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) aggregation causes

mitochondrial dysfunction during oxidative stress-induced cell death. *Journal of Biological Chemistry, 292*, 4727–4742. https://doi.org/10.1074/jbc.M116.759084

Nilsson, M., Jakobsen, T. H., Givskov, M., Twetman, S., & Tolker-Nielsen, T. (2019). Oxidative stress response plays a role in antibiotic tolerance of Streptococcus mutans biofilms. *Microbiol. (United Kingdom), 165*, 334–342. https://doi.org/10.1099/mic.0.000773

Oktyabrskii, O. N., & Smirnova, G. V. (2012). Redox potential changes in bacterial cultures under stress conditions. *Microbiology, 81*, 131–142. https://doi.org/10.1134/S0026261712020099

Ordiales, E., Benito, M. J., Martín, A., Casquete, R., Serradilla, M. J., de Córdoba, M., & G.,. (2013). Bacterial communities of the traditional raw ewe's milk cheese "Torta del Casar" made without the addition of a starter. *Food Control, 33*, 448–454.

Oruc, O., Cetĩn, O., Onal Darilmaz, D., Yüsekdag, Z.N., 2021. Determination of the biosafety of potential probiotic Enterococcus faecalis and Enterococcus faecium strains isolated from traditional white cheeses. Lwt 148, 111741. https://doi.org/10.1016/j.lwt.2021.111741.

Owens, R.A., O'keeffe, G., Smith, E.B., Dolan, S.K., Hammel, S., Sheridan, K.J., Fitzpatrick, D.A., Keane, T.M., Jones, G.W., Doyle, S., 2015. Interplay between gliotoxin resistance, secretion, and the methyl/methionine cycle in Aspergillus Fumigatus. Eukaryot. Cell 14, 941–957. https://doi.org/10.1128/EC.00055-15.

Padilla, P., Andrade, M. J., Peña, F. J., Rodríguez, A., & Estévez, M. (2021). An in vitro assay of the effect of lysine oxidation end-product, α-aminoadipic acid, on the redox status and gene expression in probiotic Lactobacillus reuteri PL503. *Amino Acids*. https://doi.org/10.1007/s00726-021-03087-4

Palócz, O., Pászti-Gere, E., Gálfi, P., & Farkas, O. (2016). Chlorogenic acid combined with lactobacillus plantarum 2142 reduced LPS-induced intestinal inflammation and oxidative stress in IPEC-J2 cells. *PLoS ONE, 11*, 1–15. https://doi.org/10.1371/journal.pone.0166642

Pereira, S. F. F., Goss, L., & Dworkin, J. (2011). Eukaryote-Like Serine/Threonine Kinases and Phosphatases in Bacteria. *Microbiology and Molecular Biology Reviews, 75*, 192–212. https://doi.org/10.1128/mmbr.00042-10

Raimondi, S., Anighoro, A., Quartieri, A., Amaretti, A., Tomás-Barberán, F. A., Rastelli, G., & Rossi, M. (2015). Role of bifidobacteria in the hydrolysis of chlorogenic acid. *Microbiologyopen*. https://doi.org/10.1002/mbo3.219

Ranjbar, S., Shahmansouri, M., Attri, P., & Bogaerts, A. (2020). Effect of plasma-induced oxidative stress on the glycolysis pathway of Escherichia coli. *Computers in Biology and Medicine, 127*, Article 104064. https://doi.org/10.1016/j.compbiomed.2020.104064

Sanders, L. M., Henderson, C. E., Hong, M. Y., Barhoumi, R., Burghardt, R. C., Carroll, R. J., … Lupton, J. R. (2004). Pro-oxidant environment of the colon compared to the small intestine may contribute to greater cancer susceptibility. *Cancer Letters, 208*, 155–161. https://doi.org/10.1016/j.canlet.2003.12.007

Sanman, L. E., Qian, Y., Eisele, N. A., Ng, T. M., van der Linden, W. A., Monack, D. M., … Bogyo, M. (2016). Disruption of glycolytic flux is a signal for inflammasome signaling and pyroptotic cell death. *Elife, 5*, 1–32. https://doi.org/10.7554/eLife.13663

Sasková, L., Nováková, L., Basler, M., & Branny, P. (2007). Eukaryotic-type serine/threonine protein kinase StkP is a global regulator of gene expression in Streptococcus pneumoniae. *Journal of Bacteriology, 189*, 4168–4179. https://doi.org/10.1128/JB.01616-06

Sato, Y., Itagaki, S., Kurokawa, T., Ogura, J., Kobayashi, M., Hirano, T., … Iseki, K. (2011). In vitro and in vivo antioxidant properties of chlorogenic acid and caffeic acid. *International Journal of Pharmaceutics, 403*, 136–138. https://doi.org/10.1016/j.ijpharm.2010.09.035

Shahidi, F., & Ambigaipalan, P. (2015). Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects - A review. *Journal of Functional Foods, 18*, 820–897. https://doi.org/10.1016/j.jff.2015.06.018

Singh, D., Chang, S.-J., Lin, P.-H., Averina, O. V, Kaberdin, V.R., Lin-Chao, S., 2009. Regulation of ribonuclease E activity by the L4 ribosomal protein of Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 106, 864–869. https://doi.org/www.pnas.org_cgi_doi_10.1073_pnas.0810205106.

Sugihara, K., Morhardt, T. L., & Kamada, N. (2019). The role of dietary nutrients in inflammatory bowel disease. *Frontiers in Immunology, 10*, 1–16. https://doi.org/10.3389/fimmu.2018.03183

Tajik, N., Tajik, M., Mack, I., & Enck, P. (2017). The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: A comprehensive review of the literature. *European Journal of Nutrition, 56*, 2215–2244. https://doi.org/10.1007/s00394-017-1379-1

Tanaka, K., Satoh, T., Kitahara, J., Uno, S., Nomura, I., Kano, Y., … Kawasaki, S. (2018). O2-inducible H2O2-forming NADPH oxidase is responsible for the hyper O2 sensitivity of Bifidobacterium longum subsp. infantis. *Scientific Reports, 8*, 10750. 10.1038/s41598-018-29030-4.

Teng, F., Nannini, E. C., & Murray, B. E. (2005). Importance of gls24 in virulence and stress response of Enterococcus faecalis and use of the Gls24 protein as a possible immunotherapy target. *Journal of Infectious Diseases, 191*, 472–480. https://doi.org/10.1086/427191

Tomas-Barberan, F., García-Villalba, R., Quartieri, A., Raimondi, S., Amaretti, A., Leonardi, A., & Rossi, M. (2014). In vitro transformation of chlorogenic acid by human gut microbiota. *Molecular Nutrition & Food Research, 58*, 1122–1131. https://doi.org/10.1002/mnfr.201300441

Tomusiak-Plebanek, A., Heczko, P., Skowron, B., Baranowska, A., Okoń, K., Thor, P. J., & Strus, M. (2018). Lactobacilli with superoxide dismutase-like or catalase activity are more effective in alleviating inflammation in an inflammatory bowel disease mouse model. *Drug Des. Devel. Ther., 12*, 3221–3233. https://doi.org/10.2147/DDDT.S164559

Tsai, K. L., Wang, S. M., Chen, C. C., Fong, T. H., & Wu, M. L. (1997). Mechanism of oxidative stress-induced intracellular acidosis in rat cerebellar astrocytes and C6 glioma cells. *Journal of Physiology, 502*, 161–174.

Utrera, M., & Estévez, M. (2012). Analysis of tryptophan oxidation by fluorescence spectroscopy: Effect of metal-catalyzed oxidation and selected phenolic compounds. *Food Chemistry, 135*, 88–93. https://doi.org/10.1016/j.foodchem.2012.04.101

Vesić, D., & Kristich, C. J. (2013). A rex family transcriptional repressor influences H2O2 accumulation by Enterococcus faecalis. *Journal of Bacteriology, 195*, 1815–1824. https://doi.org/10.1128/JB.02135-12

Wang, Z., Clifford, M. N., & Sharp, P. (2008). Analysis of chlorogenic acids in beverages prepared from Chinese health foods and investigation, in vitro, of effects on glucoseabsorption in cultured Caco-2 cells. *FoodChemistry, 108*, 369–373.

Wang, Z., Zhai, X., Sun, Y., Yin, C., Yang, E., Wang, W., & Sun, D. (2020). Antibacterial activity of chlorogenic acid-loaded SiO2 nanoparticles caused by accumulation of reactive oxygen species. *Nanotechnology, 31*. https://doi.org/10.1088/1361-6528/ab70fb

Yang, H., He, M., & Wu, C. (2021). Cross protection of lactic acid bacteria during environmental stresses: Stress responses and underlying mechanisms. *Lwt, 144*, Article 111203. https://doi.org/10.1016/j.lwt.2021.111203

Zhang, H., Zhang, C., Liu, H., Chen, Q., & Kong, B. (2021). Proteomic response strategies of Pediococcus pentosaceus R1 isolated from Harbin dry sausages to oxidative stress. *Food Biosci., 44*, Article 101364. https://doi.org/10.1016/j.fbio.2021.101364

Zhang, Y., Wang, Y., Chen, D., Yu, B., Zheng, P., Mao, X., … He, J. (2018). Dietary chlorogenic acid supplementation affects gut morphology, antioxidant capacity and intestinal selected bacterial populations in weaned piglets. *Food & Function, 9*, 4968–4978. https://doi.org/10.1039/c8fo01126e

Zhou, A., He, Z., Redding-Johanson, A. M., Mukhopadhyay, A., Hemme, C. L., Joachimiak, M. P., … Zhou, J. (2010). Hydrogen peroxide-induced oxidative stress responses in Desulfovibrio vulgaris Hildenborough. *Environmental Microbiology, 12*, 2645–2657. https://doi.org/10.1111/j.1462-2920.2010.02234.x

Zuber, P. (2004). Spx-RNA Polymerase Interaction and Global Transcriptional Control during Oxidative Stress. *Journal of Bacteriology, 186*, 1911–1918. https://doi.org/10.1128/JB.186.7.1911-1918.2004