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Original article

Resveratrol protects *Lactobacillus reuteri* against H_2O_2- induced oxidative stress and stimulates antioxidant defenses through upregulation of the *dhaT* gene

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ABSTRACT

Understanding of the mechanisms implicated in the protective role of probiotic bacteria is of the utmost scientific interest. This study provides original insight into the genetic and molecular basis of the responses of *Lactobacillus reuteri* PL503 against hydrogen peroxide (H_2O_2)-induced oxidative stress. Six experimental groups were considered depending on the addition and concentration of H_2O_2 and resveratrol: 1. CONTROL (*L. reuteri* in MRS broth); 2. H_2O_2 (*L. reuteri* in MRS broth + 0.5 mM H_2O_2); 3. LRES (*L. reuteri* in MRS broth + 20 μM resveratrol); 4. HRES (*L. reuteri* in MRS broth + 100 μM resveratrol); 5. H_2O_2-LRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 20 μM resveratrol); 6. H_2O_2-HRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 100 μM resveratrol). Three replicates were incubated at 37 °C for 24 h in microaerophilic conditions sampled at 12, 16, 20 and 24 h. The NADH-dependent-oxidoreductase encoded by the *dhaT* gene is a plausible candidate to be strongly implicated in the antioxidant response of *L. reuteri*. Resveratrol (100 μM) is found to protect *L. reuteri* against protein carbonylation plausibly through various mechanisms including direct scavenging of reactive oxygen species (ROS), upregulation of the *dhaT* gene and promoting the synthesis of sulfur containing compounds. The hypothesis formulated on the ability of *L. reuteri* to detoxify H_2O_2 and its underlying mechanism needs to be clarified. Furthermore, the consequences of protein carbonylation as a reflection of oxidative damage to bacteria and its role in the responses of bacteria to oxidative stress need to be further investigated.

1. Introduction

The colon is known to be particularly susceptible to oxidative stress owing to an intense generation of reactive oxygen species (ROS) that frequently exceed antioxidant capabilities [1]. Some dietary components and, very particularly, red meat has been identified as a source of pro-oxidant species [2] and has been further found linked to the onset of colorectal cancer (CRC) by the International Agency for Research of Cancer (IARC) [3]. In this scenario, ROS and other reactive species naturally present in red meat (hydrogen peroxide, transition metals and heme molecules, among others) or formed during its digestion (carbonyls, and nitroso-compounds, among others) cause oxidative and nitrosative damage to proteins and lipids from the intestinal mucosa [4]. An enduring chemical injury at this location leads to pathological conditions in which oxidative stress is known to play a relevant role, including inflammatory bowel disease (IBD), ulcerative colitis and the aforementioned CRC [5,6]. In relation to the onset of oxidation-driven diseases in human individuals, the oxidation of proteins is highlighted as a remarkable molecular feature of most pathological conditions as protein oxidation leads to dysfunction, impairment of physiological patterns and inflammation [7]. Recently, Estévez & Luna [4] emphasized the transfer of dietary protein oxidation products from the lumen (luminal oxidative stress) to the intestinal mucosa and from there to the bloodstream (organic oxidative stress), leading to alteration in target internal organs. In both, foods and living systems, the hydroxyl radical ($HO^{\bullet}$) typically formed from the Fenton reaction (Eq. (1)) in the presence of hydrogen peroxide (H_2O_2) and transition metals (M) (Fe and Cu, among others) is identified as the most efficient radical species in biological systems and causes a variety of oxidative modifications in proteins, including carbonylation, cross-linking and aggregation, loss of

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functional groups, among others [7].

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

Probiotic bacteria are a natural protective barrier between potential harmful effects of dietary components and the intestinal mucosa. The protective role of microbiota on this and other processes is gaining increasing interest owing to their abilities to counteract the luminal oxidative stress in humans [8]. Probiotics are designated as "live microbes which, when administered in adequate amounts, confer a health benefit to the host" [9]. *Lactobacillus reuteri* is a natural colonizer of the gastrointestinal tract in humans and animals and has been widely used as a dietary supplement to enhance human gut health [10]. Oral administration of *L. reuteri* reduces gastrointestinal disorders and infections and contributes to a balanced colonic microbiota [10]. According to the mechanisms related to its probiotic effects, *L. reuteri* has been reported to protect against oxidative stress and inhibits the accretion of oxidation products in the lumen [11]. While the benefits of *L. reuteri* against oxidative stress and gut disorders are documented [12], the molecular mechanisms implicated in the responses of this probiotic bacterium under specific pro-oxidant conditions (such as those induced by a hydroxyl-radical generating system) are not defined yet.

On the other hand, certain dietary components with antioxidant properties may also be used to neutralize the pro-oxidant action of ROS and avoid and/or alleviate the symptoms of bowel disorders [8]. Phytochemicals with assorted bioactivities have been profusely studied for their potential health benefits in the gut. The review article rendered by Biasi et al. [13] includes scientific evidences of the ability of wine components to regulate inflammation and redox-signaling in intestinal cells. Phenolic compounds such as resveratrol, are able to act directly, as free-radical scavengers and indirectly, modulating gene expression and promoting the endogenous antioxidant defenses and protection against IBD and CRC through the activation of the NF-kB transcription factor. Interestingly, wine phenolic compounds have also been praised as prebiotics as they may be able to exert benefits to the colonic bacterial population. Hence, both wine phenolics and probiotics may protect against luminal oxidative stress and reduce intestinal injury.

The study of the molecular interactions between dietary oxidation products, phytochemicals and human probiotic bacteria requires innovative methods in molecular biology. Proteins act as modulators and executors of most biological functions and hence, their oxidative damage induced by ROS, leads to dysfunction and hence, to impaired physiological processes, and depending on extent of damage, to a pathological condition [14]. From a medical perspective, the study of protein oxidation is essential to understand the molecular basis of diseases in which oxidative stress plays a major role. To this regard, little information is available on the biological responses of probiotic bacteria to specific oxidative species and in particular, in the presence of bioactive compounds such as resveratrol. On the same line, the analysis of gene expression is a valuable and highly specific tool to understand how external factors precisely influence on particular biological functions and metabolic routes. For instance, advanced genomic tools have been crucial to reveal the molecular basis of the beneficial effects of resveratrol on tumorigenic colon cells [15] and also to understand the antiproliferative effects of *L. reuteri* on human myeloid leukemia-derived cells [16]. The genetic and molecular responses of *L. reuteri* in the presence of resveratrol are, to our knowledge, unknown.

This study is a preliminary approximation to the understanding of the protective effect that the combination of *L. reuteri* bacterium and resveratrol may have against H_2O_2-induced oxidative stress. To provide mechanistic insight, molecular and genetic responses of *L. reuteri* to the oxidative threat were analyzed.

2. Material and methods

2.1. Chemicals and microorganism

All chemicals and reagents used in this study were of ACS analytical grade and purchased from Sigma Chemicals (Sigma-Aldrich, Germany), Scharlab S.L. (Spain), Pronadisa (Conda Laboratory, Spain), Applied Biosystems (USA), Epicentre (USA) and Acros Organics (Spain). *L. reuteri* PL503 was isolated from pig faeces and identified by 16S rRNA sequencing by Ruiz-Moyano et al. [17].

2.2. Experimental setting

The strain used in this study, *L. reuteri* PL503, was maintained as glycerol freezer stocks at −80 °C. For preparing working cultures, this strain was consecutively grown twice in Man Rogosa Sharpe (MRS; Conda Laboratory) broth plus 0.5% acetic acid 10% (v/v) at 37 °C for 24 h each. Six experimental groups were considered depending on the addition and concentration of H_2O_2 and resveratrol: 1. CONTROL (*L. reuteri* in MRS broth); 2. H_2O_2 (*L. reuteri* in MRS broth + 0.5 mM H_2O_2); 3. LRES (*L. reuteri* in MRS broth + 20 μM resveratrol); 4. HRES (*L. reuteri* in MRS broth + 100 μM resveratrol); 5. H_2O_2-LRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 20 μM resveratrol); 6. H_2O_2-HRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 100 μM resveratrol). Three replicates were carried out for each treatment. Experimental tubes were inoculated with 100 μL of the last overnight culture of *L. reuteri* PL503 in MRS broth and incubated at 37 °C for up to 24 h in microaerophilic conditions. Samples of the cultures were collected in four times (12, 16, 20 and 24 h) from the inoculation. For further protein analyses, culture medium was removed by washing with a phosphate buffered saline (PBS, pH 7.4) solution twice. For count of viable cells, 100 μL of *L. reuteri* PL503 were scattered on MRS agar at the same time and conditions as the experimental tubes.

2.3. Gene expression

After incubation time, 1 mL of each treatment was immediately frozen and stored at −80 °C until RNA extraction procedure, which was performed using the MasterPure™ RNA purification kit (Epicentre, USA) following manufacturer's instructions. To remove genomic DNA contamination, samples were diluted to a concentration of 0.1 μg/μL and treated with DNase I (Thermo Fisher Scientific, USA). Pure RNA was eluted in 35 μl TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA) and kept at −80 °C until required. The RNA concentration (ng/μL) and purity (A_{260}/A_{280} ratio) were spectrophotometrically determined using a 1.5 μL aliquot on the Nanodrop 2000 (Thermo Scientific, USA).

Next, two-step reverse transcription real-time PCR (RT-qPCR) were performed to conduct gene expression studies. For this, the synthesis of cDNA was firstly performed using about 500 ng of total RNA following manufacturer instructions of the PrimeScript™ RT Reagent kit (Takara Bio Inc., Japan). The obtained cDNA was stored at −20 °C until further use in qPCR reactions.

The qPCR reactions were carried out on an Applied Biosystems ViiA™ 7 Real-Time System (Applied Biosystems) in MicroAmp optical 96-well reaction plates sealed with optical adhesive covers (Applied Biosystems). The SYBR Green technology was used. The reaction mixtures of a final volume of 12.5 μL consisted of 2.5 μL of cDNA template, 6.25 μL of SYBR® *Premix Ex Taq*™ (Takara Bio Inc.), 0.625 μL of ROX™ Reference Dye (Takara Bio Inc.), 2.37 μL of sterile ultrapure water and 300 mM of each primer pair (Table 1). The qPCR methods for amplification of *uspA*, *dhaT* and 16S rRNA genes were conducted with the following thermal cycling conditions: a single step of 10 min at 95 °C, 40 cycles at 95 °C for 15 s and annealing temperature, 55 or 60 °C, for 30 s. After the final qPCR cycle, melting curves were performed by heating the products from 60 °C to 99 °C and the values of the melting temperatures were checked to ensure the truthfulness of the results and

Table 1
Sequences of primers used for reverse transcription real-time PCR assays to conduct gene expression analyses.

Primers	Gene	Nucleotide sequence (5′- 3′)	Annealing temperature	References
uspALr-F1	*uspA*	CTTGGGTAGCGTTCACCATT	60 °C	This study
uspALr-R1		TGAAAAAGCGGTTGACACTG	60 °C	This study
LS67	*dhaT*	TGACTGGATCCTAATTTGGTCCTGGTGTTATTGC	60 °C	Schaefer et al., 2010
LS68		TGACTGAATTCTTCCGGATCTTAGGGTTAGG	60 °C	Schaefer et al., 2010
Lr16S_F	16S rRNA	CCGCTTAAACTCTGTTGTTG	55 °C	This study
Lr16S_R		CGTGACTTTCTGGTTGGATA	55 °C	This study

specificity of the primer pairs. Threshold cycle (C_T) values represent the PCR cycle in which an increase in fluorescence, over a defined threshold, first occurred for each amplification plot. All samples were analyzed in triplicate, including control sample consisting of adding sterile ultrapure water instead of template cDNA, and qPCR reactions were repeated at least twice. The relative gene expression ratio was calculated using the $2-\frac{\Delta\Delta C}{T}$ method reported by Livak & Schmittgen [18]. The 16S rRNA gene was used as endogenous control for the relative quantification of the expression of the *uspA* and *dhaT* genes. The experimental group CONTROL was used as calibrator at each sampling time.

2.4. Synthesis of α-aminoadipic semialdehyde (AAS) standard compound

N-Acetyl-L-AAS was synthesized from Nα-acetyl-L-lysine using lysyl oxidase activity from egg shell membrane following the procedure described by Akagawa et al. [19]. Briefly, 10 mM Nα-acetyl-L-lysine was incubated with constant stirring with 5 g egg shell membrane in 50 mL of 20 mM sodium phosphate buffer, pH 9.0 at 37 °C for 24 h. The egg shell membrane then was removed by centrifugation and the pH of the solution adjusted to 6.0 using 1 M HCl. The resulting aldehydes were reductively aminated with 3 mmol ABA (4- aminobenzoic acid) in the presence of 4.5 mmol sodium cyanoborohydride ($NaBH_3CN$) at 37 °C for 2 h with stirring. Then, ABA derivatives were hydrolyzed by 50 mL of 12 M HCl at 110 °C for 10 h. The hydrolysates were evaporated at 40 °C to dryness. The resulting AAS-ABA was purified by using silica gel column chromatography and ethyl acetate/acetic acid/water (20:2:1, v/v/v) as elution solvent. The purity of the resulting solution and authenticity of the standard compounds obtained following the aforementioned procedures have been checked by using MS and ^{1}H NMR [19,20].

2.5. Quantification of AAS

Five hundred microliters of culture were dispensed in 2 mL microtubes and treated with cold 10% TCA (Trichloroacetic acid) solution. Each microtube was vortexed and then subjected to centrifugation at 5000 rpm for 5 min at 4 °C. The supernatants were removed and the pellets were incubated with the following freshly prepared solutions: 0.5 mL 250 mM 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 6.0 containing 1 mM diethylenetriaminepentaacetic acid (DTPA), 0.5 mL 50 mM ABA in 250 mM MES buffer pH 6.0 and 0.25 mL 100 mM $NaBH_3CN$ in 250 mM MES buffer pH 6.0. The tubes were vortexed and then incubated in water bath at 37 °C for 90 min. The samples were stirred every 15 min. After derivatization, samples were treated with a cold 50% TCA solution and centrifuged at 10000 rpm for 10 min. The pellets were then washed twice with 10% TCA and diethyl ether-ethanol (1:1). Finally, the pellets were treated with 6 N HCl and kept in an oven at 110 °C for 18 h until completion of hydrolysis. The hydrolysates were dried *in vacuo* in a centrifugal evaporator. The generated residue was reconstituted with 200 µL of milliQ water and then filtered through hydrophilic polypropylene GH Polypro (GHP) syringe filters (0.45 µm pore size, Pall Corporation, USA) for HPLC analysis.

A Shimadzu 'Prominence' HPLC apparatus (Shimadzu Corporation, Kyoto, Japan), equipped with a quaternary solvent delivery system (LC-20AD), a DGU-20AS on-line degasser, a SIL-20A auto-sampler, a RF-10A XL fluorescence (FLD) detector, and a CBM-20A system controller, was used. An aliquot (1 µL) from the reconstituted protein hydrolysates was injected and analyzed in the above mentioned HPLC equipment. AAS-ABA was eluted in a Cosmosil $5C_{18}$-AR-II RP-HPLC column (5 µm, 150 × 4.6 mm) equipped with a guard column (10 × 4.6 mm) packed with the same material. The flow rate was kept at 1 mL/min and the temperature of the column was maintained constant at 30 °C. The eluate was monitored with excitation and emission wavelengths set at 283 and 350 nm, respectively. Standards (0.1 µL) were run and analyzed under the same conditions. Identification of both derivatized semialdehydes in the FLD chromatograms was carried out by comparing their retention times with those from the standard compounds. The peak corresponding to AAS-ABA was manually integrated from FLD chromatograms and the resulting areas plotted against an ABA standard curve with known concentrations that ranged from 0.1 to 0.5 mM. Results were expressed as nmol of carbonyl compound per mg of protein.

2.6. Analysis of protein thiols

To avoid possible contamination with thiols from the medium, 250 µL culture was washed twice with PBS and with ethanol:ethyl acetate (1:1), the pellet was resuspended in 250 µL of guanidine hydrochloride and added to the cuvette in a final volume of 1.250 µL of guanidine hydrochloride. Absorbance was measured at 324 nm, pre and post addition of 250 µL of 4 DPS (4,4′-Dipyridyl disulfide) in 12 mM HCl. Results were expressed as µmol of free thiol groups per mg of protein.

2.7. Statistical analysis

Data from the analysis (n = 3) were collected and subjected to statistical analyses. In order to assess the effect of different concentrations of resveratrol in the presence of H_2O_2 on the genetic and chemical expression of the bacterium an analysis of variance (ANOVA) was applied (SPSS v. 15.5). A Tukey test was applied when ANOVA found significant differences between treatments. The statistical significance was set at $p \leq 0.05$.

3. Results and discussion

3.1. Genetic responses of L. reuteri to H_2O_2-induced oxidative stress

The counts of *L. reuteri* during the whole experimental assay show that the bacterium remained stable and that the sublethal dose of H_2O_2 applied (0.5 mM) did not compromise the survival of this bacterium. This is, indeed, an indication that *L. reuteri* exerted mechanisms to counteract the pro-oxidant actions of H_2O_2. In the present study, these actions were firstly assessed by the analysis of the expression of stress-related genes. Fig. 1A shows the relative expression ($2_T^{-\Delta\Delta C}$) of the *uspA* gene during the incubation period (37 °C/24 h) of *L. reuteri* challenged with H_2O_2 and resveratrol. The universal stress protein A (*uspA*) superfamily comprehends an ancient and conserved group of proteins found in assorted microorganisms, insects and plants. Although the regulation of the *uspA* gene has been tried to be ascertained in bacteria

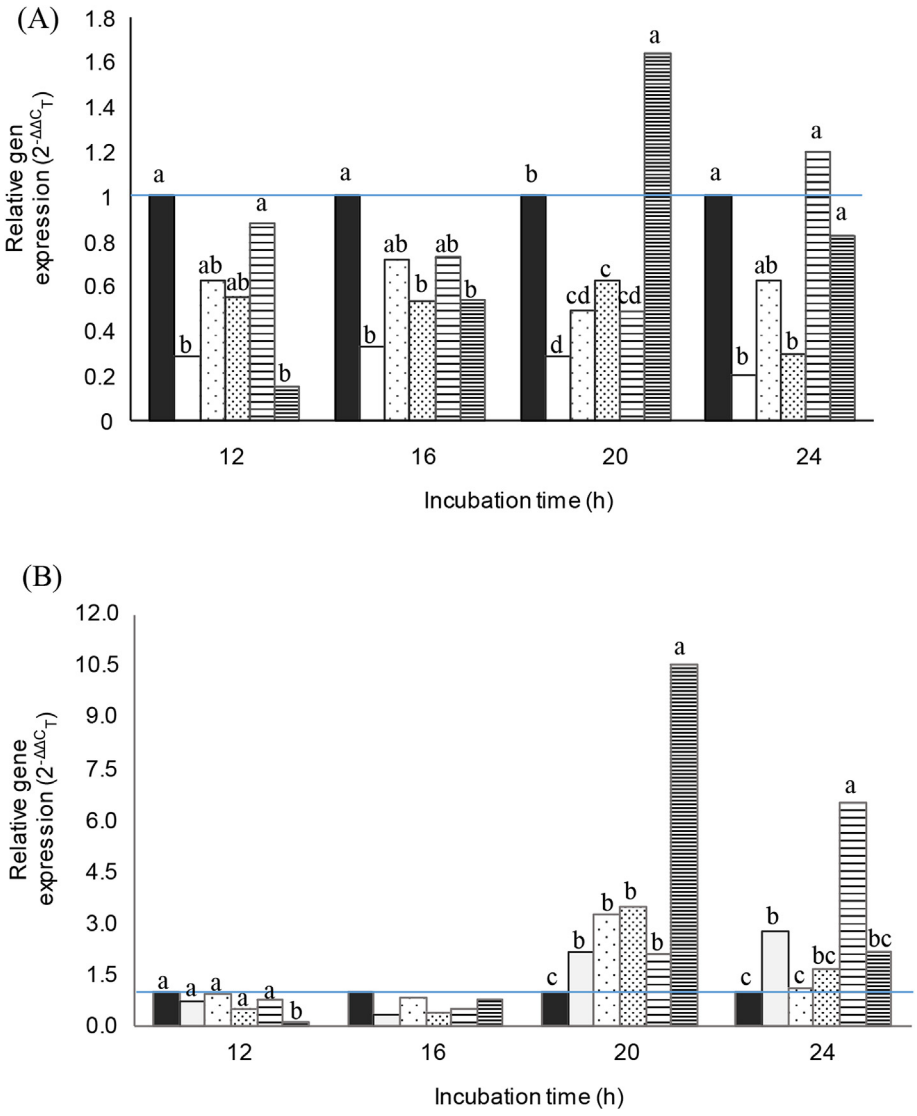

Fig. 1. Relative expression of the u*spA* (A) and *dhaT* (B) genes in *Lactobacillus reuteri* during an incubation period for up to 24 h. Blue line at $2_T^{-\Delta\Delta C} = 1$ denotes standarized expression rate for CONTROL group at each sampling time (Calibrator). $2_T^{-\Delta\Delta C} < 1$ denotes suppression of gene expression; $2_T^{-\Delta\Delta C} > 1$ denotes activation of gene expression. Different letters on top of bars indicate significant differences between treatments within a sampling point. Experimental groups: ■ CONTROL (*L. reuteri* in MRS broth); □ H_2O_2 (*L. reuteri* in MRS broth + 0.5 mM H_2O_2); ⚀ LRES (*L. reuteri* in MRS broth + 20 μM resveratrol); ⚄ HRES (*L. reuteri* in MRS broth + 100 μM resveratrol); ⚅ H_2O_2-LRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 20 μM resveratrol); ▤ H_2O_2-HRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 100 μM resveratrol). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(namely *Escherichia coli*), the precise roles of Usp proteins remain unclear; yet, they seem to be linked to struggle against DNA-damaging agents [21]. Oxidative stress may be considered as a major cause of cell damage through various mechanisms including metabolic pathway disruptions, spontaneous mutations and bacteriostatic/bactericidal effects. Lactic acid bacteria (LAB) are particularly sensitive to ROS such as H_2O_2 due to the lack of catalases and other enzymes associated with the defense against oxidative stress. Unexpectedly, the incubation of *L. reuteri* in the presence of H_2O_2 led to a down regulation of the *uspA* gene during the entire assay. This observation is, however, consistent with findings obtained by Oberg et al. [22] who observed a significant inhibition of the *uspA* gene expression in *Bifidobacterium longum* exposed to a H_2O_2-induced stress. These authors only found upregulation of an ATP-dependent metalloprotease which was hypothesized to act in response to the oxidative damage to membrane proteins. To similar conclusions came Jin et al. [23] who studied the tolerance of the same *Bifidobacterium* to organic acids using a genomic approach. Unfortunately, results from these previous studies did not provide insight into the underlying antioxidant mechanisms of LAB. Based on our initial hypothesis of resveratrol being a prebiotic and potential enhancer of the probiotic effects of LAB, this phytochemical was tested at two biologically relevant concentrations (20 and 100 μM) alone or in combination with H_2O_2. The incubation of *L. reuteri* with resveratrol also led to a downregulation of the *uspA* gene expression though the effect was not as remarkable as for H_2O_2. No dose effect was observed in this regard though the high concentration of resveratrol (100 μM) led to a suppression of the *uspA* gene similar to that displayed by H_2O_2. Only the combination of H_2O_2 with 100 and 20 μM of resveratrol at 20 and 24 h of incubation, respectively, led to a significant increase of the *uspA* gene expression, reflecting a yet indefinite potential synergistic effect of both chemical species on the response of *L. reuteri* to a pro-oxidative stress.

Unlike the heterogeneous group of proteins encoded by the *uspA* gene, the expression of the other gene under study (*dhaT*) leads to the synthesis of a single protein with definite function, the 1,3-propanediol oxidoreductase (1,3-PDO). This enzyme has lately attracted considerable attention given its ability to produce 1,3-propanediol (1,3-PD) from glycerol under anaerobic conditions (Fig. 2A). In a first step, a coenzyme B12-dependent glycerol dehydratase, catalyzes the formation of 3-hydroxypropionaldehyde (3-HPA) from glycerol and in a second step, 3-HPA is reduced to 1,3-PD [24]. It is worth highlighting that, in this latter reaction, NADH is regenerated into NAD^+, which is required to ferment glucose through its main carbohydrate fermentation pathway (6-phosphogluconate/phosphoketolase; 6-PG/PK) [24]. Hence, supplementation with glycerol and the activation of this route have been found to stimulate the ability of *L. reuteri* to grown on the presence of carbohydrates [24]. Furthermore, this route plays a major role in the probiotic properties of *L. reuteri* as the intermediate product

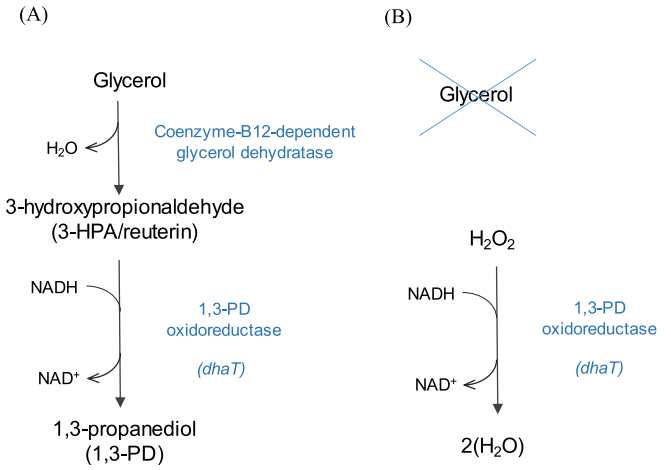

Fig. 2. Production of 3-HPA (reuterin) and 1,3-PD from glycerol in *Lactobacillus reuteri* in accordance to the description made by Talarico et al. [24] (A) and proposed mechanism by which *dhaT*–encoded 1,3-PD-NAD + oxidoreductase may detoxify hydrogen peroxide (B).

(3-HPA) is also referred as to reuterin, which is known to be excreted in copious amounts to the surrounding environment, imparting strong antimicrobial properties [25].

Highly remarkable changes in the expression of this gene were observed during the incubation of *L. reuteri* as affected by the addition of H_2O_2 and two concentrations of resveratrol (Fig. 1B). During the first 16 h, the effects shifted between no significant influence on the *dhaT* gene expression (i.e. LRES at 12 and 16 h) to a moderate down-regulation of such gene (i.e. H_2O_2-HRES at 12 h). During the second half of the incubation assay, the effect of the incubation conditions changed dramatically as an upregulation of the gene under study was observed in all the experimental groups (except LRES at 24 h). The sole addition of the pro-oxidant threat (0.5 mM H_2O_2) led to an over-expression of the *dhaT* gene which is a reflection that 1,3-PDO may be implicated in some kind of response to counteract the harmful effect of H_2O_2. Interestingly, *L. reuteri* was grown in the absence of glycerol in the present study, so the activation of the route previously described (Fig. 2A) to generate NAD^+ from 3-HPA seems unlikely. While the compound identified as the main substrate of the 1,3-DPO was plausibly absent, the gene encoding for such enzyme was being upregulated under oxidative stress conditions. We propose that the NAD^+-dependent activity of the 1,3-PDO may be able to detoxify H_2O_2 in the presence of NADH in accordance to the pathway depicted in Fig. 2B. Whereas this extent may need *in vitro* confirmation, this would be, to our knowledge the first time that the *dhaT* gene and 1,3-PDO are reported to be implicated in the antioxidant defense of LAB against H_2O_2. Kang et al. [26] highlighted a NADH oxidase-NADH peroxidase system as a common oxidative stress resistance mechanism for *Lactobacillus panis* grown in aerobic culture. In such conditions and using O_2 as electron acceptor, an NADH oxidase enzyme produced up to 100 μM of H_2O_2, with this pro-oxidant species being subsequently detoxified by a NADH peroxidase activity that authors ascribed to be decoded by an *npx* gene. While the *in vitro* NADH peroxidase activity was intense in aerobic culture (ca. 200 μM H_2O_2 decomposed per minute) and negligible in microaerobic conditions (non-detected), the expression of the *npx* gene did not seem to be affected by the culture conditions (relative expression of the *npx* gene: 1.00 ± 0.10 vs 1.16 ± 0.28 in microaerobic and aerobic conditions, respectively), suggestive of the lack of connection between such peroxidase activity and the gene expression. Interestingly, the authors found limited 1,3-PD production by *L. panis* in the aerobic conditions even though glycerol was supplied. It is reasonable to hypothesize that a H_2O_2-dependent peroxidase activity of 1,3-PDO would have contributed to detoxify H_2O_2 while the usage of 3-HPA (from glycerol metabolism) as preferential substrate played a secondary role. The lack of specific substrate in NADH-dependent enzymes has been described in literature when involved in redox-reactions [27] and other LAB such as *B. longum* has been found to lack NADH peroxidase and employs a NADH oxidoreductase to protect itself against H_2O_2-induced oxidative stress [28]. These observations provide strength to our hypothesis.

The incubation of *L. reuteri* with resveratrol induced an over-expression of the *dhaT* gene to a larger extent than H_2O_2 (20 h) and no dose-dependent effect was observed (Fig. 1B). The ability of resveratrol to protect against oxidative stress in biological systems has been profusely documented and the underlying mechanisms involve not only its radical-scavenging activity but also modulation of gene expression to improve the endogenous antioxidant and immune defenses of the biological system [13]. As for the objective of the present study, the impact of resveratrol on the redox homeostasis of the gut is of particular relevance and authors have described the ability of this phytochemical to promote the growth and health of *Bifidobacterium* and *Lactobacillus* spp. at the expense of pathogens such as *Clostridium* [29]. The upregulation of the *dhaT* gene by resveratrol was extraordinarily enlarged when this phytochemical was provided together with H_2O_2. The relative expression of the *dhaT* gene was 11 and 7 times higher in H_2O_2-HRES at 20 h and H_2O_2-LRES at 24 h than the CONTROL group, illustrating the clear protective effect of resveratrol against oxidative stress through gene expression regulation and that such protective effect is dependent on the concentration of both, H_2O_2 and resveratrol. The striking resveratrol-driven response of *L. reuteri* against H_2O_2 was more intense and appeared earlier (20 h) when the concentration of the phytochemical was higher (100 μM). The gene expression responses observed in the present study may account for some of the prebiotic effects attributed to resveratrol. Authors such as Jang & Surth [30], Konyalioglu et al. [31] and Bosutti & Degens [32] have proven the protective effect of resveratrol on H_2O_2-induced oxidative stress in assorted human and rat cells, and alike the present study, observed a dose-dependent interaction of both molecules and reported the ability of the phytochemical to strengthen the endogenous antioxidant defenses by gene expression regulation.

3.2. Chemical responses of *L. reuteri* to H_2O_2-induced oxidative stress

Oxidative stress causes damage to biomolecules and proteins are preferential targets of ROS. The damage caused to proteins by ROS under pro-oxidative conditions has been highlighted as one of the most remarkable causes of aging and disease in humans [4,33]. In a recent review, Ezraty et al. [34] reported that oxidative damage has a devastating effect on the structure and functionality of proteins and may even lead to cell death. This is particularly applicable to anaerobic LAB as they lack catalases and other regular antioxidant enzymes to neutralize the pernicious effects of ROS [35]. Interestingly, the oxidative damage to proteins is also involved in the bacterial response to oxidative stress [34] as proteins are activated by oxidative means to trigger specific antioxidant mechanisms. Despite the undeniable significance of protein oxidation in bacterial biology, scientific literature lacks the description of the oxidative damage to bacterial proteins challenged under pro-oxidant conditions. In the present study we aimed to quantify AAS as a specific marker of the oxidative damage to proteins and free thiols as relevant redox-active moieties in proteins.

3.2.1. Protein carbonylation

The evolution of the concentration of AAS in *L. reuteri* during the incubation assay (37 °C/24 h) is shown in Table 2. AAS is formed in biological systems as a result of the oxidative deamination of lysine residues in proteins, is emphasized as the most abundant protein carbonyl and commonly used in biology and medicine as a marker of oxidative stress, aging and disease [36,37]. Ballesteros et al. [38] also proposed protein carbonylation as a reflection of bacterial senescence and that, as occurs in eukaryotes, oxidized proteins accumulate in non-

Table 2
Concentration of α-aminoadipic semialdehyde (AAS; nmol/mg protein) in *Lactobacillus reuteri* during incubation assay (37 °C/24 h).

	CONTROL	H_2O_2	LRES	HRES	H_2O_2-LRES	H_2O_2-HRES	p^A
0 h	$0.30^{v} \pm 0.03$	$0.31^{v} \pm 0.04$	$0.32^{v} \pm 0.03$	$0.27^{v} \pm 0.04$	$0.29^{v} \pm 0.03$	$0.27^{v} \pm 0.04$	ns
12 h	$0.32^{d,v} \pm 0.04$	$1.89^{a,x} \pm 0.23$	$0.50^{c,w} \pm 0.12$	$0.41^{cd,w} \pm 0.10$	$0.99^{b,w} \pm 0.15$	$0.95^{x} \pm 0.29$	***
16 h	$0.45^{d,vw} \pm 0.13$	$2.84^{a,y} \pm 0.31$	$1.06^{b,x} \pm 0.21$	$0.58^{cd,wx} \pm 0.18$	$0.96^{b,w} \pm 0.18$	$0.62^{c,w} \pm 0.22$	***
20 h	$1.48^{b,x} \pm 0.29$	$3.44^{a,z} \pm 0.55$	$1.77^{b,y} \pm 0.39$	$0.79^{c,x} \pm 0.22$	$1.54^{b,x} \pm 0.39$	$1.84^{b,y} \pm 0.41$	***
24 h	$0.71^{b,w} \pm 0.23$	$1.27^{a,w} \pm 0.28$	$0.99^{b,x} \pm 0.31$	$0.38^{c,w} \pm 0.08$	$0.81^{b,w} \pm 0.21$	$0.85^{b,wx} \pm 0.24$	**
p^B	***	***	***	**	***	***	

Data expressed as means ± standard deviations of three replicates.
Experimental groups: 1. CONTROL (*L. reuteri* in MRS broth); 2. H_2O_2 (*L. reuteri* in MRS broth + 0.5 mM H_2O_2); 3. LRES (*L. reuteri* in MRS broth + 20 μM resveratrol); 4. HRES (*L. reuteri* in MRS broth + 100 μM resveratrol); 5. H_2O_2-LRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 20 μM resveratrol); 6. H_2O_2-HRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 100 μM resveratrol).
[A] Significance from ANOVA denotes differences between means from the same row. **: $p \leq 0.01$; ***: $p \leq 0.001$; ns: no significant.
[B] Significance from ANOVA denotes differences between means from the same column. **: $p \leq 0.01$; ***: $p \leq 0.001$.
Superscripts (a-d) within the same row denote significant differences between experimental groups.
Superscripts (v-z) within the same column denote significant differences between sampling times.

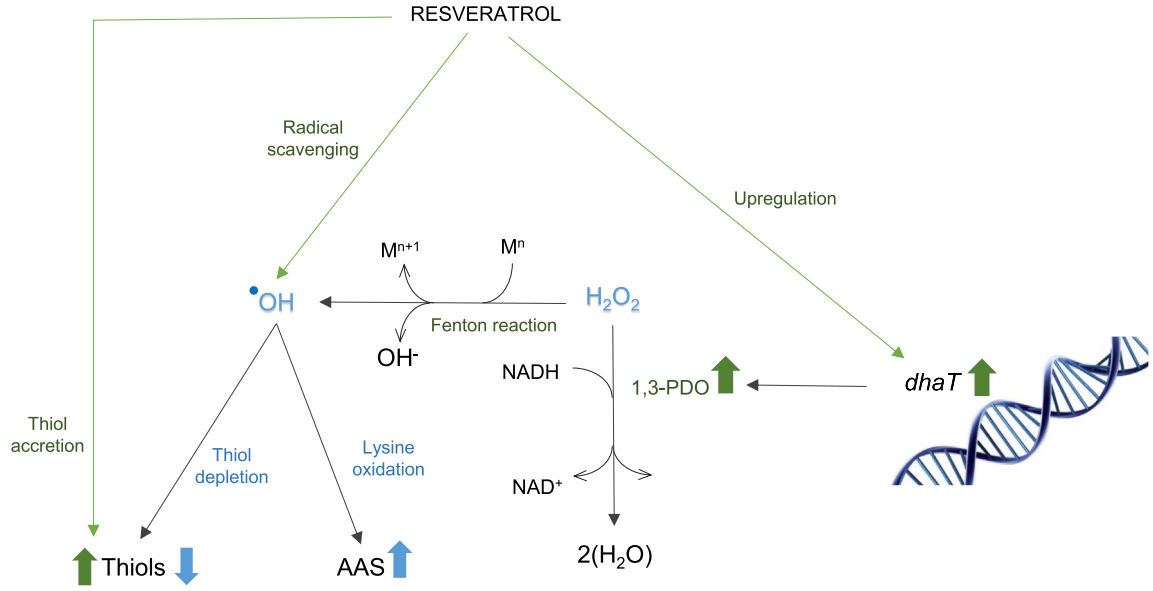

Fig. 3. General scheme of the proposed mechanisms by which resveratrol may protect *Lactobacillus reuteri* proteins against hydrogen peroxide-induced oxidative stress.

proliferating bacteria. This may explain the evolution of AAS in CONTROL group of *L. reuteri*. The concentration of AAS increased up to 1.48 nmol/mg protein at 20 h following incubation and decreased afterwards, by the end of the assay. While the increase of AAS precisely reflects the oxidation of lysine residues by reactive species [20,37], the further decline may respond to the involvement of this carbonyl in subsequent reactions, more specifically in the formation of Schiff base structures by reaction with other protein amino groups [14]. As expected, the incubation with H_2O_2 promoted the oxidative deamination of lysine residues to yield the corresponding carbonyl compound. The concentration of AAS in these colonies was significantly higher than in the CONTROL counterparts at all sampling times (except initial point). H_2O_2 has been efficiently used to promote *in vitro* the carbonylation of assorted proteins such as bovine serum albumin [37], human serum albumin [39], α-lactoglobulin [40], myoglobin [41], and myofibrillar proteins [42], among others. In the presence of transition metals, H_2O_2 yields hydroxyl radical which is able to abstract an electron from the carbon neighboring the amino group in lysine residues, triggering the subsequent deamination [37]. To our knowledge, this is the first attempt to quantify AAS in LAB challenged with H_2O_2 as previous studies have used the routine and more unspecific dinitrophenylhydrazine (DNPH) method to estimate the total amount of protein carbonyls [38]. While AAS has been reported in bacteria before [43], never was it related to oxidative stress. The addition of resveratrol had contradictory effects on the formation of AAS as low concentrations had a pro-oxidant action and the high concentration led to a significant reduction of protein carbonylation as compared to the CONTROL (20 and 24 h). Both pro- and antioxidant actions of resveratrol have been reported in literature and the dose is known to play a relevant role. While both concentrations were able to activate the *dhaT* gene, only the highest dose displayed an antioxidant action. While pro-oxidant effects are ascribed to the involvement of resveratrol in ROS generation, the protective effect observed at high concentrations may be principally attributed to direct redox effects of the resveratrol such as its ability to neutralize ROS [44]. In clear connection with the gene expression regulation observed for the *dhaT* gene, the addition of resveratrol to *L. reuteri* challenged with H_2O_2 led to a significant protection of proteins against carbonylation. The fact that the concentration of AAS in LRES is higher than that in H_2O_2-LRES proves that the underlying mechanisms of resveratrol as a protective molecule in *L. reuteri* are highly dependent on the presence of H_2O_2. While the protection of *L. reuteri* against protein carbonylation by resveratrol in H_2O_2-containing cultures was noticiable already at 12 h, the remarkable effect of resveratrol on the *dhaT* gene took place in subsequent stages of the incubation assay

(Fig. 1B). In clear connection with the interesting considerations made by Ezraty et al. [34] the accumulation of carbonyls, as a irreversible modification in oxidized proteins, triggered the H_2O_2-induced *dhaT* upregulation by resveratrol (Fig. 1B). Carbonyls may not only be the irresversible products of the oxidative stress on bacterial proteins, they may also act as signaling molecules to induce the activation of specific pathways aimed to control senescence and impaired functionality. Likewise, resveratrol (both concentrations) may not only be effective against protein carbonylation by neutralizing ROS in challenged cultures, the stilbene may also promote the expression of genes related to endogenous antioxidant defenses at particular carbonyl concentrations (> 1 nmol/mg protein) (Fig. 3).

3.2.2. Free thiols

The amino acids cysteine (Cys) and methionine (Met) are particularly sensitive to oxidation owing to the electron-rich sulfur atom in their side chain. While thiol depletion is a typical feature in oxidized proteins and this may lead to dysfunction, sulfur-containing amino acids are known to act as antioxidant moieties in proteins due to its sacrificial oxidation in the presence of ROS [7]. According to this theory, thiols with irrelevant biological significance are readily oxidized under oxidative stress and as a result, avoid the oxidative degradation of other valuable residues, namely amino groups from lysine [7]. Furthermore, unlike lysine oxidation and the formation of AAS, certain oxidation mechanisms involving free thiols are reversible and hence, under reducing environments, proteins may be repaired to preserve its integrity and functionality [34]. In bacteria, this mechanism of proteins repair may be related to virulence: counteracting the pernicious effect of ROS in proteins allows their survival [34]. In LAB and other potential pro-biotic bacteria, this mechanism may also be linked to their ability to neutralize reactive species and protect themselves and the host against their toxic effects.

Table 3 shows the concentration of free thiols in *L. reuteri* during the incubation assay (37 °C/24 h). No significance changes in thiol concentration was observed in CONTROL samples reflecting that the lack of external sources of oxidative stress enabled a paired balance between thiol oxidation and repair/*de novo* synthesis of proteins that allowed a stable quantity of sulfur-containing compounds during the entire assay. Conversely, the incubation in the presence of H_2O_2 caused a gradual and significant loss of free thiols. H_2O_2 can directly react with Cys and Met to yield sulfenic acid and methionine sulfoxide, respectively, or be decomposed through the Fenton reaction into hydroxyl radical which readily and efficiently oxidizes sulfur amino acids [7,34]. The depletion of thiols, however, was not so intense to compromise the survival of *L. reuteri* (Complementary Table 1). Even if free thiols could have acted as antioxidants and be lost as a sacrificial protection, when protein redox capabilities are wasted, other amino acid residues are oxidized, which is compatible with the increase of lysine-derived protein carbonyls shown in Table 2. In general, growing *L. reuteri* with resveratrol diminished the depletion of thiols which emphasizes the protective effect against oxidative stress. Only a depletion of thiols in the first stages of the incubation in LRES denotes a pro-oxidant effect which is again compatible with the results observed for this incubation system in Table 2. At the end of the incubation assay (24 h), the concentration of thiols in LRES and HRES is similar to that in CONTROL. In H_2O_2-LRES and H_2O_2-HRES, the evolution of free thiols followed a different trend: an initial depletion at 12 h was followed by a significant increase so that the concentration of free thiols in H_2O_2-HRES and H_2O_2-LRES was significantly higher than in CONTROL at 20 and 24 h, respectively. The timely coincidence between the accretion of thiol groups in these experimental groups with the remarkable expression of the *dhaT* gene in the same groups suggest a plausible connection between the genetic response of *L. reuteri* against the H_2O_2-induce oxidative stress and *de novo* synthesis of sulfur containing proteins/peptides (Fig. 3). While the implicated genes and underlying molecular mechanisms remain indefinite, the hypothesis of considering thiol accretion as means to protect against oxidative stress is highly plausible given the well-known role of such moieties as redox-active compounds and elements of antioxidant protection in biological systems, including LAB [28]. The present results indicate that the biological effects of resveratrol on *L. reuteri* (thiols accretion, the *dhaT* gene overexpression) require the occurrence of an oxidation threat (e.g. H_2O_2). This observation may indicate that resveratrol (or more likely an oxidation derivative) may act as an signaling molecule that would trigger the antioxidant response of *L. reuteri* against oxidative stress. The delay between the addition of the resveratrol and the observed effects (~20–24 h) would provide strength to this hypothesis. This extent, however, requires further confirmation.

4. Conclusions

The beneficial effects of probiotic bacteria on human gut health may be, at least, partly attributed to the ability of these bacteria to protect the intestinal mucosa against pernicious chemical compounds. Luminal oxidative stress is believed to be an underlying cause to assorted colonic pathologies and hence, the understanding of the mechanisms implicated in the protective role of probiotic bacteria is of the utmost scientific interest. This study provides preliminary but sound and original findings on the genetic and molecular basis of the responses of *L. reuteri* against H_2O_2-induced oxidative stress. The NADH-dependent-oxidoreductase encoded by the *dhaT* gene is a plausible candidate to be strongly implicated in the antioxidant response of *L. reuteri*. Resveratrol (100 μM) is found to protect *L. reuteri* against protein carbonylation plausibly through various mechanisms including direct scavenging of

Table 3
Concentration of free thiols (μmol/mg protein) in *Lactobacillus reuteri* during incubation assay (37 °C/24 h).

	CONTROL	H_2O_2	LRES	HRES	H_2O_2-LRES	H_2O_2-HRES	p^A
0 h	10.97 ± 0.41	$11.58^{x} \pm 0.56$	$11.50^{x} \pm 0.65$	$11.04^{x} \pm 0.84$	$11.14^{x} \pm 0.95$	$11.10^{x} \pm 0.75$	ns
12 h	$9.76^{bc} \pm 0.51$	$9.63^{bc,y} \pm 0.48$	$8.80^{c,z} \pm 0.43$	$11.01^{a,x} \pm 0.78$	$9.93^{b,y} \pm 0.65$	$9.58^{bc,y} \pm 0.64$	**
16 h	$10.05^{bc} \pm 0.45$	$9.18^{c,yz} \pm 0.59$	$9.51^{bc,yz} \pm 0.57$	$10.78^{ab,x} \pm 0.45$	$10.17^{bc,xy} \pm 0.68$	$11.07^{a,x} \pm 0.58$	*
20 h	$10.84^{b} \pm 0.65$	$8.71^{c,yz} \pm 0.61$	$10.28^{b,y} \pm 0.61$	$10.61^{b,xy} \pm 0.65$	$10.58^{b,x} \pm 0.64$	$12.11^{a,x} \pm 0.78$	**
24 h	$10.20^{bc} \pm 0.55$	$8.48^{d,z} \pm 0.51$	$10.11^{bc,y} \pm 0.59$	$9.94^{c,y} \pm 0.44$	$11.13^{a,x} \pm 0.71$	$10.87^{ab,xy} \pm 0.47$	***
p^B	ns	***	**	**	**	***	

Data expressed as means ± standard deviations of three replicates.
Experimental groups: 1. CONTROL (*L. reuteri* in MRS broth); 2. H_2O_2 (*L. reuteri* in MRS broth + 0.5 mM H_2O_2); 3. LRES (*L. reuteri* in MRS broth + 20 μM resveratrol); 4. HRES (*L. reuteri* in MRS broth + 100 μM resveratrol); 5. H_2O_2-LRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 20 μM resveratrol); 6. H_2O_2-HRES (*L. reuteri* in MRS broth + 0.5 mM H_2O_2 + 100 μM resveratrol).
[A] Significance from ANOVA denotes differences between means from the same row. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ns: no significant.
[B] Significance from ANOVA denotes differences between means from the same column. *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$.
Superscripts (a-d) within the same row denote significant differences between experimental groups.
Superscripts (x-z) within the same column denote significant differences between sampling times.

ROS, upregulation of the *dhaT* gene and promoting the synthesis of sulfur containing compounds with protective effect. Along with this novel information, the present study provides grounds for future studies as the hypotheses formulated on i) the ability of *L. reuteri* to detoxify H_2O_2 and ii) the ability of resveratrol to trigger an antioxidant defense in *L. reuteri* in the presence of an oxidation threat, need to be clarified. Furthermore, protein carbonylation as a reflection of oxidative damage to bacteria and its consequences, as well as the role of protein carbonyls as signaling molecules implicated in the responses of bacteria to oxidative stress, require further elucidation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2019.02.023.

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