

# Molecular mechanisms of the disturbance caused by malondialdehyde on probiotic *Lactobacillus reuteri* PL503

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## Summary

This study aimed to provide insight into the molecular and genetic mechanisms implicated in the responses of *Lactobacillus reuteri* against the oxidative stress induced by malondialdehyde (MDA) by analysing protein oxidation and assessing the *uspA* and the *dhaT* genes. Four experimental groups were evaluated depending on the concentration of MDA added in Man, Rogosa and Sharpe (MRS) broth: Control (*L. reuteri*), 5  $\mu\text{M}$  (*L. reuteri* + 5  $\mu\text{M}$  MDA), 25  $\mu\text{M}$  (*L. reuteri* + 25  $\mu\text{M}$  MDA) and 100  $\mu\text{M}$  (*L. reuteri* + 100  $\mu\text{M}$  MDA). Three replicates were incubated at 37 °C for 24 h in microaerophilic conditions and sampled at 12, 16, 20 and 24 h. The upregulation of the *uspA* gene by *L. reuteri* indicates the recognition of MDA as a potential DNA-damaging agent. The *dhaT* gene, encoding a NADH-dependent-oxidoreductase, was also upregulated at the highest MDA concentrations. This gene was proposed to play a role in the antioxidant response of *L. reuteri*. The incubation of *L. reuteri* with MDA increased the production of ROS and caused thiol depletion and protein carbonylation. *L. reuteri* is proposed to detoxify pro-oxidative species while the underlying mechanism requires further elucidation.

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## Introduction

Oxidative stress is a redox deregulation typically caused by an imbalance between pro-oxidants and the antioxidant defences, which is manifested as damage to molecules of biological significance by radical and non-radical species (Finkel and Holbrook, 2000). The biological consequences of the oxidative damage to lipids, proteins and the DNA, involve impaired physiological processes and the onset of assorted health disorders. Profuse literature supports the implication of persistent oxidative stress on severe health disorders such as diabetes (Asmat *et al.*, 2016), neurodegenerative and cardiovascular diseases (Lin and Beal, 2006), and several types of cancer (Valko *et al.*, 2005; Reuter *et al.*, 2010). The colon has been identified to be particularly sensitive to oxidative stress (Sanders *et al.*, 2014) and such condition is involved in the onset of a number of pathological conditions at this location, including ulcerative colitis, Crohn's and inflammatory bowel diseases, and colorectal cancer (Gackowski *et al.*, 2002; Zhu and Li, 2012). The intestinal mucosa is permanently exposed to the pro-oxidant action of dietary lipid and protein oxidation products (~ luminal oxidative stress) which may contribute to impair the redox status of the bowel (Estévez and Luna, 2017). Malondialdehyde (MDA), a remarkable lipid oxidation product in muscle foods and oxidized oils, is able to bind to DNA leading to the formation of etheno-modified DNA bases, with these adducts being found in organs with diseases related to enduring inflammatory conditions that may eventually cause malignancies (Bartsch and Nair, 2005). Owing to the pathological effects of luminal oxidative stress, dietary antioxidants have been proposed to inhibit and/or alleviate the symptoms of some of these health disorders (Spyropoulos *et al.*, 2011). On this line, supplementation with probiotic bacteria has been proposed as a feasible strategy to counteract the oxidative stress in the gastrointestinal tract (GIT) in humans (Spyropoulos *et al.*, 2011).

Probiotics are defined as 'live microbes which, when administered in adequate amounts, confer a health benefit to the host' (FAO, 2002). As a natural colonizer of the GIT tract, *Lactobacillus reuteri* has been widely used as a dietetic supplement to promote gut health in humans (Hjern *et al.*, 2020; Wang *et al.*, 2020). Oral

administration of *L. reuteri* decreases the onset and severity of inflammatory and infectious disorders in the GIT and contributes to a balanced colonic microbiota (Wang *et al.*, 2020). In relation to the underlying mechanisms of its probiotic effects, *L. reuteri* has been found to diminish colonic oxidative stress by reducing the formation and accumulation of oxidation products in the lumen (Amaretti *et al.*, 2013). The positive effects of *L. reuteri* on the oxidative and health status of human colon are documented (Petrella, 2016) and include protection against a number of pathological conditions such as colorectal cancer (Bistas *et al.*, 2020), inflammatory bowel diseases (Wang *et al.*, 2020) and cardiovascular disorders (Kazemian *et al.*, 2020). Yet, the precise biochemical and genetic responses of *L. reuteri* against pro-oxidative conditions caused by luminal oxidation products (such as MDA) are not fully understood.

Pathogenesis of MDA not only involves the formation of DNA adducts, this lipid peroxidation product is known to bind to proteins and induce a number of oxidative modifications (Esterbauer *et al.*, 1993). From a medical standpoint, the accumulation of oxidized proteins is a pathological hallmark of ageing and chronic diseases (Estévez and Luna, 2017) and protein oxidation is known to play central role in the pathogenesis of gut disorders driven by oxidative stress (Gackowski *et al.*, 2002; Zhu and Li, 2012). Though much less studied than in humans, the occurrence of protein oxidation in bacteria has also been linked to impaired growth and senescence (Ezraty *et al.*, 2017). On this line, the modifications induced by MDA in bacterial proteins and their biological consequences, are unknown. Furthermore, the precise biological mechanisms activated by *L. reuteri* in response to an MDA-induced oxidative stress are poorly documented. In this regard, the study of the regulation of particular stress-related genes seems crucial to comprehend the influence of external sources of oxidative stress on particular metabolic pathways and biological functions. Previous reports have documented that particular genes such as *uspA* and the *dhaT* are activated in *L. reuteri* in response to an oxidative threat caused by reactive oxygen species (ROS; Arcanjo *et al.*, 2019). The universal stress protein A (*uspA*) superfamily is an ancient and conserved group of proteins found in assorted microorganisms, insects and plants (Kvint *et al.*, 2003). The precise roles of *Usp* proteins in biological systems remain unclear; yet, they seem to be involved in the defence against DNA-damaging agents (Kvint *et al.*, 2003). The *dhaT* gene expression leads to the synthesis of a 1,3 propanediol oxidoreductase (1,3-PDO), which was proposed by Arcanjo *et al.* (2019) to be involved in the protection of *L. reuteri* against oxidative stress. Arcanjo *et al.* (2019) also reported that protein carbonylation could play a relevant role as indicator

of oxidative damage in bacteria and as a potential signalling mechanism. Yet, the connection between oxidative stress, gene expression and protein oxidation in probiotic bacteria is poorly understood.

This study aimed to provide insight into the molecular and genetic mechanisms involved in the responses of *L. reuteri* against MDA-induced oxidative stress by analysing protein oxidation and assessing the *uspA* and the *dhaT* genes.

## Results

### *Regulation of uspA and dhaT genes by L. reuteri in response to MDA*

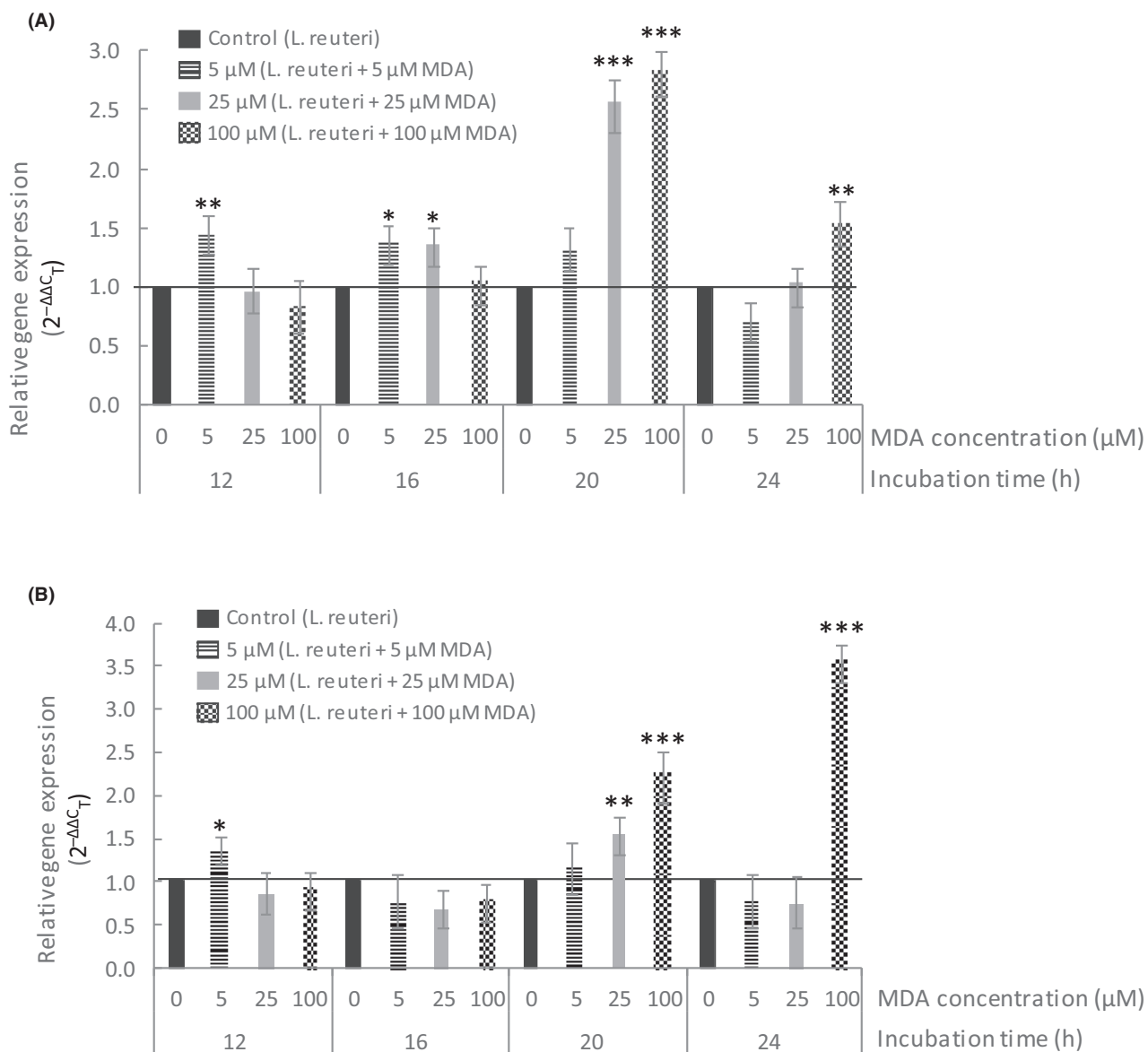
Fig. 1A shows the relative expression ( $2^{-\Delta\Delta C_T}$ ) of the *L. reuteri* PL503 *uspA* gene during the incubation assay in the presence of different concentrations of MDA. The exposure to MDA caused an upregulation of the *uspA* gene, as shown in Fig. 1A. This event had a dose-effect (12 h) which affected not only the intensity of the gene overexpression but also the time-frame of the occurrence of such biological response. The lowest concentration (5  $\mu\text{M}$ ) apparently led to a fast and limited response already at 12 h. The relative expression of the *uspA* gene in *L. reuteri* PL503 incubated with 25 and 100  $\mu\text{M}$  of MDA showed a similar response 4 h later, while such expression had a peak after 20 h of incubation being significantly more intense with 25 and 100  $\mu\text{M}$  of MDA than in the absence or 5  $\mu\text{M}$  of MDA.

Overall, no significant changes in the relative expression of the *dhaT* gene were observed during the first two samplings. Yet, a dose-dependent effect of the incubation with MDA on the *dhaT* gene expression was found at 20 h of incubation (Fig. 1B). In particular, the relative transcription of the *dhaT* gene significantly increased in the presence of 25 and 100  $\mu\text{M}$  of MDA at 20 h. At the final sampling, the relative expression of the *dhaT* gene significantly increased in the bacterium challenged with the highest concentration of MDA.

### *Induction of oxidative stress in L. reuteri by MDA*

To investigate the ability of MDA to induce oxidative stress in *L. reuteri* PL503, ROS generation was assessed in bacteria by flow cytometry. Figure 2 shows how MDA exposure increased the percentage of bacteria suffering from oxidative stress in a dose-dependent manner. To assess the oxidative damage caused by MDA incubation in *L. reuteri* PL503, both lipid and protein oxidation markers were quantified.

The initial concentration of TBARS found in the cultures accurately reflects the three levels of MDA used for challenging *L. reuteri* PL503 (Fig. 3A). The basal TBARS concentration in CONTROL cultures

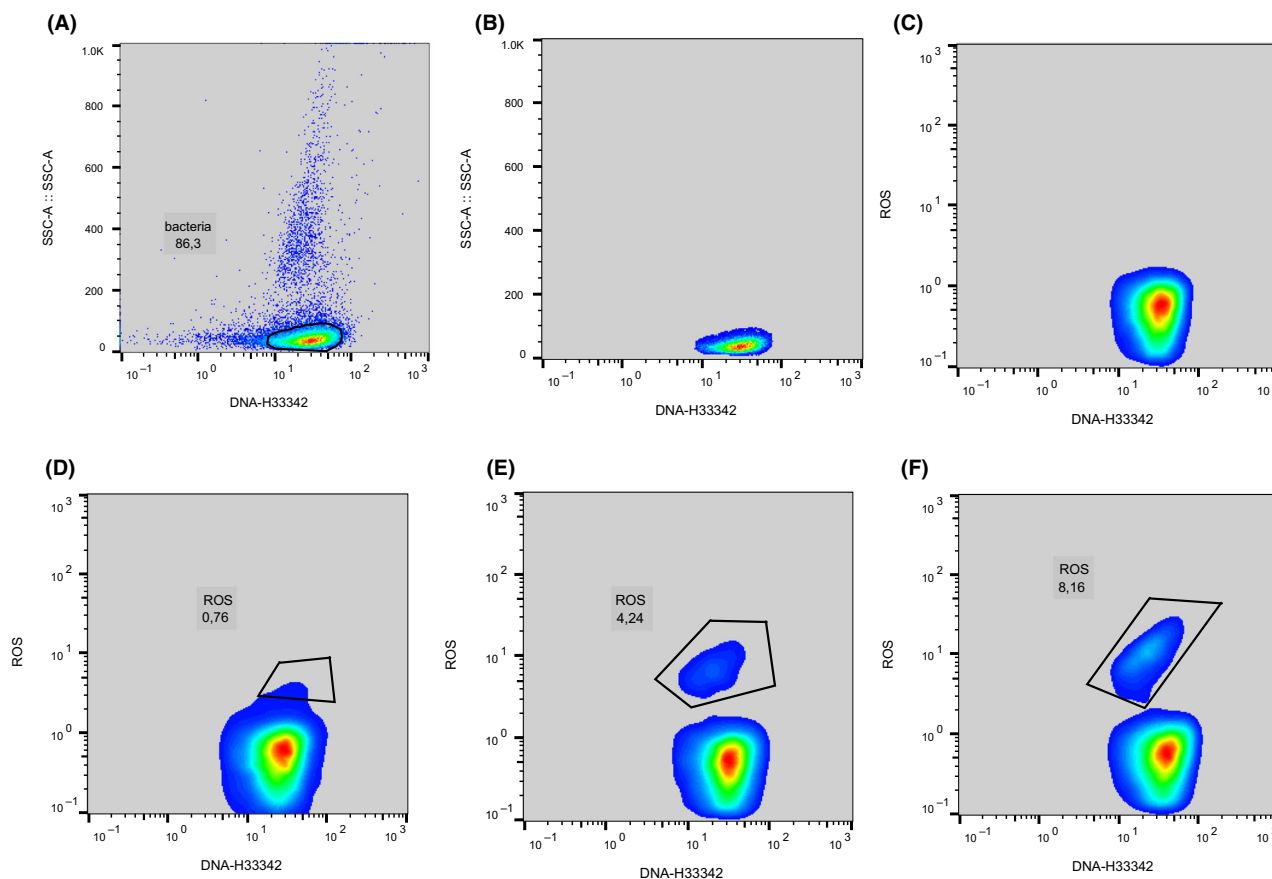


**Fig. 1.** Relative expression ( $2^{-\Delta\Delta C_T}$ ) of the *uspA* (A) and *dhaT* (B) genes in *Lactobacillus reuteri* PL503 as affected by increasing concentrations of malondialdehyde (MDA) for up to 24 h. Means for each experimental group are calculated from analyses applied to three biological replicates and all analyses were technically duplicated. Black line at  $2^{-\Delta\Delta C_T} = 1$  denotes standardized expression rate for CONTROL group at each sampling time (calibrator).  $2^{-\Delta\Delta C_T} < 1$  denotes suppression of gene expression;  $2^{-\Delta\Delta C_T} > 1$  denotes activation of gene expression. Asterisks on top of bars denote significant differences in paired Student's *t*-tests performed to compare each MDA concentration with CONTROL [MDA = 0]: \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; and \*\*\* $P \leq 0.001$ .

( $\sim 1 \text{ mg l}^{-1}$ ) could be the result of the occurrence of lipid peroxidation in the bacterium under physiological conditions and did not change significantly during the assay ( $P > 0.05$ ). In the cultures challenged with MDA, the initial concentration significantly decreased ( $P < 0.05$ ) in the range of 22–26% with this depletion being a likely reflection of the reaction of MDA with other biomolecules.

The evolution of the concentration of allysine, a recognized marker of protein oxidation, was assessed in *L. reuteri* PL503 during the incubation period (37 °C/24 h)

and results are shown in Fig. 3B. The evolution of allysine in CONTROL group of *L. reuteri* PL503 shows a significant increase ( $P < 0.05$ ) from 0.7 to 1.65 nmol allysine per mg protein. As compared to CONTROL, the exposure to MDA caused a significant increase ( $P < 0.001$ ) in the concentration of allysine in proteins from *L. reuteri* PL503 and such increase followed a dose-dependent fashion for 16 h, suggesting a direct implication of MDA in the carbonylation of proteins from *L. reuteri* PL503. After that sampling, the behaviour varied between experimental groups. In *L. reuteri* PL503



**Fig. 2.** Production of reactive oxygen species (ROS) in *Lactobacillus reuteri* PL503 grown in MRS broth with increasing concentrations of malondialdehyde (MDA) for 24 h. A: DNA containing bacterium (debris and nonbacterium events are removed); B: Gate for bacterium; C-F: X-axis bacterium (DNA +) Y-axis ROS; C: CONTROL; D: *L. reuteri* PL503 incubated with 5  $\mu\text{M}$  MDA; E: *L. reuteri* PL503 incubated with 25  $\mu\text{M}$  MDA; F: *L. reuteri* PL503 incubated with 100  $\mu\text{M}$  MDA. Analyses were applied to three biological replicates and all analyses were technically duplicated.

grown in the presence of 5 and 25  $\mu\text{M}$  of MDA, the increase of alllysine was sustained during the complete assay reaching the highest concentration at 24 h (3.7 and 5.2 nmol alllysine/mg protein respectively). When exposed to the highest MDA concentration (100  $\mu\text{M}$ ) the concentration of alllysine peaked at 16 h after which a decrease was observed so that basal levels ( $< 1$  nmol alllysine  $\text{mg}^{-1}$  protein) were found at the end of the incubation period. In the present study, the formation of Schiff bases (Fig. 3C) was dependent on the presence of MDA. Yet, it is worth noting that no clear dose-dependent effect was observed and that the evolution of its concentration during the assay was erratic.

To verify whether MDA is able or not to induce alllysine formation via an oxidative deamination mechanism similar to that exerted by other dicarbonyls (i.e. glyoxal), MDA (0.25  $\mu\text{M}$ ) was incubated with assorted proteins (5  $\text{mg ml}^{-1}$ ) at 37  $^{\circ}\text{C}$  (HSA, HH) and 80  $^{\circ}\text{C}$  (LAC) for 24 h. As compared to CONTROL groups (protein suspensions with no added MDA), protein suspensions incubated with MDA had significantly lower concentrations of

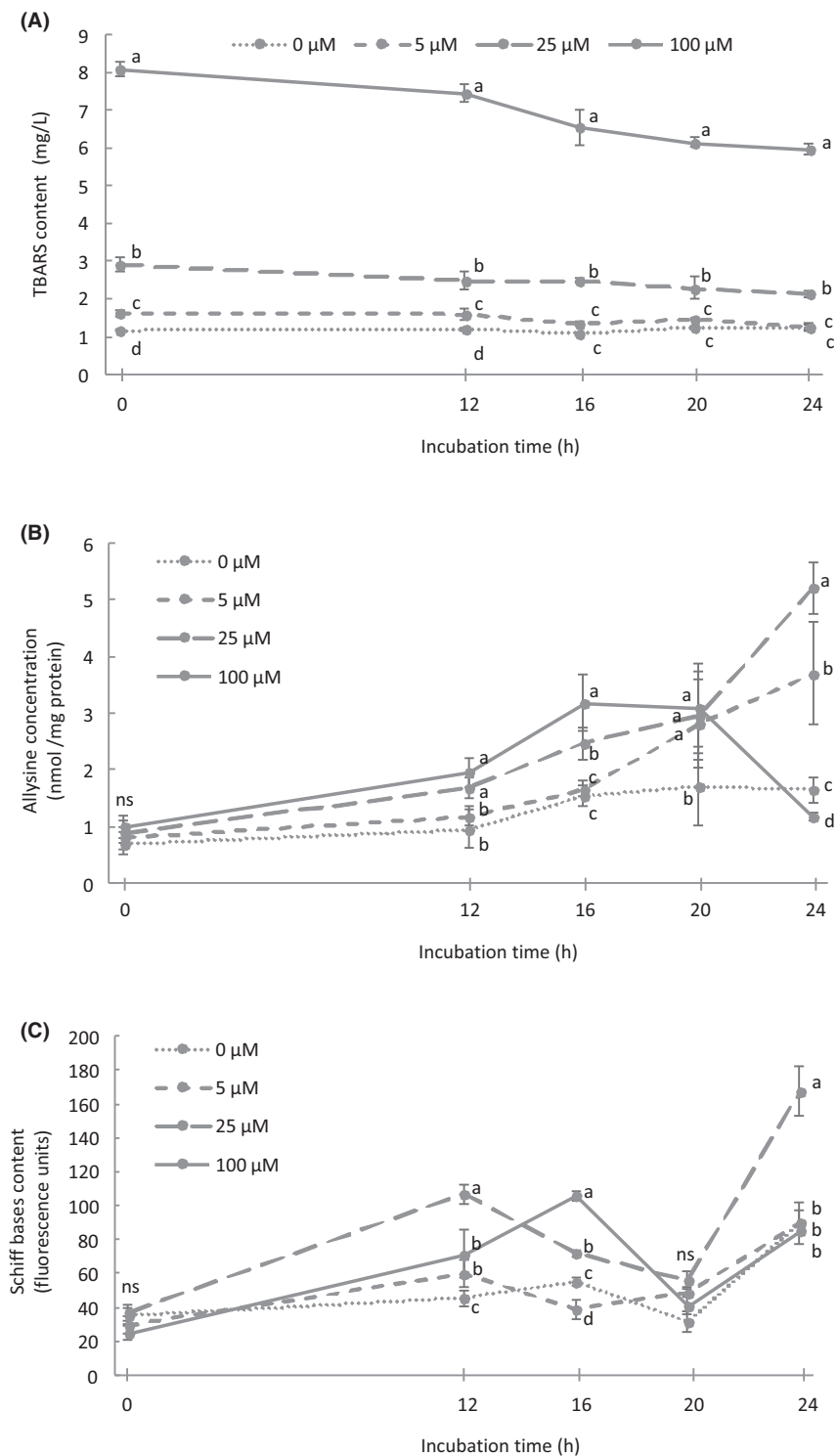
allysine (Fig. 4A). Conversely, MDA caused a significant increase in the concentration of Schiff bases (Fig. 4B).

The concentration of free thiols in *L. reuteri* PL503 during the incubation assay (37  $^{\circ}\text{C}/24$  h) is shown in Fig. 5. No significant changes in the thiol concentration were observed in CONTROL samples during the first 12 h. During the second half of the assay, a quantitatively small but significant increase of thiols was detected. The incubation of *L. reuteri* PL503 in the presence of MDA caused a significant dose-dependent increase of free thiols during the first 16 h of assay. In the following sampling points, the evolution of thiols remained stable in bacteria treated with 100  $\mu\text{M}$  of MDA and significantly declined in bacteria exposed to 5 and 25  $\mu\text{M}$  of MDA.

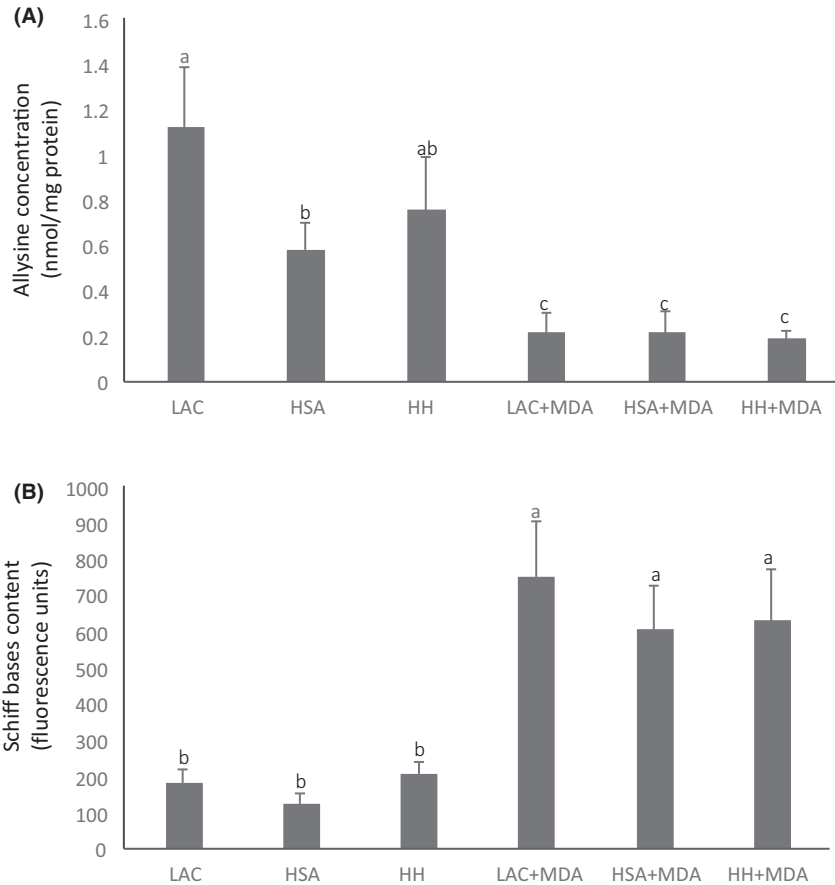
## Discussion

### *Regulation of uspA and dhaT genes by L. reuteri in response to MDA*

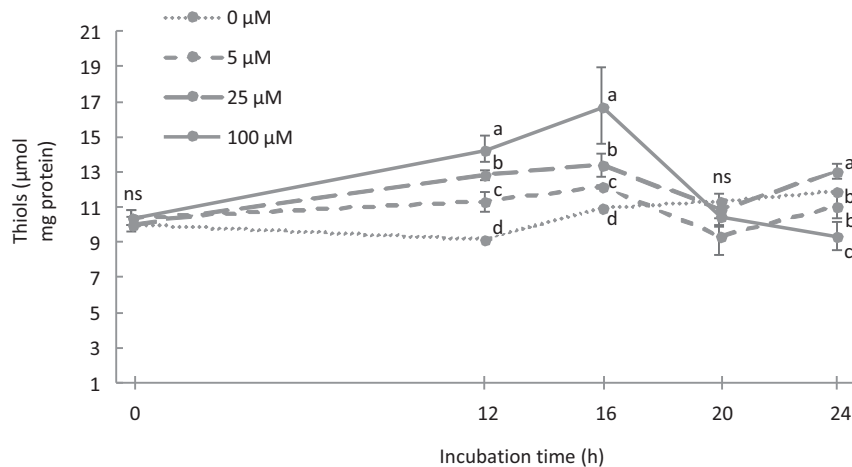
The increasing applied doses of MDA (5  $\mu\text{M}$ , 25  $\mu\text{M}$ , 100  $\mu\text{M}$ ) did not compromise the survival of *L. reuteri*, as



**Fig. 3.** Concentration of TBARS (A); allisine (B) and Schiff bases (C) (means  $\pm$  standard deviation) in *Lactobacillus reuteri* PL503 grown in MRS broth with increasing concentrations of malondialdehyde (MDA) during an incubation period for up to 24 h. Means for each experimental group are calculated from analyses applied to three biological replicates and all analyses were technically duplicated. Different letters at the same sampling time denote significant differences between means within the same sampling point in ANOVA ( $P \leq 0.05$ ).



**Fig. 4.** Concentration of alllysine (A) and Schiff bases (B) (means  $\pm$  standard deviation) in human serum albumin (HSA), human haemoglobin (HH) and  $\beta$ -lactoglobulin (LAC) ( $5 \text{ mg ml}^{-1}$ ) after incubation with MDA ( $0.25 \mu\text{M}$ ) at  $37^\circ\text{C}$  (HSA, HH) and  $80^\circ\text{C}$  (LAC) for 24 h. Means for each experimental group are calculated from analyses applied to three biological replicates and all analyses were technically duplicated. Different letters denote significant differences between means within the same sampling point in ANOVA ( $P \leq 0.05$ ).



**Fig. 5.** Concentration of free thiols (means  $\pm$  standard deviation) in *Lactobacillus reuteri* PL503 grown in MRS broth with increasing concentrations of malondialdehyde (MDA) during an incubation period for up to 24 h. Means for each experimental group are calculated from analyses applied to three biological replicates and all analyses were technically duplicated. Different letters at the same sampling time denote significant differences between means within the same sampling point in ANOVA ( $P \leq 0.05$ ).

the counts remained stable during the entire experimental assay (37 °C/24 h). This finding reflects the ability of *L. reuteri* to activate mechanisms to neutralize the potential harmful effects of the sublethal concentrations of this lipid peroxidation product. In the present study, these mechanisms were firstly assessed by the analysis of the relative transcription of stress-related genes.

#### *uspA* gene

Considering the role of the *uspA* gene in the defence against DNA-damaging agents (Kvint *et al.*, 2003), the upregulation of such gene as a response to the challenge of MDA was expected. MDA is known to form adducts with assorted biomolecules, including the DNA (Esterbauer *et al.*, 1993). Marnett (1999) reported that MDA reacts with DNA to form adducts to deoxyguanosine and deoxyadenosine. However, the major adduct to DNA is a pyrimidopurine called M1G, which has been also identified in human liver, white blood cells, pancreas and breast tissues, and is considered a significant contributor to cancer linked to dietary factors (Niedernhofer *et al.*, 2003). It is hence, relevant to find out whether colonic microbiota is able to detoxify and/or counteract the noxious effects of MDA. In bacteria, M1G has been also found to lead to mutagenesis, which is repaired by the nucleotide excision repair pathway (Marnett, 1999). The threat of such mutagenesis could have caused an upregulation of the *uspA* gene, as shown in Fig. 1A. These results are, however, divergent to those reported by Oberg *et al.* (2015) who observed a significant inhibition of the *uspA* gene expression in *Bifidobacterium longum* exposed to a hydroxyl radical generating system. Conversely, an ATP-dependent metallo-protease was found to be upregulated to likely protect membrane proteins against radical-mediated oxidative damage. The specificity of the genetic responses of a given bacterium challenged with different pro-oxidant threats seems plausible since the damage caused by MDA via adduct formation to biomolecules (including proteins and DNA) is fairly different from the severe oxidative damage caused by ROS.

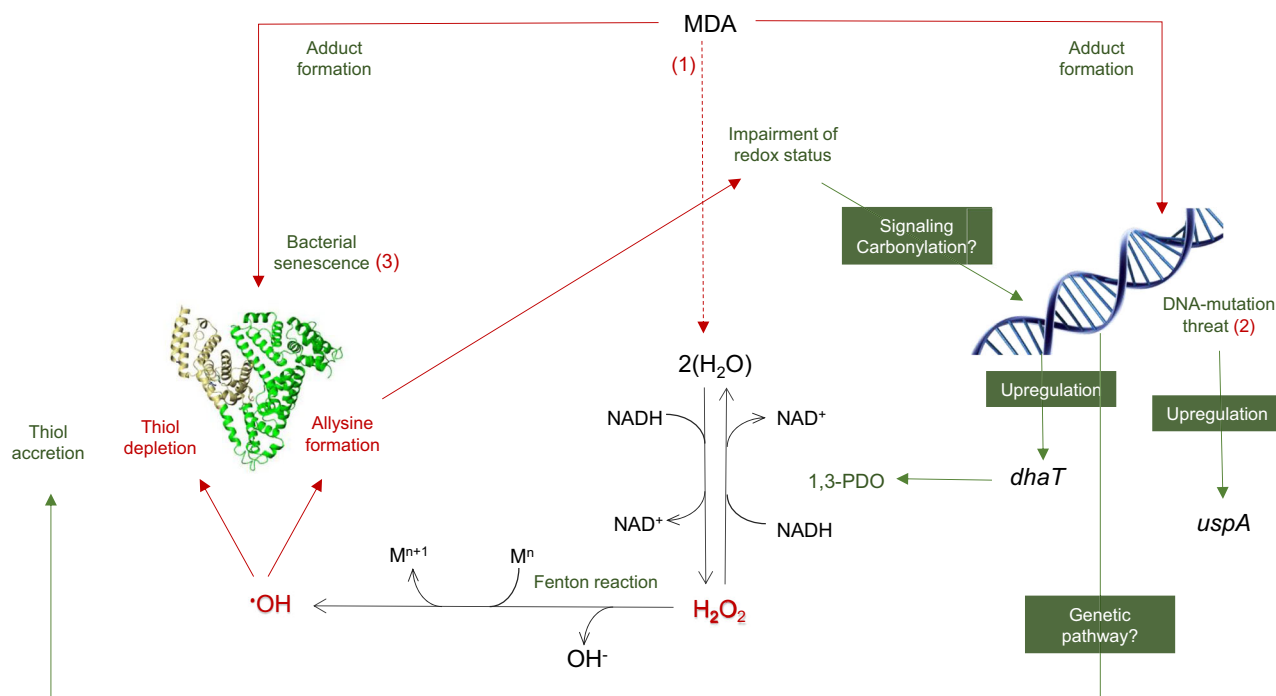
#### *dhaT* gene

While the *uspA* gene encodes an assorted collection of proteins, the expression of the *dhaT* gene leads to the synthesis of a single protein with definite function, the 1,3 propanediol oxidoreductase (1,3-PDO; Schaefer *et al.*, 2010). This enzyme plays a relevant role in stress situations involving energetic demand since 1,3-PDO facilitates the main carbohydrate fermentation pathway (6-phosphogluconate/phosphoketolase; 6-PG/PK) through the production of NAD<sup>+</sup> (required for glucose fermentation)

from NADH in the conversion of 3-hydroxypropionaldehyde (3-HPA) (its substrate) into 1,3 propanediol (1,3-PDO) under anaerobic conditions. Additionally, 3-HPA, also known as reuterin, is known to be profusely excreted to the surrounding environment under stress situations, imparting strong antimicrobial properties (Schaefer *et al.*, 2010).

The underlying mechanisms by which *L. reuteri* may try to protect against MDA-induced biological damage through the activation of the 3-HPA pathway should be a matter of thoroughly analysis. The 3-HPA pathway, as previously reported, requires the presence of glycerol, commonly added as growth promoter in *Lactobacillus* cultures (Talarico *et al.*, 1988). Since *L. reuteri* from the present experiment had no access to glycerol, and the production of 3-HPA, preferential target of 1,3-PDO, may not be present in the media, the purpose of the upregulation of the gene encoding the 1,3-PDO remains indefinite. It is clear, however, that such enzyme may be implicated in a protection mechanism against MDA-induced oxidative stress that reasonably involves the NADH/NAD<sup>+</sup> redox pair and that 3-HPA may not be the unique substrate for 1,3-PDO. In a previous study (Arcanjo *et al.*, 2019), in which *L. reuteri* was challenged with H<sub>2</sub>O<sub>2</sub> (0.5 mM) in the absence of glycerol, a similar effect on the expression of the *dhaT* gene was observed. The authors hypothesized whether the NAD<sup>+</sup>-dependent activity of the 1,3-PDO may be able to detoxify H<sub>2</sub>O<sub>2</sub> in the presence of NADH in accordance to the pathway proposed in Fig. 6. Interestingly, *Lactobacillus* spp. have been also found to be able to generate H<sub>2</sub>O<sub>2</sub> and other ROS via implication of NAD(P)H oxidoreductases (Hertzberger *et al.*, 2014). The incubation of *L. reuteri* PL503 in the presence of MDA led to increase the production ROS as shown in Fig. 2. The analysis of the bacterium with flow cytometry showed that increasing concentrations of MDA led to accumulative collection of cells with significant generation of ROS. These results originally prove that MDA induces the formation of diverse radical species in *L. reuteri* PL503 plausibly via production of H<sub>2</sub>O<sub>2</sub>, the most common source of hydroxyl radical in biological systems (Davies, 2005). The production of H<sub>2</sub>O<sub>2</sub> by *Lactobacillus* spp., already reported in literature, seems to be promoted by the presence of electron acceptors such as O<sub>2</sub> and fructose (Mane, 2016). MDA, as a potent electron acceptor, may also have such effect on *L. reuteri* PL503 supporting the connection between the lipid peroxidation product, the impairment of the redox status of the cell, the production of H<sub>2</sub>O<sub>2</sub> and the upregulation of the gene encoding a NADH-dependent oxidoreductase (Fig. 6). However, the underlying mechanism by which MDA would promote ROS formation in *L. reuteri* requires further elucidation. Although *Lactobacillus* spp. can produce antimicrobial





(1) MDA-treated bacteria were found to have higher concentration of hydrogen peroxide and ROS. While mechanisms are yet to be elucidated, the formation of hydrogen peroxide by a NADH-dependent mechanism is proposed in accordance to Hertzberger et al. (2014).

(2) MDA is known to adduct to DNA bases and induce mutation in bacteria (Draper et al. 1986).

(3) MDA-mediated protein carbonylation has been found to cause impairment of biological processes and senescence in bacteria (Ezraty et al. 2017).

**Fig. 6.** General scheme of the proposed mechanisms whereby malondialdehyde (MDA) damages biomolecules in *Lactobacillus reuteri* PL503 (red lines) and mechanism by which the bacterium may protect against MDA-induced oxidative stress (green lines).

peptides, bacteriocins and several organic compounds, releasing H<sub>2</sub>O<sub>2</sub> seems to be central for antimicrobial and restorative processes (Singh *et al.*, 2018). This has been also highlighted as a relevant probiotic mechanism as ROS production by *Lactobacillus* spp. has been shown to promote epithelial restitution during colitis and alleviate inflammation in human mucosa (Singh *et al.*, 2018). Finally, physiological production of radical species induced by pro-oxidative compounds such as MDA can contribute cells to minimize oxidative stress. This mechanism may involve radical species acting as signalling molecules or inducing subtle modifications in proteins that may, in turn, act as signalling molecules that could eventually enhance endogenous antioxidant mechanisms in the bacterium and in the host (Martín and Suarez, 2010). While the H<sub>2</sub>O<sub>2</sub> production in *Lactobacillus* spp. is well documented, the mechanisms are not definite though it may involve the conjunction of NADPH and oxidoreductase enzymes (proposed mechanism in Fig. 6). The overexpression of the *dhaT* gene, alleged to protect against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress, may respond to the necessity of the bacterium to counteract the potential damage that such pro-oxidant species may exert in its own biomolecules.

The pathogenesis of particular chemical species depends on their ability to establish molecular interactions with biomolecules from the host and as a result, impair biological processes. MDA is known to exert harmful effects by adducting biomolecules such as DNA and proteins (Bartsch and Nair, 2005). While the adducts with DNA and the corresponding MDA-induced mutations have been known for some time in bacteria (Draper *et al.*, 1986), the impact of MDA on proteins of biological relevance is not so well understood. Protein oxidation is not only mediated by ROS, as oxidizing lipids and final lipid oxidation products have been also identified as potential initiators of oxidative reactions in proteins (Davies, 2005). The damage caused to proteins in pro-oxidative environments has been emphasized as one of the most salient causes of ageing and disease in humans (Davies, 2005; Estévez and Luna, 2017). Protein oxidation also has devastating effects on the structure and functionality of bacteria which may even lead to bacterial senescence and cell death (Ezraty *et al.*, 2017). Interestingly, the oxidative damage to proteins plays a role in the bacterial response to oxidative stress (Ezraty *et al.*, 2017) as proteins are activated by oxidative means to trigger specific antioxidant mechanisms. In



order to provide further insight into the effects of MDA on *L. reuteri*, protein oxidation was assessed through the quantification of a specific protein carbonyl (allysine) and the formation of Schiff bases in bacterial proteins. Free thiols, as relevant redox-active moieties in proteins, were also quantified.

#### Protein oxidation

Allysine is the most abundant protein carbonyl in biological systems and typically used as indicator of the oxidative damage to proteins (Estévez, 2011). In tissues from mammals, a concentration of 1 nmol carbonyls per mg protein has been reported as physiological while significant increases are commonly reflecting oxidative stress conditions (Akagawa *et al.*, 2002). This is, to our knowledge, the first time that such specific protein oxidation product is quantified in cultured bacteria as marker of oxidative stress. Measuring protein carbonylation using the routine spectrophotometric DNPH method, Ballesteros *et al.* (2001) proposed this expression of the oxidative damage to proteins as a reflection of bacterial senescence as oxidized proteins accumulate in non-proliferating bacteria. This is consistent with the evolution of allysine in CONTROL group of *L. reuteri* PL503. The present results show that allysine, formed in bacteria, as in eukaryotes, may be used as a reliable indicator of protein oxidation. Allysine is formed in proteins as an outcome of oxidative deamination of the  $\epsilon$ -amino group in lysine residues and that oxidation pathway can be initiated by i) radical species (i.e. hydroxyl radical) (Utrera and Estevez, 2013) or by dicarbonyls from the Maillard reaction (i.e. glyoxal/methylglyoxal) (Akagawa *et al.*, 2002). MDA was found to promote the formation of allysine in *L. reuteri* PL503 and we performed additional analyses to find out the underlying mechanism of such oxidative damage. It is known that MDA reacts with  $\epsilon$ -amino group in lysine residues but the formation of allysine as an outcome of such reaction is not described in the literature. The additional assay carried out with human and bovine proteins (Fig. 4A,B) confirmed that MDA is not able to induce the oxidative deamination of alkaline amino acids via the Maillard mechanism previously reported. Instead, the reaction of MDA with such residues is known to yield Schiff bases and stable protein crosslinks (Requena *et al.*, 1997; Estévez *et al.*, 2019). While the formation of such fluorescent structures was the most likely fate of MDA residues in isolated human and bovine proteins, the evolution of Schiff bases in bacteria was irregular and considerably low as compared to other studies carried out in animal proteins (Utrera and Estevez, 2013). It is then reasonable to hypothesize that the significant decrease in MDA,

previously stated (Fig. 3A), may respond to reactions with other biomolecules, including DNA, which would explain the fast and dose-dependent overexpression of the *uspA* gene, involved in the defence against DNA-damaging agents. These results suggest that MDA promoted allysine formation in *L. reuteri* PL503 through mechanisms that likely involve a ROS-mediated pathway. This hypothesis is supported by the increase of ROS detected by flow cytometry in the bacterium treated with MDA (Fig. 2). This mechanism may involve the previous formation of  $H_2O_2$  and its subsequent decomposition through the Fenton reaction into hydroxyl radicals (Proposed mechanism depicted in Fig. 6). The radical-mediated oxidative deamination of lysyl residues is, so far, the most plausible mechanism behind the formation of allysine in *L. reuteri* PL503 incubated with MDA.

The biological significance of protein carbonylation should be another issue of discussion. As an irreversible modification in proteins in a pro-oxidative environment, protein carbonylation is typically regarded as a reaction of negative biological consequences. Carbonylated proteins can be dysfunctional and may be tagged to removal as their accumulation causes impaired homeostasis that leads to chronic dysfunction and apoptosis (Shacter, 2000). On the other hand, carbonylated proteins may also act as signalling molecules, which may trigger specific pathways, aimed to preserve homeostasis control senescence (Shacter, 2000). Both circumstances may be applied to the present experiment. The initial increase in protein carbonyls in the MDA-treated bacterium (16 h) was followed by a notable decrease of allysine in *L. reuteri* PL503 exposed to the highest MDA concentration. In these samples, at concentrations around 3 nmol allysine  $mg^{-1}$  protein, such bacterium may have activated the *dhaT* gene, clearly noticeable in the following sampling times. The NADH-dependent oxidoreductase decoded by this gene may have contributed to detoxify pro-oxidant species such as  $H_2O_2$  and hence, inhibiting the enduring carbonylation observed in *L. reuteri* treated with lower doses of MDA (Fig. 6). The allysine decline by the end of the assay in *L. reuteri* PL503 treated with 100  $\mu M$  MDA can only respond to the removal of carbonylated proteins through a mechanism (not identified in the present study) that could have been likely activated along with the *dhaT* pathway. It is worth noting that these mechanisms were not present in the bacterium incubated with 5 and 25  $\mu M$  as allysine concentrations higher than 3 nmol allysine  $mg^{-1}$  protein were only reached at the end of the assay and regrettably the events that could have happened in hypothetical further sampling times are ignored. The hypothesis that the *dhaT* gene could have been activated by  $H_2O_2$  and/or the effect of the former

on protein carbonylation is supported by previous considerations made by Ezraty *et al.* (2017) and Arcanjo *et al.* (2019). The latter authors, in particular, observed how the accumulation of carbonyls in *L. reuteri* PL503, as a irreversible modification in oxidized proteins, triggered the upregulation of the *dhaT* gene in the presence of H<sub>2</sub>O<sub>2</sub> and resveratrol.

### Free thiols

Sulfur-containing amino acids such as cysteine (Cys) and methionine (Met) are particularly sensitive to oxidation and thiol depletion is a typical feature in oxidized proteins. While the oxidation of thiols in the active site of enzymes may lead to dysfunction, irrelevant sulfur-containing amino acids are known to act as endogenous antioxidants by offering a sacrificial loss to ROS and protecting other amino acids with relevant biological significance (Estévez *et al.*, 2020). This double role of thiols was investigated in the present experiment. A paired balance between thiol oxidation and repair/*de novo* synthesis of proteins occurred during the first 12 h in CONTROL bacteria owing to the absence of oxidative stress. During the second half of the assay, the timely coincidence of thiol accretion with the increase of carbonylation in CONTROL samples may respond to a physiological strategy to keep a balanced redox status in a senescent cell. The pro-oxidant changes induced by MDA in bacteria, including the formation of protein carbonyls, plausibly triggered the accretion of thiol groups by *de novo* synthesis of sulfur-containing proteins/peptides and as a result, protect the bacterium against potential pro-oxidant threats (Fig. 5). Thiols are typically regarded as elements of antioxidant protection in eukaryotes and also, in lactic acid bacteria (Schaefer *et al.*, 2010; Xiao *et al.*, 2011). Yet, the underlying molecular mechanisms behind the synthesis of thiol-containing species remain indefinite and needs further elucidation.

The depletion of thiols observed in the following sampling points may respond to two divergent circumstances depending on the concentration of MDA. In cultures with 100 µM MDA, the control of the oxidative threat through a timely genetic response (overexpression of the *uspA* and particularly the *dhaT* genes, among others) allowed lowering the amount of protein carbonyls to physiological levels and hence, the accretion of thiols may not be required anymore and was also lowered at basal levels. In the groups of *L. reuteri* PL503 treated with 5 and 25 µM, an insufficient genetic response did not avoid MDA-induced ROS generation and the subsequent protein carbonylation, and hence, the drop in thiols may respond to the consumption of such moieties in the sacrificial loss aforementioned. In probiotic bacteria such as *L. reuteri*, this mechanism may contribute to their

capability to counteract ROS and hence, defend themselves and the host against oxidative stress.

Enclosing, the present study provides original insight into the molecular and genetic responses of *L. reuteri* PL503 to the toxic effects of MDA, one of the most common lipid peroxidation products. This bacterium is able to detoxify MDA and hence, exert a potential health benefit in the gastrointestinal tract. Contributing to identify some of the underlying genetic and biochemical mechanisms facilitate the development targeted prophylactic and treatment strategies involving this and other probiotic bacteria. While the *uspA* and *dhaT* genes have been found to be strongly affected by MDA and likely play a relevant role in the response of the probiotic bacteria to oxidative stress, other genes and metabolic pathways may have been affected. Therefore, further genomic studies are required to unveil which other genes and metabolic routes may be involved in the responses of probiotic bacteria to the oxidative stress induced by MDA. The health benefits of dietary supplementation may be proven in further clinical studies.

## Experimental procedures

### Chemicals and raw material

All chemicals and reagents used in this study were of American Chemical Society (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 isolated from pig faeces was previously identified by 16S rRNA gene sequencing (Ruiz-Moyano *et al.*, 2008).

### Experimental setting

Stock cultures of *L. reuteri* PL503 were stored at −80 °C in Man, Rogosa and Sharpe (MRS) broth supplied with glycerol to a final concentration of 20% (v/v). Before experimental use, *L. reuteri* PL503 was subcultured twice under microaerophilic conditions at 37 °C for 24 h in MRS broth supplemented with 0.5% of diluted acetic acid (10%, v/v). Four experimental groups were considered depending on the concentration of MDA added: CONTROL (*L. reuteri*), 5 µM (*L. reuteri* + 5 µM MDA), 25 µM (*L. reuteri* + 25 µM MDA) and 100 µM (*L. reuteri* + 100 µM MDA). 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. The bacterial counts ranged from 9.6 to 10 log cfu ml<sup>−1</sup> during the entire assay and no significant changes were observed

between experimental groups. 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 counting of viable cells, 100  $\mu\text{l}$  of *L. reuteri* PL503 was inoculated on MRS agar at the same sampling time and conditions as the experimental tubes.

#### Gene expression studies

**Extraction of RNA.** After each incubation time, 1 ml samples of each treatment were frozen and stored at  $-80\text{ }^{\circ}\text{C}$ . RNA was extracted using the MasterPure™ RNA purification kit (Epicentre), which includes DNase treatment, as described by the manufacturer. Pure RNA was eluted in 35  $\mu\text{l}$  Tris (200 mM) EDTA (20 mM) buffer (pH 8) and kept at  $-80\text{ }^{\circ}\text{C}$  until required. The RNA concentration ( $\text{ng } \mu\text{l}^{-1}$ ) and purity ( $A_{260}/A_{280}$  ratio) were spectrophotometrically determined using a 1.5  $\mu\text{l}$  aliquot on the Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA).

#### cDNA synthesis

cDNA was synthesized using about 500 ng of total RNA following the instructions of the PrimeScript™ RT Reagent kit (Takara Bio Inc., Kusatsu, Japan). cDNA was stored at  $-20\text{ }^{\circ}\text{C}$  until further use.

#### Real-time PCR analysis of gene expression

The *uspA* and *dhaT* genes were selected for relative expression studies using real-time PCR (qPCR). The 16S gene was used as reference gene. The qPCR assays were performed and monitored in a ViiA™ 7 Real-Time system (Applied Biosystems, Foster City, CA, USA) using MicroAmp optical 96-well reaction plates, sealed with optical adhesive covers (Applied Biosystems). qPCR results were analysed using the Software ViiA™ 7 RUO v1.2.4. (Applied Biosystems). The SYBR Green technology was used. The reaction mixture (final volume, 12.5  $\mu\text{l}$ ), contained 2.5  $\mu\text{l}$  of cDNA, 6.25  $\mu\text{l}$  of SYBR® Premix Ex Taq™ (Takara Bio Inc.), 0.625  $\mu\text{l}$  of ROX™ Reference Dye (Takara Bio Inc.) and 300 mM of each primer pair. Some primers were specifically developed for this study such as *uspALr-F1* (CTTGGGTAGCGTTCACCATT) and *uspALr-R1* (TGA AAAAGCGGTTGACACTG) for the *uspA* gene (annealing temperature:  $60\text{ }^{\circ}\text{C}$ ) and *Lr16S\_F* (CCGC TAAACTCTGTTGTTG) and *Lr16S\_R* (CGTGACT TTCTGGTTGGATA) for the 16S gene (annealing temperature:  $55\text{ }^{\circ}\text{C}$ ). The primers LS67 (TGA CTGGATCC-TAATTTGGTCTCGGTGTTATTGC) and LS68

(TGA CTGAATTCTTCCGGATCTTAGGGTTAGG) were designed in accordance to Schaefer *et al.* [50] for the *dhaT* gene (annealing temperature:  $60\text{ }^{\circ}\text{C}$ ).

The qPCR programme consisted of initial denaturation step at  $95\text{ }^{\circ}\text{C}$  for 10 min at  $95\text{ }^{\circ}\text{C}$ ; 40 cycles at a denaturation temperature of  $95\text{ }^{\circ}\text{C}$  for 15 s and annealing/extension temperatures of  $55\text{ }^{\circ}\text{C}$  and  $60\text{ }^{\circ}\text{C}$  for the 16S and target genes, respectively, during 30 s. After the final qPCR cycle, a melting curve was included by heating the product from  $60$  to  $99\text{ }^{\circ}\text{C}$  and continuous measurement of the fluorescence was performed to verify the qPCR products. All samples were analysed in triplicate, including control sample consisting of adding sterile ultrapure water instead of cDNA. The expression ratio was calculated using the  $2^{-\Delta\Delta C_T}$  method reported by Livak and Schmittgen (2001). The calibrator sample corresponded to the value of the expression of the experimental group CONTROL at each sampling time.

**Study of *in vitro* reactivity of MDA with proteins.** In order to evaluate the ability of MDA to induce carbonylation in proteins, three proteins, namely, human serum albumin (HSA), human haemoglobin (HH) and bovine  $\beta$ -lactoglobulin (LAC) ( $5\text{ mg ml}^{-1}$ , final concentration) were dissolved in 100 mM phosphate buffer pH 6.5 and incubated separately with MDA ( $0.25\text{ } \mu\text{M}$ ) at  $37\text{ }^{\circ}\text{C}$  (HSA, HH) and  $80\text{ }^{\circ}\text{C}$  (LAC in an oven at constant stirring for 24 h. Proteins were selected based on their previously reported susceptibility to oxidative damage (Luna and Estévez, 2018, 2019). MDA concentration was set at preliminary tests aimed to find sublethal concentrations within the range found in colonic digests. Samples were taken at 24 h for the quantification of allysine and Schiff bases. The preparation of six experimental units corresponding to the reaction units (HSA-MDA, HH-MDA and LAC-MDA) and the corresponding controls (proteins without MDA) were replicated three times in corresponding independent assay and all analyses were repeated three times in each same experimental unit (9 measurements per analysis and per treatment to calculate means and standard deviations).

#### Analytical procedures

**ROS generation by flow cytometry analyses.** Flow cytometry detection of ROS in *L. reuteri* PL503 was performed as determined using previous published protocols (Ortega-Ferrusola *et al.*, 2017; Peña *et al.*, 2018). In brief, *L. reuteri* PL503 ( $1 \times 10^6$ ) was extended in 1 ml of PBS and stained with CellRox Deep Red (ThermoFisher, Waltham, MA, USA;  $5\text{ } \mu\text{M}$ ; excitation and emission wavelengths, 644 and 645 nm respectively) for detection of the bacterium producing

ROS, and Hoechst 33342 (0.5  $\mu$ M) (excitation and emission wavelengths, 345 nm and 488 nm respectively; Sigma, Steinheim, Germany) to identify the bacterium and remove debris from the analysis. After thorough mixing, the cell suspension was incubated at room temperature in the dark for 25 min, washed in PBS and the samples were immediately run on the flow cytometer. Flow cytometry analyses were conducted using a Cytoflex<sup>®</sup> flow cytometer (Beckman Coulter, Brea, CA, USA) equipped with violet, blue and red lasers. The instrument was calibrated daily 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 analysed 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.

*Synthesis of allysine standard compound.* N-Acetyl-L-AAS (allysine) was synthesized from N $\alpha$ -acetyl-L-lysine using lysyl oxidase activity from egg shell membrane following the procedure described by Akagawa *et al.* (2002). Briefly, 10 mM N $\alpha$ -acetyl-L-lysine was incubated under 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 was then 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<sub>3</sub>CN) at 37 °C for 2 h under stirring. Then, ABA derivatives were hydrolysed by 50 ml of 12 M HCl at 110 °C for 10 h. The hydrolysates were evaporated at 40 °C *in vacuo* to dryness. The resulting allysine-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 were checked by using MS and <sup>1</sup>H NMR (Estévez *et al.*, 2009).

#### Quantification of allysine

Five hundred microlitres of culture were dispensed in 2 ml microtubes and treated with cold (4 °C) 10% Trichloroacetic acid (TCA) solution. Each microtube was vortexed and then subjected to centrifugation at 600 *g* 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<sub>3</sub>CN 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 (4 °C) 50% TCA solution and centrifuged at 1200 *g* for 10 min. The pellets were then washed twice with 10% TCA and diethyl ether-ethanol (1:1). Finally, the pellet was treated with 6N 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  $\mu$ l of milliQ water and then filtered through hydrophilic polypropylene GH Polypro (GHP) syringe filters (0.45  $\mu$ m pore size, Pall Corporation, USA) for HPLC analysis.

A Shimadzu 'Prominence' HPLC apparatus (Shimadzu Corporation, Japan), equipped with a quaternary solvent delivery system (LC-20AD), a DGU-20AS online degasser, a SIL-20A auto-sampler, a RF-10A XL fluorescence detector, and a CBM-20A system controller, was used. An aliquot (1  $\mu$ l) from the reconstituted protein hydrolysates was injected and analysed in the above-mentioned HPLC equipment. AAS- ABA was eluted in a Cosmosil 5C<sub>18</sub>-AR-II RP-HPLC column (5  $\mu$ m, 150  $\times$  4.6 mm) equipped with a guard column (10  $\times$  4.6 mm) packed with the same material. The flow rate was kept at 1 ml min<sup>-1</sup> 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  $\mu$ l) were run and analysed 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 allysine-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 mMn (Utrera *et al.*, 2011). Results were expressed as nmol of allysine per mg of protein.

#### Analysis of Schiff bases

The formation of Schiff bases (SB) was assessed using a LS-55 PerkinElmer fluorescence spectrometer (PerkinElmer, Waltham, MA, USA). Prior to the analysis, reaction mixtures were diluted (1:20) with 8 M urea in 100 mM sodium phosphate buffer, pH 7. SB was excited at 350 nm and the emitted fluorescence recorded at 450 nm. The excitation and emission slit widths were set at 10 nm and the speed of data collection while

scanning was of 500 nm per min. The height of the peaks corresponding to SB spectra was recorded. After taking into consideration the applied dilutions, the results were expressed as fluorescence units.

#### Analysis of protein thiols

To avoid possible contamination with thiols from the medium, 250  $\mu$ l of each *L. reuteri* PL503 culture were washed twice with PBS and with ethanol:ethyl acetate (1:1). The pellet was resuspended in 250  $\mu$ l of guanidine hydrochloride and added to the cuvette in a final volume of 1250  $\mu$ l of guanidine hydrochloride. Absorbance was measured at 324 nm, pre and post addition of 250  $\mu$ l of 4 DPS (4,4'-Dipyridyl disulfide) in 12 mM HCl. Results were expressed as  $\mu$ mol of free thiol groups per mg of protein.

#### Analysis of thiobarbituric-reactive substances

Malondialdehyde and other thiobarbituric-reactive substances (TBARS) was measured in all samples from 200  $\mu$ l of each *L. reuteri* PL503 culture, adding 500  $\mu$ l thiobarbituric acid (0.02 M) and 500  $\mu$ l trichloroacetic acid (10%), incubating 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.

#### Statistical analysis

Data from the analysis ( $n = 3$ ) were collected and subjected to statistical analysis. The effect of different concentrations of MDA and incubation times on the chemical measurements, analyses of variance (ANOVA) was applied [SPSS v. 15.5, IBM (Endicott, NY, USA)]. The effect of MDA on the gene expression ( $\Delta\Delta C_T$  values) was analysed using paired Students' *t*-tests (SPSS v. 15.5). The statistical significance was set at  $P \leq 0.05$ .

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#### Author contributions

M.E. conceived the project and finalized the manuscript. M.E., M.J.A and A.R. designed the experiments and analysed the data. P.P. collected the samples, conducted the PCR and alllysine analyses and drafted the manuscript. F.J.P. conducted the cell cytometry analysis. All authors contributed to interpreting the data, reviewed and approved the submission of the manuscript.

#### Conflict of interest

The authors declare that they have no conflict of interest.

#### Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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