Effect of respiration and manganese on oxidative stress resistance of *Lactobacillus plantarum* WCFS1

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*Lactobacillus plantarum* is a facultatively anaerobic bacterium that can perform respiration under aerobic conditions in the presence of haem, with vitamin K₂ acting as a source of menaquinone. We investigated growth performance and oxidative stress resistance of *Lb. plantarum* WCFS1 cultures grown in de Man, Rogosa and Sharpe (MRS) medium without and with added manganese under fermentative, aerobic, aerobic with haem, and respiratory conditions. Previous studies showed that *Lb. plantarum* WCFS1 lacks a superoxide dismutase and requires high levels of manganese for optimum fermentative and aerobic growth. In this study, respiratory growth with added manganese resulted in significantly higher cell densities compared to the other growth conditions, while without manganese added, similar but lower cell densities were reached. Notably, cells derived from the respiratory cultures showed the highest hydrogen peroxide resistance in all conditions tested, although similar activity levels of haem-dependent catalase were detected in cells grown under aerobic conditions with haem. These results indicate that oxidative stress resistance of *Lb. plantarum* is affected by respiratory growth, growth phase, haem and manganese. As levels of haem and manganese can differ considerably in the raw materials used in fermentation processes, including those of milk, meat and vegetables, the insight gained here may provide tools to increase the performance and robustness of starter bacteria.

INTRODUCTION

Lactic acid bacteria such as *Lactobacillus plantarum* are widely used to produce various types of fermented foods. Several strains of *Lb. plantarum*, referred to as probiotics, are used and have been investigated for their potential health properties. Probiotics are defined as live microorganisms that are beneficial for health of the host (Fuller, 1989; FAO/WHO, 2001). Such benefits include reduced diarrhoea symptoms and stimulation of the immune system. *Lb. plantarum* WCFS1, which was isolated from human saliva, is one of the most widely studied probiotic strains and its genome has been sequenced (Kleerebezem *et al.*, 2003). The function of this strain in the gastrointestinal tract has been investigated extensively (Vesa *et al.*, 2000; Vaughan *et al.*, 2005; Marco *et al.*, 2007) and the host–microbe interaction is also being investigated (Meijerink *et al.*, 2010).

*Abbreviations*: ETC, electron transport chain; q-PCR, quantitative real-time PCR; ROS, reactive oxygen species; SOD, superoxide dismutase.

A supplementary figure is available with the online version of this paper.
pH (Ingham et al., 2008) and oxidative stress (Serrano et al., 2007). However, the *Lb. plantarum* oxidative stress response and survival capacity have not to our knowledge been investigated under respiratory growth conditions.

One of the side effects of growing under respiratory conditions is the possibility that reactive oxygen species (ROS) such as superoxide or hydroxyl radicals are generated due to leakage of electrons from intermediates of the ETC (Kohanski et al., 2007). These ROS can cause significant damage to various cellular structures such as DNA and proteins (Imlay & Linn, 1988). Most bacteria contain a specific enzyme, superoxide dismutase (SOD), which catalyses the conversion of superoxide to hydrogen peroxide. However, *Lb. plantarum* lacks a SOD enzyme and instead this bacterium accumulates high intracellular concentrations of manganese to scavenge superoxide and convert it to hydrogen peroxide (*H₂O₂*) during fermentative and aerobic growth (Archibald & Fridovich, 1981). Indeed, under these conditions *Lb. plantarum* WCFS1 requires high concentrations of manganese in the growth medium, e.g. 0.24 mM added manganese in de Man, Rogosa and Sharpe (MRS) medium, to obtain optimal growth conditions (Groot et al., 2005). The impact of manganese on *Lb. plantarum* performance during aerobic with haem and respiratory growth conditions has not previously been assessed. Notably, concentrations of manganese in the different niches occupied by *Lb. plantarum*, including soil, plant rhizosphere, and foods of plant and animal origin, vary considerably, ranging from 180 nM to 9 mM (Reuther & Labanauskas, 1966; Chapman, 1966; Labanauskas, 1966; Wang et al., 2008). As the given concentrations reflect total manganese present, the amount of manganese that can be sequestered by *Lb. plantarum* can be low in the different environments. *H₂O₂* can subsequently be decomposed by the action of catalases. Although lactic acid bacteria are generally considered to be catalase-negative, catalase activity has been reported for several species, including members of the genera *Lactobacillus*, *Pediococcus* and *Leuconostoc* (Delwiche, 1961; Knauf et al., 1992; Abriouel et al., 2004). There are two major classes of catalases: true or haem-dependent catalase, and manganese-containing catalase or pseudocatalase (Kono & Fridovich, 1983a, b). *Lb. plantarum* WCFS1 contains a specific haem-dependent catalase (Kleerebezem et al., 2003; Brooojmans et al., 2009a, b).

Respiratory growth of *Lb. plantarum* probably results in higher intracellular production of ROS and *H₂O₂* compared with aerobic growth with haem, and fermentative growth. It is possible that respiratory-grown cells adapt to these oxidizing compounds by inducing oxidative stress mechanisms. Induction of these mechanisms during growth could therefore result in more robust cells that are better equipped to resist the adverse conditions encountered in the gastro-intestinal tract or during preparation or storage of cells for starter cultures. For *Lactococcus lactis*, it has been shown that growth under respiratory conditions resulted in an increased survival capacity during long-term storage, and resistance was suggested to be linked to reduced intracellular oxygen concentrations (Dwat et al., 2001; Rezaiki et al., 2004). However, *Lc. lactis* differs significantly from *Lb. plantarum* because it contains a functional SOD enzyme but no catalase enzyme.

In this study, we investigated the difference in growth and oxidative stress resistance between respiratory-grown cultures, aerobically grown cultures with and without haem, and fermentatively grown cultures of *Lb. plantarum* WCFS1 in MRS medium without and with added manganese. We also investigated whether differences in *H₂O₂* resistance could be linked to the expression and/or activity of the *Lb. plantarum* haem-dependent catalase.

**METHODS**

**Bacterial strain and culture conditions.** *Lb. plantarum* WCFS1 was cultivated in MRS broth (de Man et al., 1960), or in modified MRS broth with manganese omitted. These media were reconstituted by adding 10 g peptone from pancreatic digestion of casein (Merck), 8 g beef extract (Sigma-Aldrich), 4 g yeast extract (Oxoid), 20 g (1 + 1) glucose monohydrate (Merck), 2 g dipotassium hydrogen phosphate (Merck), 1 g Tween 80 (Merck), 2 g diammonium citrate (Merck), 5 g sodium acetate (Merck), 0.2 mg magnesium sulfate heptahydrate (Merck), with or without 0.04 g manganese(II) sulfate monohydrate (Merck) to 1 litre of demineralized water. For the induction of ETC activity, haem and vitamin K₂ (both Sigma-Aldrich) were added to a final concentration of 10 μg ml⁻¹ and 50 μg ml⁻¹, respectively (Brooojmans et al., 2009a, b). To prepare pre-cultures, 10 ml MRS was statically incubated overnight at 30 °C. Pre-cultures were washed once with PBS (pH adjusted to 7.4 with HCl), resuspended in a similar volume of PBS and inoculated into the different media (1%, v/v). Cultures were grown under static (fermentative) or shaking (aerobic) conditions at 30 °C. Aerobic growth was performed by incubation in a shake flask (100 ml) at 200 r.p.m. Cell densities were monitored by measuring OD₆₀₀.

**Assessment of stress survival.** The stress survival of exponential-phase cultures (OD₆₀₀ ~0.7) and stationary-phase cultures (25 h) was assessed after exposure to *H₂O₂* and plumbagin. *H₂O₂* [30% (w/v) stock solution; Merck] was added to 15 ml cultures to give a final concentration of 0.2% (w/v) for exponential-phase cells and 0.5% (w/v) for stationary-phase cells. Samples were taken 0, 10, 20 and 30 min after exposure, serially diluted in peptone physiological salt solution [1 g neutralized bacteriological peptone (Oxoid) and 8.5 g NaCl to 1 litre of demineralized water], and appropriate dilutions were plated on MRS agar plates [1.5% (w/v) bacteriological agar; Merck]. Plates were incubated for 72 h at 30 °C and colonies were enumerated. Stress survival of cells was also assessed after exposure to plumbagin. Plumbagin (10 mg ml⁻¹ stock solution; Sigma-Aldrich) was added to 2 ml cultures to give a final concentration of 10 μg ml⁻¹ and the culture was incubated at 30 °C with shaking at 200 r.p.m. Samples were taken 60 min after exposure; enumeration was performed as described for *H₂O₂*-stressed cells.

All stress survival experiments were performed in three biologically independent replicates. Means and standard deviations were calculated and statistically significant differences were determined using Student’s t tests (P < 0.05).

**Catalase activity absorbance assay.** The catalase activity absorbance assay was performed as described previously (den Besten et al., 2009). Briefly, cells were washed in PBS and then diluted (1:10, v/v) in PBS supplemented with *H₂O₂* (final concentration, 40 mM). The decrease in absorbance at 240 nm was measured over time at 30 °C. 

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with a spectrophotometer (Spectramax Plus 384; Molecular Devices). One unit of catalase activity was defined as a decrease in the absorbance at 240 nm of 1 unit min⁻¹. The rate of decrease for each sample was corrected for the amount of cells added to the assay buffer (absorbance at 600 nm and the assay dilution factor, as cell suspensions were diluted 1:10 when they were exposed to H₂O₂). For all experimental conditions, three biologically independent replicates were performed.

RNA isolation. Total RNA was isolated from the unstressed cultures grown under different conditions. Cells were pelleted by centrifugation (15,000 g, 30 s, 4 °C). The cell pellet was resuspended in 1 ml TRI Reagent (Ambion), snap-frozen in liquid nitrogen, and stored at −80 °C until use. Frozen samples were thawed, transferred to Lysing MatrixB tubes (MP Biochemicals) and homogenized twice for 2 min at speed 6.0 in a FastPrep-24 instrument (MP Biochemicals). RNA isolation was performed as described in the TRI Reagent manufacturer’s protocol. A DNase treatment was performed on the RNA samples using TURBO DNase (Ambion), and the DNase was inactivated with the DNase inactivation agent (Ambion). After this treatment, 10 µl sodium acetate solution (3 M, pH 5.2) and 250 µl 96 % (v/v) ethanol were added to 100 µl RNA and samples were stored at −80 °C overnight. RNA was pelleted and washed with 70 % (v/v) ethanol. Finally, RNA was dissolved in 50 µl nuclease-free water. Isolated RNA was quantified and the quality was checked by measuring the absorbance at 260 and 280 nm on an Eppendorf biophotometer. RNA isolations were performed in two biologically independent replicates.

cDNA synthesis and quantitative real-time PCR (q-PCR). First-strand cDNA synthesis was performed with Superscript III Reverse transcriptase (Invitrogen) following the manufacturer’s protocol, using 2 µg total RNA for each sample. q-PCRs were performed on a 7000 sequence detection system (Applied Biosystems) and analysed with 7000 system SDS software. The reactions contained 10 µl 2 × SYBR green PCR Master Mix (Applied Biosystems), 1 µl of each primer (16S rRNA-fwd TGATCCTGGCTCAGGACGAA and 16S rRNA-rev TGCAAGCACC-AATCAATACCA for 16S rRNA, and kat-fwd TGGGGAAT and kat-rev TTTCCGGGCAACTTGTGAG for kat), 6 µl water and 2 µl cDNA. For each primer set a calibration curve was generated to calculate the efficiency of the PCRs. Expression of the kat target gene was normalized using 16S rRNA as internal standard. q-PCR experiments were performed in two independent experiments using two replicates for each sample.

RESULTS

Effect of manganese and ETC on growth of Lb. plantarum WCFS1

To investigate the effect of manganese and oxygen utilization on the growth performance of Lb. plantarum WCFS1, growth experiments were performed in MRS broth with and without added manganese under the following conditions:
fermentation (static; micro-anaerobic), aerobic (shaking), aerobic (shaking) with haem, and respiration (shaking with haem and vitamin K₂) (Fig. 1). With added manganese no significant differences in growth between the four growth conditions were observed in the early exponential phase (up to 8 h) (Fig. 1a). However, from 12 h onwards, the cell density of the respiratory-grown culture was significantly higher than the those of the other cultures (P<0.05, t test). The mean (±SD) final cell densities (OD₆₀₀) after 25 h of growth for the fermentative, aerobic, aerobic with haem, and respiratory growth conditions were 6.55±0.03, 6.79±0.66, 6.67±0.26, and 8.50±0.29, respectively, with only the last being significantly different from the others.

Growth of Lb. plantarum WCFS1 is associated with a decrease in pH of the medium, and a similar decrease in pH was observed for all growth conditions up to 16 h (Fig. 1b). However, pH during respiratory growth then increased, which resulted in a significantly higher pH after 25 h for the respiratory-grown culture (pH 4.45±0.11) compared with the fermentative (pH 3.87±0.07), aerobic (pH 3.97±0.09), and aerobic with haem conditions (pH 3.99±0.09) (P<0.05, t test). The increase in pH for the respiratory growth culture might be explained by the conversion of lactate to acetate after glucose depletion, as observed previously for Lc. lactis (Gaudu et al., 2002).

To investigate the role of manganese in the different growth conditions, cultures were grown in MRS broth without added manganese. No differences in growth (Fig. 1a) or pH (Fig. 1b) between the four growth conditions were observed, but the cultures grown without added manganese reached a lower cell density than cultures with added manganese (P<0.05, t test). These results extend earlier observations with Lb. plantarum that showed a requirement for high concentrations of manganese to reach optimum growth under fermentative and aerobic without haem conditions (Archibald & Duong, 1984).

Effect of manganese and ETC on H₂O₂ resistance

To investigate the effect of manganese and oxygen utilization on the resistance to oxidative stress, exponential- and stationary-phase cells of cultures grown in MRS with and without added manganese were exposed to H₂O₂ (Figs 2 and 3). Exponential-phase cells from the respiratory-grown culture grown in MRS with manganese showed higher survival after exposure to 0.2 % (w/v) H₂O₂ for 10 and 20 min compared with exponential-phase cells of the fermentative, aerobic and aerobic with haem cultures (P<0.05, t test) (Fig. 2a). Furthermore, higher survival of stationary-phase cells from both the respiratory and aerobic with haem cultures grown in MRS with...
manganese was observed after 30 min exposure to 0.5% (w/v) H_2O_2 compared with stationary-phase cells of the fermentative and aerobic cultures (P<0.05, t test) (Fig. 2b).

Notably, exponential-phase respiratory-grown cells in MRS without manganese showed higher survival after exposure for 20 min to 0.2% (w/v) H_2O_2 compared with exponential-phase cells of the fermentative, aerobic, and aerobic with haem cultures (P<0.05, t test) (Fig. 3a). Also, stationary-phase cells of the respiratory-grown cultures at 20 min were more resistant to 0.5% (w/v) H_2O_2 compared with stationary-phase cells from the other conditions (P<0.05, t test) (Fig. 3b). Interestingly, exponential-phase cells of the respiratory-grown cultures after 20 min and of the other conditions after 10 min with added manganese were more sensitive to H_2O_2 exposure than cells derived from cultures without added manganese (P<0.05, t test) (Figs 2a and 3a). These results suggest that respiratory growth results in increased resistance to H_2O_2 exposure and that the contribution of manganese is dependent on the growth phase at which the cells were harvested and subsequently tested for resistance. In a separate experiment, the cells were also exposed to plumbagin, a superoxide generator. Exposure of harvested cells from the fermentative, aerobic without and with haem, and respiratory-grown cultures revealed resistance of stationary-phase cells (data not shown), whereas exponential-phase cells grown in the absence of manganese showed higher resistance to plumbagin (see Supplementary Fig. S1, available with the online version of this paper). This is consistent with our data obtained with H_2O_2 stress, suggesting that growth at low manganese levels may result in activation of alternative stress resistance mechanisms.

**Impact of catalase on growth-mode-dependent H_2O_2 resistance**

As catalase is one of the most important enzymes for bacterial resistance against H_2O_2, we investigated catalase gene (kat) expression and catalase activity in the different growth conditions (Fig. 4). No differences in expression of kat were observed in exponential-phase cells derived from the different cultures (Fig. 4a). However, except for fermentation conditions, expression of kat was significantly induced in the stationary phase of growth in media without and with added manganese (P<0.05, t test). These results suggest that oxygen influx (shaking), which is the common factor that is absent during fermentative growth, results in the induction of kat expression. Catalase activity was also determined for the different growth conditions. In exponential-phase cells grown under the aerobic with haem and respiratory conditions, catalase activity was significantly higher compared with the fermentative and aerobic growth conditions without and with added manganese (P<0.05, t test) (Fig. 4b). Similar results were observed for catalase activity in stationary-phase cells, although the catalase activity in cells from the aerobic with haem and respiratory growth conditions was significantly.
induced compared with activity in exponential-phase cells ($P<0.05$, t test). However, catalase activity in respiratory-grown cells was similar to that in cells grown aerobically with haem, although in most conditions higher resistance against hydrogen peroxide was observed for respiratory-grown cells. These results suggest that, besides catalase, additional factors might be involved in the resistance of respiratory-grown cells to H$_2$O$_2$ exposure. Also, for the respiratory-grown cultures catalase activity was higher without manganese added for both exponential- and stationary-phase cells compared with cultures grown with added manganese ($P<0.05$, t test). These results indicate that manganese availability affects haem-dependent catalase activity and H$_2$O$_2$ resistance of exponential- and stationary-phase cells of *Lb. plantarum* WCFS1 to different extents.

**DISCUSSION**

We investigated the growth performance and oxidative stress resistance of *Lb. plantarum* WCFS1 under respiratory, aerobic with haem, aerobic, and fermentative growth conditions in MRS medium without and with added manganese. Previously, it has been shown that respiratory growth of *Lb. plantarum* resulted in higher biomass production and a higher final pH compared with fermentative growth (Brooijmans et al., 2009a, b). These results are in agreement with our observations for respiratory growth in the presence of manganese. However, no differences in *Lb. plantarum* WCFS1 biomass production or final pH were observed with cultures grown without added manganese, and final cell densities were significantly lower than those reached with added manganese. It has previously been shown that *Lb. plantarum* requires high concentrations of manganese to reach optimum growth in fermentative and in aerobic conditions without haem (Archibald & Duong, 1984; Groot et al., 2005). As *Lb. plantarum* WCFS1 was capable of growth in MRS broth without added manganese, it is possible that trace amounts of manganese were introduced with the yeast extract, beef extract and/or peptone fractions added to the MRS medium.

To investigate the influence of the different growth modes on oxidative stress resistance, exponential- and stationary-phase cells of the different cultures were exposed to H$_2$O$_2$. In general, both exponential- and stationary-phase cells of the respiratory-grown cultures showed highest resistance against H$_2$O$_2$. *Lb. plantarum* WCFS1 contains a specific
haem-dependent H$_2$O$_2$-degrading catalase (Kleerebezem et al., 2003; Brooijmans et al., 2009a, b), but not an additional manganese-dependent catalase, as described for other lactobacilli (Kono & Fridovich, 1983a). Therefore, we investigated the role of this haem-dependent catalase in the resistance of cells derived from the various growth modes against H$_2$O$_2$. Higher expression and activity of catalase were indeed observed under respiratory growth compared with fermentative growth. Expression of kat appeared to be specifically dependent on induced oxygen influx (shaking), while catalase activity was dependent on the addition of haem to the growth medium. Nevertheless, the respiratory-grown cultures tended to be more resistant to H$_2$O$_2$ than aerobic with haem cultures, which showed similar levels of catalase expression and activity. However, catalases convert H$_2$O$_2$ to water and oxygen, thereby increasing intracellular oxygen levels, which in turn may cause oxidative damage. The increased resistance to H$_2$O$_2$ of the respiratory-grown cultures compared with those grown aerobically with haem might therefore be related to the rapid consumption of oxygen by the active ETC (Fig. 5), as previously suggested for increased long-term survival of respiratory-grown Lc. lactis cultures compared with aerobic cultures (Rezaiki et al., 2004). Alternatively, increased resistance of the respiratory-grown Lb. plantarum culture might be related to the induction of oxidative stress resistance mechanisms as a result of formation of ROS, such as superoxide radicals, due to leakage of electrons from ETC intermediates (Imlay & Fridovich, 1991; González-Flecha & Demple, 1995; Kohanski et al., 2007). Previous studies with fermentative and aerobically grown Lb. plantarum showed that cells accumulate high levels of intracellular manganese to scavenge for superoxide radicals, as this bacterium does not contain a SOD enzyme that could prevent the damaging activities of these oxidizing radicals (Archibald & Fridovich, 1981). In our study, stationary-phase cells grown with high manganese showed higher resistance to H$_2$O$_2$ than cells grown with low manganese, with haem-catalase-producing aerobic with haem and respiratory-grown cells showing the highest resistance. However, exponential-phase cells of Lb. plantarum WCF$^1$ grown without added manganese showed higher resistance to H$_2$O$_2$ than exponential-phase cultures grown with added manganese, with again the haem-dependent catalase-producing aerobic with haem and respiratory cultures showing the highest resistance. Notably, a similar observation was made for cells exposed to plumbagin, a superoxide generator. The low manganese levels might activate other Lb. plantarum oxidative stress resistance mechanisms, including thioredoxin reductases, glutathione reductases, NADH oxidases and NADH peroxidases (Murphy & Condon, 1984; Kleerebezem et al., 2003; Serrano et al., 2007). In addition, a role for the alternative sigma factor 54 (rpoN) in regulating the mannose phosphotransferase system in H$_2$O$_2$ resistance has recently been identified (Stevens et al., 2010).

In conclusion, this study has shown that respiratory growth of Lb. plantarum results in increased cell densities and increased oxidative stress resistance. Furthermore, a growth-phase-dependent influence of manganese levels on H$_2$O$_2$ and plumbagin resistance was detected. These results may be relevant for industrial applications and may provide a basis to assess the impact of respiratory-like growth on the robustness of starter bacteria and probiotics.

**Fig. 5.** Schematic representation of the effect of oxygen utilization, haem and manganese availability on (haem-dependent) catalase expression/activity and H$_2$O$_2$ resistance of Lb. plantarum WCF$^1$. See text for details.

**REFERENCES**


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