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ORIGINAL ARTICLE

Temperature and respiration affect the growth and stress resistance of *Lactobacillus plantarum* C17

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Abstract

Aims: The aim of the study is to gain further insight on the respiratory behaviour of *Lactobacillus plantarum* and its consequences on stress tolerance.
Methods and Results: We investigated the effect of temperature and respiration on the growth and stress (heat, oxidative, freezing, freeze-drying) response of *Lact. plantarum* C17 during batch cultivations. Temperature as well as respiration clearly affected the physiological state of cells, and generally, cultures grown under respiratory conditions exhibited improved tolerance of some stresses (heat, oxidative, freezing) compared to those obtained in anaerobiosis. Our results revealed that the activities in cell-free extracts of the main enzymes related to aerobic metabolism, POX (pyruvate oxidase) and NPR (NADH peroxidase), were significantly affected by temperature. POX was completely inhibited at 37°C, while the activity of NPR slightly increased at 25°C, indicating that in *Lact. plantarum*, the temperature of growth may be involved in the activation and modulation of aerobic/respiratory metabolism.
Conclusions: We confirmed that respiration confers robustness to *Lact. plantarum* cells, allowing a greater stress tolerance and advantages in the production of starter and probiotic cultures.
Significance and Impact of the Study: This is the first study on respiratory metabolism on a strain other than the model strains WCFS1; novel information on the role of temperature in the modulation of aerobic/respiratory metabolism in *Lact. plantarum* is presented.

Introduction

Lactobacillus plantarum is a lactic acid bacterium (LAB) used as starter, adjunctive and/or probiotic culture in the production of many fermented and functional foods to improve shelf-life, organoleptic properties and human health (Siezen *et al.* 2011). The ability to cope with environmental factors is essential for its performance, and the type of metabolism may affect its fitness in food and nonfood applications. Several works on the stress response of *Lact. plantarum* have been published, but most of them have been carried out under anaerobic conditions. More recently, different authors (Lechardeur *et al.* 2011; Pedersen *et al.* 2012) have reported that oxygen availability and the presence of heme and menaquinone in the growth medium induce in some LAB species (*Lactococcus lactis*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Lact. plantarum*) a range of physiological changes (production of cytochromes; activation of an electron transport chain, ETC.; synthesis of antioxidant enzymes; ATP generation due to the production of acetate; improved utilization of available sugars and thus higher growth rate and increased biomass; lower intracellular O_2; improved long-term and oxidative survival), which, directly or indirectly, result in the expression of a phenotype with enhanced technological and stress response properties.

The shift towards respiratory pathway has been studied, characterized and exploited in *L. lactis* (Pedersen *et al.* 2005), but in *Lact. plantarum*, with the exception of

some studies about the model strain WCFS1 (Goffin *et al.* 2006; Brooijmans *et al.* 2009; Watanabe *et al.* 2012a,b; Zotta *et al.* 2012), data on the aerobic lifestyle are not extensive. Additionally, although several studies stressed the positive effect of aerobiosis/respiration on the strain performances, the mechanism of control and induction of aerobic/respiratory metabolism in *Lact. plantarum* is not yet completely understood.

Regulation of aerobic pathway takes place primarily at the level of POX (pyruvate oxidase) activity, which leads the oxidation of pyruvate into acetate with production of hydrogen peroxide and ATP generation by acetate kinase. As POX expression is controlled by availability of oxygen, hydrogen peroxide and carbon catabolite repression (Goffin *et al.* 2006), we recently investigated the effect of aerobiosis and inactivation of *ccpA* gene on the growth, stress tolerance (Zotta *et al.* 2012) and changes in proteome (Mazzeo *et al.* 2012) in *Lact. plantarum* WCFS1, to better understand the role of *ccpA* in regulation of aerobic metabolism and stress response. Our study showed that inactivation of *ccpA* dramatically affected the growth and stress resistance of *Lact. plantarum*, confirming the necessity to investigate and clarify the regulation of some proteins involved in aerobiosis and in stress survival.

Moreover, Watanabe *et al.* (2012a,b) evaluated the effect of respiration on growth and stress (oxidative and acid) resistance of *Lact. plantarum* WCFS1, concluding that, in contrast with *L. lactis,* the respiratory behaviour does not always offer robustness to harmful conditions: respiring cells were more resistant to oxidative stress (Watanabe *et al.* 2012a) but more sensitive to acid stress (Watanabe *et al.* 2012b). However, they did not investigate the consequence of respiratory metabolism on the survival to heat stress and long-term storage, which could be of practical relevance in biotechnological applications as well as in the preparation and distribution of starter and probiotic cultures.

Because the properties of micro-organisms are generally strain-specific and based on the above-mentioned considerations, we decided to investigate the effect of temperature (for which little data are present in literature: Zwietering *et al.* 1990, 1994), aeration and hemin-menaquinone supplementation on the growth, on the activity of oxygen-related enzymes and stress response of the strain *Lact. plantarum* C17 in batch fermentations.

Material and methods

Strains and culture conditions

Lactobacillus plantarum C17 was isolated from Caciocavallo cheese. The strain was selected for this study because of its stress tolerance (Parente *et al.* 2010), functional properties (A. Guidone, T. Zotta, R.P. Ross, C. Stanton, M.C. Rea, E. Parente, A. Ricciardi, personal communication), its capability for aerobic metabolism (Guidone *et al.* 2013) and its performance as an adjunct in cheese making (Ciocia *et al.* 2013).

The strain was maintained as freeze-dried stocks in reconstituted 11% (w/v) skim milk containing 0·1% (w/v) ascorbic acid (RSM) in the culture collection of Scuola di Scienze Agrarie, Forestali, Alimentari e Ambientali, Università degli Studi della Basilicata.

For batch cultivations and routine propagation (16 h, 35°C), a complex basal medium WMB (Zotta *et al.* 2012) was used.

Batch fermentations

The kinetics of growth of *Lact. plantarum* C17 was investigated during batch fermentations (2 replicates each) carried out in WMB (controlled pH 6·5) under anaerobic (nitrogen 0·1 v/v min^{-1}) and respiration (air 0·2 v/v min^{-1}, with 2·5 $\mu g\ ml^{-1}$ hemin and 1 $\mu g\ ml^{-1}$ menaquinone) conditions at either 25°C (suboptimal temperature) or 35°C (optimal temperature). Bioreactors (1 l working volume, Applikon, Schiedam, the Netherlands) were inoculated (1% v/v) with an overnight anaerobic or aerobic WMB preculture, as appropriate. Agitation was performed using a magnetic stirrer (200 rpm, Heidolph Instruments GmbH & Co., Schwabach, Germany), while pH was controlled (*ez*Control controller, Applikon) by automatic addition of sterile 1 : 1 Na_2CO_3/NaOH solution (4 eq l^{-1}) and foam by adding 1% (v/v) antifoam A solution. Concentration of dissolved oxygen (DO,%) was measured by a polarographic electrode (Applisens, Applikon).

Samples were aseptically withdrawn to measure the absorbance at 650 nm (A_{650}), and at the end of exponential growth phase, cells were enumerated by pour plating in WMB with 1·2% agar (WMA, pH 6·8, 35°C, 48 h, in Generbox jars; bioMérieux SA, Marcy-l'Etoile, France, with AnaeroGen bags, Oxoid, Ltd., Basingstoke, Hampshire, UK). A standard curve relating A_{650} and plate counts was obtained by liner regression and used to estimate the number of cells. Growth curve parameters (lag time, growth rate and asymptote) were estimated with the dynamic primary model of Baranyi and Roberts (1994) using the DMFit v 2.0 program (Baranyi and Le Marc 1996).

Chemical and biochemical analyses

Gravimetric measurement (cell dry weight, measured after drying the washed biomass at 105°C for 24 h) of biomass was used to estimate the maximum biomass yield ($Y_{X/S}$, $g\ g^{-1}$, net biomass production divided by total sugar consumed) during the growth.

The 3,5-dinitrosalicylic acid (DNSA) method (Miller 1959) was used to measure the residual glucose (as reducing sugars) in culture supernatants, while enzymatic kits (R-Biopharm AG, Darmstadt, Germany) were used to quantify the production of lactic and acetic acids. The amount of hydrogen peroxide (H_2O_2) in supernatants and catalase activity in whole cells were measured as described by Risse *et al.* (1992).

The activities of the enzymes related to aerobic metabolism (pyruvate oxidase, POX; NADH oxidase, NOX; NADH peroxidase, NPR) were measured in cell-free extracts (FastPrep-24 Instrument; MP Biomedicals, Santa Ana, CA, USA) using spectrophotometric assays (Quatravaux *et al.* 2006) at both 25 and 37°C (*in vitro* assay temperature).

Oxygen uptake

The oxygen uptake of both exponential and stationary phase cells was measured by resuspending (A_{650} = 1·0) whole cells recovered by centrifugation (12000 ***g***, 5 min) in oxygen-saturated 20 mmol l^{-1} phosphate buffer pH 7·0 (PB7) containing 5·5 mmol l^{-1} glucose and by monitoring (polarographic electrodes; BioXpert 2 software, Applikon) the decrease in oxygen concentration (DO,%) for 15 min at 35°C.

The concentration of dissolved oxygen (DO,%) was transformed into μmol l^{-1} using Henry's law and the specific oxygen uptake rate (μmol O_2 min^{-1} g^{-1} of biomass) was calculated.

Stress treatments

Stress tolerance was evaluated on both exponential (E) and stationary (S) growth phases.

Tolerance of heat stress was evaluated as described in Zotta *et al.* (2012) by exposing cell suspensions (A_{650} = 1·0) in PB7 at 55°C for 0, 5, 10, 15 and 30 min and estimating the number of survivors by pour plating in WMA (35°C, 48 h, anaerobiosis). Kinetics of inactivation were fitted using a Weibull model (van Boekel 2002) with SYSTAT 13 software.

Tolerance of freezing (cells resuspended in RSM, A_{650} = 1·0, storage at −20°C in 50% w/w glycerol solution, thawing) and freeze-drying (cells resuspended in RSM, A_{650} = 1·0, freeze-drying and storage at −20°C) treatments was evaluated by pour plating on WMA (35°C, 48 h, anaerobiosis) after 30 and 90 days of storage.

Batch cells were exposed (30 min, 35°C) to different H_2O_2 concentrations (from 0·8 to 0·0015 mol l^{-1}) and the survivors (if any) were grown in WMB (pH 6·8, 16 h, 35°C, microplate experiment) to evaluate the resistance to oxidative stress.

Statistical analysis

All statistical and graphic analyses were performed using SYSTAT 13.0 for Windows (Systat Software Inc., Richmond, CA, USA).

Results

Growth and metabolite production

The kinetics of growth of *Lact. plantarum* C17 was modelled using the D-model (Baranyi and Roberts 1994), which provided an excellent fit for all fermentations (R^2 from 0·982 to 0·991).

Growth parameters and metabolite production are shown in Table 1. As expected, cultivations at 25°C decreased (ca. 42%) the maximum specific growth rates (μ_{max}) compared to the levels obtained at 35°C. Respiration (aeration and supplementation with hemin and menaquinone) did not affect growth rate, but eliminated the lag phase at 35°C (0 h compared to 0·75 h measured in anaerobiosis) and increased the adaptation period at 25°C (2·5 h compared to 1·5 h measured in anaerobiosis). Biomass yield ($Y_{X/S}$) was not significantly affected by the presence of oxygen and addition of cofactors.

Sugar was almost completely consumed in stationary phase (from 1·4 g l^{-1} of residual glucose in aerobiosis at 25°C, maximum value, to 0·6 g l^{-1} of residual glucose in anaerobiosis at 35°C, minimum value). The lactic acid yield ($Y_{P/S}$) during anaerobic growth was 96% and 85%, respectively, at 35 and 25°C. On the contrary, when oxygen, hemin and menaquinone were used, lactic acid concentration greatly decreased, suggesting a possible conversion to acetate (the main product of aerobic metabolism) or to CO_2. As a result, an increase in acetic acid concentration was measured under respiratory growth in the stationary phase; however, small amounts (from 0·97 to 1·35 g l^{-1}) of this metabolite were detected in anaerobiosis and during the exponential phase as well.

The time course of dissolved oxygen concentration under aerobic conditions at 25 and 35°C was similar; oxygen consumption apparently increased during the exponential phase, peaked at the beginning of the stationary phase and then decreased (Fig. 1). At 25°C, the levels of dissolved oxygen (DO,%) in stationary phase was slightly lower (30%) than at 35°C (45%).

H_2O_2 (another important product of POX activity) concentration was always very low (0·07–0·02 mmol l^{-1}), because of high catalase activity (15–16 mkatal g^{-1} of biomass) detected, at both temperatures, in the stationary phase of respiratory growth.

Table 1 Growth parameters and metabolic production during batch fermentations

Growth condition	Growth phase	μ_{max} (h^{-1})	S/S_0 (%)	$Y_{X/S}$	$Y_{P/S\ (lactic\ acid)}$	Lactic acid (g l^{-1})	Acetic acid (g l^{-1})
35°C, AN	E	0·771 ± 0·03	21·2	0·08 ± 0·01	0·59 ± 0·01	2·61 ± 0·07	0·97 ± 0·10
	S		96·7	0·12 ± 0·01	0·96 ± 0·01	18·85 ± 0·11	1·43 ± 0·11
35°C, AE	E	0·772 ± 0·03	25·1	0·06 ± 0·02	0·23 ± 0·04	1·27 ± 0·11	1·07 ± 0·05
	S		94·3	0·11 ± 0·01	0·63 ± 0·00	12·09 ± 0·20	6·65 ± 0·31
25°C, AN	E	0·456 ± 0·02	18·5	0·07 ± 0·01	0·50 ± 0·02	1·94 ± 0·07	1·03 ± 0·12
	S		94·8	0·12 ± 0·00	0·85 ± 0·02	16·35 ± 0·07	1·20 ± 0·04
25°C, AE	E	0·455 ± 0·02	18·9	0·06 ± 0·01	0·21 ± 0·04	0·88 ± 0·07	1·35 ± 0·09
	S		92·7	0·13 ± 0·00	0·77 ± 0·00	14·53 ± 0·11	2·20 ± 0·06

Mean values ± standard error are shown.
μ_{max} = maximum specific growth rate, estimate value ± standard error; R^2 ranged from 0·982 to 0·991.
S/S_0 = Fermentation efficiency (consumed glucose, g/initial glucose, g,*100).
$Y_{X/S}$ = Biomass yield coefficient (biomass yield, g, relative to total sugar consumed, g).
$Y_{P/S}$ = Lactic acid production coefficient (lactic acid produced, g, relative to total sugar consumed, g).
Growth condition: AN, anaerobic growth condition; AEHM, aerobic growth condition in presence of 2·5 μg ml−1 haemin and 1 μg ml−1 menaquinone.
Growth phase: E, exponential phase; S, stationary phase.

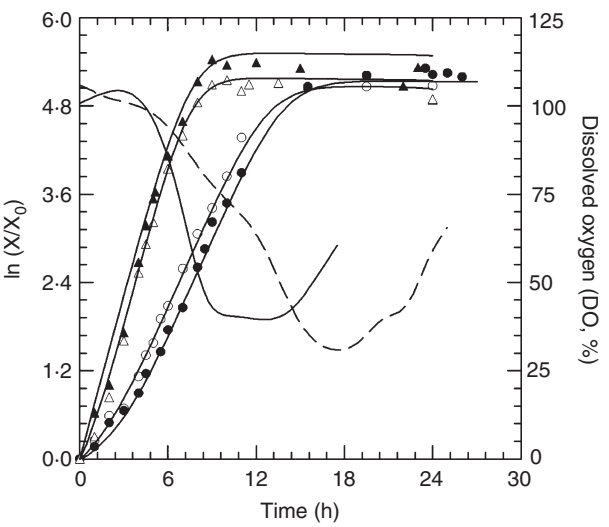

Figure 1 Kinetics of growth (lnX/X_0, biomass mg l^{-1}) and dissolved oxygen concentration (DO,%) of *Lactobacillus plantarum* C17 grown under anaerobic (nitrogen 0·1 v/v min^{-1}) and aerobic (air 0·2 v/v min^{-1}, with 2·5 μg ml^{-1} hemin and 1 μg ml^{-1} menaquinone) conditions, at both 25 and 35°C. ○, growth at 25°C in anaerobiosis; ●, growth at 25°C in aerobiosis; △, growth at 35°C in anaerobiosis; ▲, growth at 35°C in aerobiosis. DO% is shown only for fermentations with air sparging: continuous line, cultivation at 35°C; dotted line, cultivation at 25°C.

Activities of enzymes involved in aerobic metabolism

The activities of POX, NOX and NPR were measured in stationary-phase cells at both 25 and 37°C, irrespectively of the growth temperature of cells. As shown in Fig. 2, our results revealed that POX and NPR, but not NOX (Tukey's HSD, $P = 0·721$), were significantly ($P < 0·005$) affected by temperature. Specifically, POX was completely inhibited (*in vitro* assay) at 37°C but active at 25°C, especially in cells grown in presence of oxygen and cofactors. Additionally, cultivation at lower temperature (25°C) seems to increase the specific enzymatic activity.

As mentioned before, NPR activity was also affected by assay temperature ($P < 0·005$), with higher activities at 25°C. As with POX, increased specific activity of NPR was also found in cells grown at 25°C, even if significant activity was detected in cultures grown at 35°C and under fermentative conditions. Only the activity of NOX seemed not to be correlated with low temperature and presence of oxygen, showing similar values at both 25 and 35°C (*in vitro* assay; $P = 0·721$) and exhibiting the highest activities in cells cultivated in anaerobiosis and optimal temperature of growth (35°C).

Oxygen uptake

Cells of *Lact. plantarum* C17 grown in aerobiosis exhibited the ability to consume oxygen in presence of 5·5 mmol l^{-1} of glucose. Cultures grown at 25°C had rates of oxygen uptake (2·94 and 4·25 μmol min^{-1} g^{-1} of biomass in E phase and S phase, respectively) higher than those measured in cells cultivated at 35°C (1·28 and 1·73 μmol min^{-1} g^{-1} of biomass in E phase and S phase, respectively).

Effect of temperature and respiratory growth on stress response

Thermal inactivation

The parameters of the kinetics of thermal inactivation were estimated with the Weibull model (Fig. 3) that provided a satisfactory fit for most trials (R^2 from to 0·895 to 0·993). As the kinetics were calculated on two

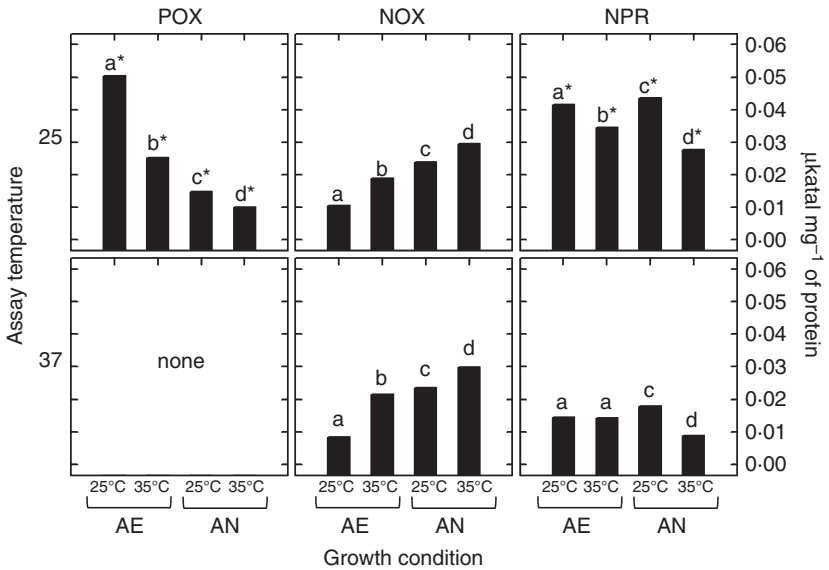

Figure 2 Activities of the main enzymes related to aerobic metabolism, measured in stationary cell-free extracts of *Lact. plantarum* C17 grown under anaerobic (nitrogen 0·1 v/v min^{-1}) and aerobic (air 0·2 v/v min^{-1}, with 2·5 μg ml^{-1} hemin and 1 μg ml^{-1} menaquinone) conditions, at both 25 and 35°C. Assay was carried out at both 25 and 37°C. POX, pyruvate oxidase; NOX, NADH oxidase; NPR, NADH peroxidase. Data were expressed as μkatal mg^{-1} of protein. As NOX and NPR, one katal corresponds to the oxidation of 1 mol of NADH per min at T°C; as POX, one katal corresponded to the production of 1 mol of H_2O_2 per min at T°C. Letters (a, b, c, d) on multiplot bars indicate significant differences (Tukey's HSD, $P < 0·005$) in enzymatic activities among growth conditions (temperature and aeration) and asterisks (*) indicates significant differences between assay temperature. Standard errors (SE) were always lower than 0·001, than SE bars are not detectable on the graph.

replicates, the goodness of fit was higher for exponential phases (R^2 from to 0·976 to 0·993) because of better control of cell growth (all samples were taken at the same optical density, $OD_{650\ nm}$=1·0), compared to the stationary phase in which (due to different sampling time) the control of physiological state of cells was more difficult and affected by several parameters. The inactivation trend was complex and varied depending on the interaction between growth phase and temperature of growth as well as aerobic parameters (Table 2). In exponential phase cells (Fig. 3), the presence of oxygen and cofactors increased the time to reach 3-log-cycle reduction (t3D) at 25°C and, to a major extent, at 35°C (Fig. 4, vertical continuous lines). Differences in reduction time due to interaction between temperature/rate/aeration and supplementation were significant ($P < 0·005$) and more marked (Fig. 4; oblique dashed lines). As expected, compared to exponential phase, t3D values were higher in stationary growth (Table 2). However, respiration reduced heat tolerance of the stationary cells grown at 35°C while protected those cultivated at 25°C, suggesting that, in stationary growth phase, the factors that affect stress response are several and related in a complex way.

Oxidative stress

In keeping with the high levels of catalase and NPR activities detected in stationary-phase cell, the resistance to H_2O_2 was significantly improved by respiratory lifestyle and growth into the stationary phase. Cultivation at 35°C slightly increased stress tolerance. Specifically, the presence of oxygen and cofactors increased the robustness of *Lact. plantarum* C17, doubling the H_2O_2 levels tolerated by cells: at 35°C, in fact, concentrations of 50 mmol l^{-1} were tolerated by the strain, compared to 25 mmol l^{-1} in anaerobiosis, while at lower temperature (25°C), 38 mmol l^{-1} of H_2O_2 was tolerated during respiratory growth and only 19 mmol l^{-1} under anaerobic growth.

Freezing and freeze-drying tolerance

Responses to freezing and freeze-drying were complex and related to the interaction among temperature, growth phase and aeration (Fig. 5). The positive effect of respiration on cell survival was clearly evident (increase from 1 to 4 log units; $P < 0·005$) in exponential phase after both 30 and 90 days of freezing. Additionally, after 1 month of storage, the temperature of growth did not affect the levels of tolerance, while for a long-term storage (90 days), the growth under optimal conditions (35°C) and the presence of oxygen and cofactors gave a net gain in the number of survivors.

As to freeze-drying process, differences in stress tolerance were not as evident as in freezing (maximum up to 1 log cycle reduction) and respiration and adaptation to lower temperature (25°C) offered a significant

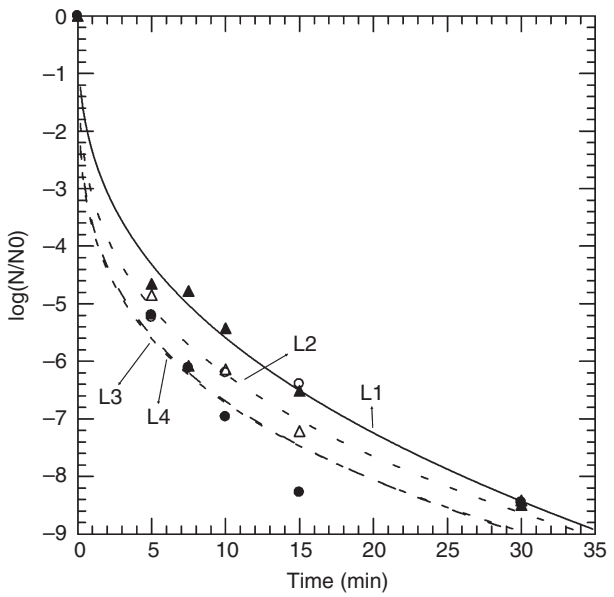

Figure 3 Kinetics of thermal inactivation (Weibull model) of exponential cells of *Lactobacillus plantarum* C17 grown under anaerobic (nitrogen 0·1 v/v/ min^{-1}) and aerobic (air 0·2 v/v min^{-1}, with 2·5 μg ml^{-1} hemin and 1 μg ml^{-1} menaquinone) conditions, at both 25 and 35°C. Survival was expressed as log (N/N_0), where N_0 and N are the number of viable cells before and after exposure to heat stress. (○), growth at 25°C in anaerobiosis; (●), growth at 25°C in aerobiosis; (△), growth at 35°C in anaerobiosis; (▲), growth at 35°C in aerobiosis. L1, growth at 35°C in aerobiosis; L2, growth at 35°C in anaerobiosis; L3, growth at 25°C in anaerobiosis; L4, growth at 25°C in aerobiosis. Averages of two independent fermentations with two replicate inactivation experiments each.

($P < 0{\cdot}005$) advantage only for the exponential phase cells after 90 days of storage.

Stationary phase cells, as expected, were intrinsically more tolerant and their survival was generally higher than that measured during exponential phase growth, although the shift towards respiration did not always provide advantages in stress resistance.

Discussion

Aerobic and respiratory metabolism have been successfully studied and exploited in *L. lactis* (Gaudu *et al.* 2003; Rezaiki *et al.* 2004; Pedersen *et al.* 2005, 2008; Brooijmans *et al.* 2007; de Felipe and Gaudu 2009) and to a lesser extent in *Lact. plantarum* (Quatravaux *et al.* 2006; Stevens *et al.* 2008; Brooijmans *et al.* 2009; Mazzeo *et al.* 2012; Watanabe *et al.* 2012a,b; Zotta *et al.* 2012). The latter species includes versatile strains that can contribute to the production of both plant and animal foods. These environments contain oxygen and heme sources, which may support respiratory growth.

In this study, we evaluated the effect of growth temperature and aerobic cultivation, under conditions (oxygen, hemin and menaquinone as cofactors) promoting respiration, on the metabolite production and response to stresses (heat, oxidative, freezing, freeze-drying) of practical relevance for the fitness of starter and probiotic *Lact. plantarum* cultures.

Cultivations at 25°C significantly reduced the growth of *Lact. plantarum* C17 compared to the optimal temperature 35°C, while the presence of oxygen and cofactors did not improve μ_{max} and biomass production, indicating that the effects of respiration on *Lact. plantarum* growth are strain-specific.

The effect of temperature on the growth and functionality of lactic acid bacteria has been considered by several authors (Zwietering *et al.* 1994; Ricciardi *et al.* 2009; Trontel *et al.* 2010; Enitan *et al.* 2011). Additionally, we

Table 2 Parameters of thermal inactivation (Weibull model, van Boekel 2002)

Growth condition	Growth phase	μ_{max} (h^{-1})	α (min)	β (–)	R^2	RMS	t3D (min)
35°C, AN	E	0·771 ± 0·03	0·001 ± 0·001	0·298 ± 0·013	0·990	0·077	0·85
	S		0·796 ± 0·134	0·735 ± 0·038	0·961	0·206	11·04
35°C, AE	E	0·772 ± 0·03	0·011 ± 0·003	0·374 ± 0·014	0·993	0·064	1·88
	S		0·033 ± 0·033	0·393 ± 0·062	0·895	0·676	4·51
25°C, AN	E	0·456 ± 0·02	0·000 ± 0·000	0·268 ± 0·047	0·983	0·499	0·38
	S		0·015 ± 0·009	0·391 ± 0·035	0·937	0·553	2·10
25°C, AE	E	0·455 ± 0·02	0·000 ± 0·000	0·268 ± 0·021	0·976	0·223	0·50
	S		0·149 ± 0·058	0·553 ± 0·044	0·925	0·656	4·91

Mean values ± standard error are shown.

μ_{max} = maximum specific growth rate, estimate value ± standard error; R^2 ranged from 0·895 to 0·993.

α = Scale parameter (min) of Weibull distribution.

β = Shape parameter (without dimension) of Weibull distribution.

RSM, root mean square.

t3D = Time (min) to reach 3-log-cycle reduction.

Growth condition: AN, anaerobic growth condition; AE, aerobic growth condition in presence of 2·5 μg ml^{-1} haemin and 1 μg ml^{-1} menaquinone.

Growth phase: E, exponential phase; S, stationary phase.

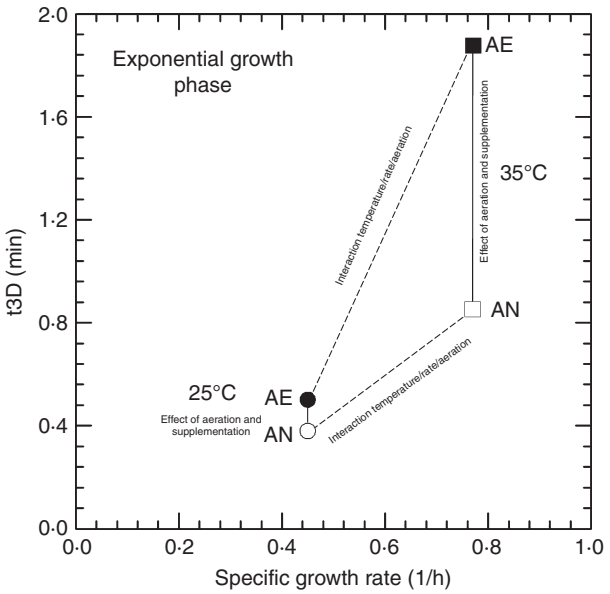

Figure 4 Plot showing the values of t3D (time to reach 3 log cycle reduction; estimated with Weibull model) as function of μ_{max} (exponential growth phase). The effect of only aeration-supplementation (vertical continuous lines) and that of interaction temperature/rate/aeration-supplementation (oblique dashed lines) are clearly evident. AN, anaerobiosis; AE, aerobiosis.

(Guidone *et al.* 2013) previously investigated the diversity and the effect of aerobic/respiratory metabolism in several (11) strains of *Lact. plantarum* group, demonstrating that presence of oxygen and cofactors differently affected the growth rate and biomass production in this species. In LAB (Pedersen *et al.* 2012), the production of additional energy (ATP) during aerobic/respiratory lifestyle (due to the conversion of pyruvate into acetate) promotes growth and survival in stationary phase. However, the production of H_2O_2 and the oxygen accumulation as well as the capability to synthesize antioxidant enzymes might change the growth behaviour of strains and may not result in an increase in biomass even if the extra ATP is produced.

The occurrence of a shift towards aerobic metabolism (pyruvate-to-acetate pathway) may be supported by the presence of higher acetate concentrations when oxygen and cofactors were used. However, stoichiometric considerations about metabolite production indicated that the amounts of acetate were higher than those possibly resulting from lactate, probably due to the presence of citrate (5 g l^{-1} tri-sodium citrate, corresponding to 19 mmol l^{-1} citrate) in the growth medium. Small amounts (16–24 mmol l^{-1}) of this metabolite were also found in anaerobic growth as well as in exponential phase of aerobic cultivations, indicating that production of acetic acid is not due solely to pyruvate oxidase/acetate kinase activities. Although in *Lact. plantarum* the citrate metabolism is mainly active at lower pH (pH 4·5–5–0; Palles *et al.* 1998), the transcriptome analysis of Stevens *et al.* (2008) on the aerobic cultures of *Lact. plantarum* WCFS1 (DO_{600} = 1; pH 6·5, 37°C, aerobiosis) suggested the conversion of citrate into pyruvate, as the citrate lyase gene (CL is the first enzyme in citrate pathway) was highly expressed in exponential phase cells.

Additionally, the same authors (Stevens *et al.* 2008) found that *poxF* gene (coding for pyruvate oxidase, POX) was expressed in exponential phase resulting in the production of acetate during early aerobic growth: these data contradict the previous observations about carbon catabolite repression of *pox* gene (Lorquet *et al.* 2004), suggesting that other factors should be taken in account in the regulation of aerobic metabolism and confirming once again that the shift mechanisms and the effect of oxygen pathway are strain-specific.

Pyruvate oxidase (POX) is the key enzyme of aerobic metabolism promoting, in presence of oxygen, the decarboxylation of pyruvate to acetyl-phosphate (acetyl-P), in turn converted to acetate by acetate kinase (Goffin *et al.* 2006; Quatravaux *et al.* 2006).

Although the genome databases suggest the presence of multiple POX-encoding genes (*pox1* or *poxD*, lp_0849; *pox2* or *poxE*, lp_0852; *pox3* or *poxF*, lp_2629; *pox4* or *poxC*, lp_3587; *pox5* or *poxB*, lp_3589) in *Lact. plantarum*, several transcriptomic and proteomic studies revealed that Pox3 and Pox5 are the main enzymes involved in pyruvate-to-acetate conversion (Goffin *et al.* 2006; Bron *et al.* 2012; Zotta *et al.* 2012). Other genes seem to be not transcribed (*pox1*) or not functional (*pox2* and *pox4*) because of low specificity with pyruvate (Goffin *et al.* 2006).

As a result of POX activity, H_2O_2 and CO_2 are also produced. In our study, H_2O_2 was not detected in the supernatant of aerobic cultures confirming the presence of heme catalase in *Lact. plantarum* (Abriouel *et al.* 2004; Guidone *et al.* 2013). The absence of H_2O_2 under fermentative conditions shows that this metabolite is strictly linked to aerobic pathway, although the small amounts of NADH peroxidase (NPR) in anaerobiosis may have contributed to its degradation.

In addition to POX, NADH oxidase (NOX) and NADH peroxidase (NPR) are also involved in aerobic catabolic pathways in lactic acid bacteria. NOX catalyses the oxidation of $NADH+H^+$ generating H_2O_2, while NPR promotes the consumption of the reactive oxygen species (H_2O_2) with water formation using an organic compound (generally $NADH+H^+$ as a donor; Quatravaux *et al.* 2006). NOX and NPR enzymes are also encoded by different loci (*nox1*; lp_0760; *nox2*, lp_0766; *nox3*,

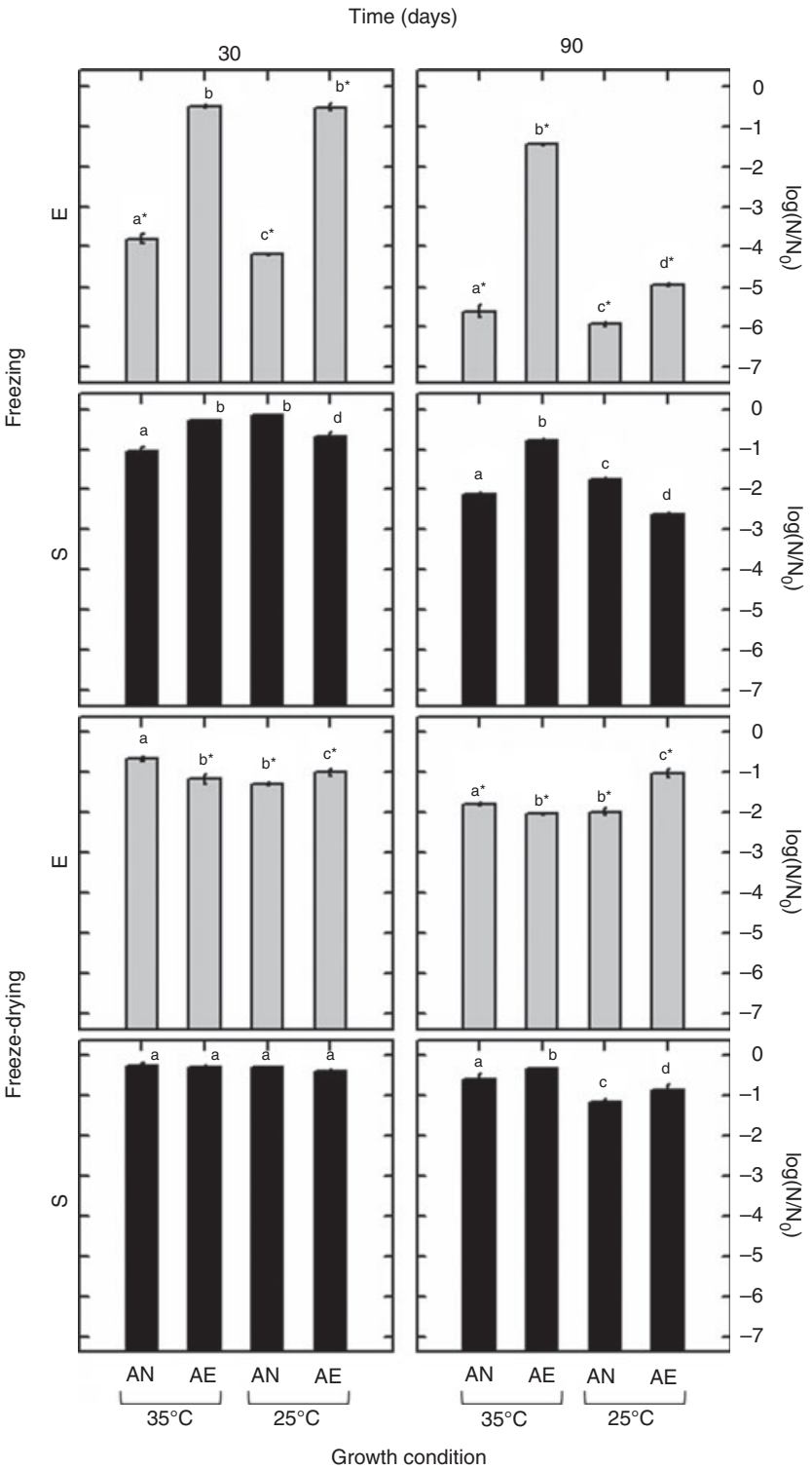

Figure 5 Multiplot showing the tolerance to freezing and freeze-drying stresses of exponential and stationary phase cells of *Lactobacillus plantarum* C17 grown under anaerobic (nitrogen 0·1 v/v min^{-1}) and aerobic (air 0·2 v/v min^{-1}, with 2·5 μg ml^{-1} hemin and 1 μg ml^{-1} menaquinone) conditions, at both 25 and 35°C. Survival was expressed as log (N/N_0), where N_0 and N are the number of viable cells before and after exposure to stresses. Letters (a, b, c, d) on multiplot bars indicate significant differences (Tukey's HSD, $P < 0{\cdot}005$) in survival among growth conditions (temperature and aeration) and asterisks (*) indicates significant differences between growth phases (E and S). Standard error bars are shown. E, exponential phase cells; S, stationary phase cells.

lp_1925; *nox4*, lp_1941; *nox5*, lp_3449; *nox6*, lp_1350; *npr1*, lp_1445 and *npr2*, lp_2544), but several studies suggest the principal involvement of *nox5* and *npr2* in the oxygen metabolism and peroxide stress (Stevens *et al.* 2010; Bron *et al.* 2012; Zotta *et al.* 2012). Our study proposes that POX and NPR have similar regulation being promoted by the same factors (oxygen, H_2O_2, low temperature), while NOX is not necessarily related to

aerobic metabolism having increased activity under fermentative cultivation.

Pyruvate oxidase activity was strongly affected by assay temperature indicating that the enzyme, even if induced by aerobiosis and glucose depletion, may not always be active *in vivo* and that its activity may also be strain-specific. Bron *et al.* (2012) found an over-expression of *pox3* and *pox5* in *Lact. plantarum* WCFS1 cultivated at 37°C in presence of O_2 compared to the aerobic growth at 28°C; however, not always the gene expression levels reflect the enzymatic activity *in vivo* conditions, because environmental factors may not regulate the genes at transcriptional level, but affect the functionality of their products (proteins, enzymes; Kang *et al.* 2013). The loss of POX stability at temperatures beyond 32°C has been demonstrated by Risse *et al.* (1992) and successively Tittmann *et al.* (1998) showed that the formation of FAD-thiamine-pyruvate oxidase ternary complex (necessary for the enzyme activation) was detected at low temperature (more at 10°C compared to 25°C). This could explain the absence of POX activity at 37°C (assay temperature) and higher levels in cells grown at a lower temperature (25°C).

Additionally, Quatravaux *et al.* (2006) showed that POX was significantly affected by aeration parameters, finding optimal levels in culture grown in presence of 30% of dissolved oxygen (DO). In our study, DO% was not controlled and was usually higher than 30%; specifically, stationary phase cells (in which enzymatic activities were measured) were harvested when DO% was around 45% and 30%, respectively, at 35 and 25°C; then, the high levels of DO% detected in stationary growth at 35°C could be an another factor that decreased the enzyme activity and, probably, a more appropriate modulation of oxygen supply is relevant to optimize the strain performance during aerobic growth. The major extent of oxygen consumption detected *in vivo* in cells grown at 25°C was also found *in vitro* assay, indicating a possible more rapid activation of respiratory pathway at low temperature.

This study underlines similar mechanisms of activation and regulation in POX and NPR, but not in NOX being not directly regulated by temperature and oxygen. However, this is not surprising because other authors (Murphy and Condon 1984; Sedewitz *et al.* 1984; Marty-Teysset *et al.* 2000) found NOX activities also in nonaerated or low-aerated cultures.

Cultivation at 25°C, generally, impaired the stress tolerance of *Lact. plantarum* C17. In a previous study (Zotta *et al.* 2012), we evaluated the effect of aerobiosis on the growth and stress tolerance (acid, heat, oxidative, starvation) of *Lact. plantarum* WCFS1, suggesting the positive role of aerobic metabolism on cell survival. However, Rezaiki *et al.* (2004) suggested that, sometime, only aeration could be toxic to the cells (due to accumulation of oxygen), while the addition of cofactors (such as hemin) during aerobic growth could provide greater benefits (in term of stress response) to cultures because of oxygen elimination by the respiration (ETC.) chain. Therefore, we decided to evaluate the effect of respiration (oxygen and supplementation of hemin and menaquinone) on stress resistance of *Lact. plantarum* C17.

The response to heat stress has been extensively characterized in *Lact. plantarum* but, with exception of our study on the model strain WCFS1 (Zotta *et al.* 2012), all data are relative to anaerobic cultures (De Angelis *et al.* 2004; Fiocco *et al.* 2010; Ricciardi *et al.* 2012). Respiratory metabolism offers a net gain in the number of survivors in exponential phase but does not always increase tolerance to heat stress in stationary conditions. Watanabe *et al.* (2012b) have demonstrated that respiratory growth negatively affects the survival to acid stress in *Lb. plantarum* WCFS1.

Oxidative stress is mainly related to the presence of oxygen. In fact, using a range of flavin dependent oxidases, LAB can produce reactive oxygen species (ROS; hydrogen peroxide H_2O_2, superoxide anion O_2^-, hydroxyl radical HO-), which are toxic to the cells.

Our results showed that, in *Lact. plantarum*, the shift towards a respiratory pathway reduces oxidative stress, compared to the traditional fermentative cultivation. Respiration, in fact, induces the synthesis of cytochromes, which can protect cells from oxidative conditions because of oxygen elimination by ET chain. This evidence was supported by some data of Rezaiki *et al.* (2004) that verified an increased oxidative tolerance in a *sod* gene mutant of *L. lactis* under respiratory state and, more recently, by Watanabe *et al.* (2012a,b) that showed, in *Lact. plantarum* WCFS1, that the induction of respiratory metabolism positively affects the survival to oxidative stress.

Drying and freeze-drying are important for the large-scale production of stable starter and/or probiotic powders at relatively low costs, but impose thermal, oxidative and osmotic stresses due to low (freezing) and high (spray drying) temperatures, accumulation of solutes during evaporation or sublimation and oxidation of membrane lipids, which could decrease the number of survivors (at levels lower than 8 log CFU) and affect the strain performances.

As for thermal inactivation, respiratory metabolism did not always increase survival to freezing and freeze-drying processes in stationary cells, giving a profile of stress tolerance very complex and confirming that, in stationary growth, the regulation of stress response is linked to several mechanisms of the general stress response (GSR), including the capability to adapt to acid and nutritional stresses.

This work adds to the knowledge about aerobic/respiratory metabolism of *Lact. plantarum* because of several reasons: first of all, we decided to use *Lact. plantarum* C17 to evaluate the responses to respiration shift in a strain with physiological properties completely different from the model WCF1; second, we highlighted other possible factors involved in the regulation of aerobic enzyme; finally, we investigated the effect of respiration on a stressors (freezing and freeze-drying) prominent in the production and distribution of starter and probiotic cultures.

Overall, we confirmed that respiration confers some physiological and metabolic advantages under unfavourable conditions, especially for exponential cultures. However, some mechanisms are not completely clear and we can suppose that carbon catabolite repression, aeration and presence of some metabolite, such as H_2O_2, are not the only factors affecting the growth via respiration and the implication of temperature could be also possible.

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