Metabolic profiling of anaerobic and respiratory cultures of *Lactobacillus plantarum* C17

IANNIELLO Rocco Gerardo[1,*], RICCIARDI Annamaria[1,2], PARENTE Eugenio[1,2], CASTIGLIONE MORELLI Maria Antonietta[3], VIGGIANI Felicia[3], ZOTTA Teresa[2]

[1] Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali, Università degli Studi della Basilicata, Potenza, Italy; [2] Istituto di Scienze dell'Alimentazione, Consiglio Nazionale delle Ricerche, Avellino, Italy;
[3] Dipartimento di Scienze, Università degli Studi della Basilicata, 85010 Potenza, Italy

INTRODUCTION

Lactobacillus plantarum is a fermentative lactic acid bacterium (LAB) used in the production of many fermented and functional foods. Recently, it has been demonstrated that respiratory metabolism results in this species in the expression of a phenotype with enhanced technological and stress response properties (increase in biomass, synthesis of antioxidant enzymes, robustness to stress conditions).

Aim of this study was to investigate the effect of anaerobiosis (AN) and respiratory promoting conditions (RS; 30% dissolved oxygen, hemin and menaquinone) on the growth, oxygen uptake, activity of oxygen-related enzymes (pyruvate oxidase, POX; NADH oxidase, NOX; NADH peroxidase, NPR), metabolic profile (^{1}H-NMR spectroscopy) and oxidative stress response (catalase, tolerance of H_2O_2 and menadione) of *L. plantarum* C17 (wild-type) and its natural oxidative stress-tolerant mutant C17-m58, using chemostat (D=0.07 h^{-1}, pH 6.5, 35°C) cultivations in chemically defined medium (CDM).

MATERIALS AND METHODS

Strains and culture conditions: *L. plantarum* C17 and its mutant C17-m58 (Zotta *et al.* 2013b) were routinely propagated (16 h, 35 °C) in a complex basal medium (WMB, Zotta *et al.* 2012) and a modified chemically defined medium (mCDM; Teusink *et al.* 2005) was used for batch and continuous cultivations.

Continuous fermentation: Chemostat cultivations were carried out in a 3 l glass fermentor (Applikon), filled with 1.5 l of mCDM, under anaerobic (AN; nitrogen 0.1 vol vol^{-1} min^{-1}) and respiratory promoting conditions (RS; air 0.2 vol vol^{-1} min^{-1}, with 2.5 μg ml^{-1} hemin and 1 μg ml^{-1} menaquinone supplementation) at 35 °C (Zotta *et al.* 2013) and constant pH 6.5.
Concentration of dissolved oxygen (DO%) was measured by a polarographic electrode and controlled at ≥30% in RS conditions by automatically varying the stirrer speed.
The bioreactor was operated batchwise until early stationary growth phase, when continuous feeding of fresh mCDM (glucose concentration, S_R= 10 g l^{-1}) was started at a flow of 60 ml h^{-1} and continuous cultures were operated at dilution rate (D) of 0.070 h^{-1}. Cultivation was carried out first in anaerobic conditions and then in respiratory ones. Sampling was started when at least three culture volumes were passed through the vessel and the optical density at 650 nm (OD_{650}) of three samples taken from the outlet medium at 30-min intervals were stable (steady-state condition)

^{1}H-NMR analyses: Consumption of substrate and production of metabolites were evaluated by ^{1}H-NMR analyses (acquisition of samples at 25 °C and 499.96 MHz on a Varian Unity INOVA 500 MHz spectrometer; identification and quantification of metabolites were performed with Chenomx NMR Suite 7.6 software, adjusting and matching all samples against the model compounds included in the Chenomx NMR reference libraries) . Un-inoculated mCDM (AN-batch, AN-continuous and RS-continuous mCDM used for both C17 and C17-58 cultivations) was used as replicates to calculate the coefficient of variation of ^{1}H-NMR spectra and verify the repeatability of analyses.

Biochemical analyses: Specific rates of oxygen uptake (μmol O_2 min^{-1} g^{-1} of CDW) were measured *in situ* in AN batch and AN or RS steady-state cultures according to Zotta *et al.* (2013a). Concentration of H_2O_2 in supernatants and catalase activity in whole cells were measured as described by Zotta *et al.* (2014). The activities of enzymes related to the aerobic metabolism (pyruvate oxidase, POX; NADH oxidase, NOX; NADH peroxidase, NPR) were measured in cell free extracts according to Zotta *et al.* (2014) at both 25 °C and 37 °C (*in vitro* assay temperature).

Stress treatments: Tolerance of oxidative stress was evaluated by exposing (30 min, 35 °C) batch and steady-state AN or RS cells (final OD_{650}=1.0) to different H_2O_2 concentrations (10 two-fold dilutions from 440 to 0.86 mmol l^{-1}) and estimating the number of survivors by pour plate counts in WMA (35 °C, 48 h, anaerobiosis).
Survival to heat stress was evaluated as described in Zotta *et al.* (2013a; 2013b), by exposing batch and steady-state AN or RS cells (final OD_{650}=1.0) at 55 °C for 0, 5, 10, 15 and 30 min in PB7 and enumerating the survivors on WMA plates (35 °C, 48 h, anaerobiosis). Kinetic of thermal inactivation was fitted using a Weibull model (van Boekel, 2002).

RESULTS

Growth performances and production of metabolites

Results of substrate consumption, metabolite production (detected with ^{1}H-NMR analyses, Fig. 1) and oxygen utilization in mCDM are reported in Table 1:

Table 1: Consumption of substrates and production of metabolites in *Lactobacillus casei* C17 and its mutant C17-m58 grown in chemically defined medium

Substrates/ Metabolites	mCDM †	Wild type C17			Mutant C17-m58		
		AN, batch* (0.31 h^{-1})	AN, chemostat* (0.07 h^{-1})	RS, chemostat* (0.07 h^{-1})	AN, batch* (0.46 h^{-1})	AN, chemostat* (0.07 h^{-1})	RS, chemostat* 0.07 h^{-1})
	mmol/l	Concentrations (C; mmol/l) and specific rates (mmol/h/g CDW) of substrate uptake (v) and metabolite production (π) ‡					
Sugar metabolism		C (v or π)	C (v or π)	C (v or π)	C (v or π)	C (v or π)	C (v or π)
Glucose	50.0 ± 5.3	**-25.91** (12.55)	**-50.00** (3.84)	**-50.00** (3.89)	**-50.00** (21.68)	**-50.00** (2.82)	**-50.00** (1.72)
Lactate	n.d.	**+50.92** (24.67)	**+67.77** (5.21)	**+52.40** (4.08)	**+63.53** (27.14)	**+58.94** (3.33)	**+61.19** (2.11)
Acetate	16.2 ± 1.4	+0.26 (0.12)	**+7.17** (0.55)	+1.11 (0.09)	**-4.21** (1.82)	**+6.18** (0.35)	+3.10 (0.11)
Pyruvate	n.d.	n.d.	n.d.	**+15.52** (1.21)	n.d.	n.d.	**+14.27** (0.49)
Formate	n.d.	n.d.	**+13.74** (1.06)	n.d.	n.d.	**+11.98** (0.68)	n.d.
Succinate	n.d.	+0.01 (0.01)	**+0.89** (0.07)	n.d.	+0.09 (0.04)	**+0.90** (0.05)	n.d.
Aminoacid metabolism							
Glutamic acid	3.5 ± 0.8	-0.27 (0.13)	-1.04 (0.08)	-0.60 (0.05)	-1.30 (0.57)	-0.96 (0.05)	-1.73 (0.06)
Leucine	3.4 ± 0.3	-0.31 (0.15)	**-2.05** (0.16)	**-1.84** (0.14)	**-1.38** (0.60)	**-1.70** (0.10)	**-0.86** (0.03)
Methionine	0.5 ± 0.1	**+0.46** (0.22)	-0.10 (0.01)	+0.10 (0.01)	+0.19 (0.08)	-0.02 (0.01)	**+0.39** (0.01)
Phenylalanine	1.6 ± 0.2	-0.06 (0.03)	-0.49 (0.03)	-0.53 (0.04)	**-0.69** (0.29)	**-0.71** (0.04)	-0.25 (0.01)
Tryptophan	0.3 ± 0.1	+0.05 (0.03)	-0.06 (0.00)	-0.25 (0.02)	-0.22 (0.10)	-0.23 (0.01)	-0.23 (0.01)
Tyrosine	1.2 ± 0.1	-0.07 (0.03)	**-0.53** (0.04)	-0.40 (0.03)	**-0.48** (0.21)	**-0.50** (0.03)	-0.25 (0.01)
Valine	2.0 ± 0.2	+0.41 (0.20)	-0.28 (0.02)	-0.37 (0.03)	-0.67 (0.29)	-0.59 (0.03)	+0.01(0.00)
Other metabolites							
Adipate	n.d.	n.d.	n.d.	**+ 0.02**	n.d.	n.d.	**+ 0.02**
Cell density and yields							
CDW (g/l)	-	0.64	0.91	0.90	1.06	1.24	2.03
$Y_{X/S}$ (biomass)	-	0.02	0.02	0.02	0.02	0.02	0.04
$Y_{P/S}$ (lactate)	-	0.98	0.68	0.52	0.63	0.59	0.61
% excess pyruvate	-	1.7	32.2	47.6	37.4	41.0	38.8
Specific rate of oxygen uptake (μmol O_2/min/g of CDW)							
O_2	-	0.0	0.0	1.27	0.0	0.0	1.58

† mCDM = un-inoculated medium; mean values ± standard error of 4 replicates are shown; n.d., not detected.
* Cultivation (AN, anaerobic growth; RS, respiratory growth in presence of air 0.2 vol/vol/min, 2.5 μg ml^{-1} hemin and 1 μg ml^{-1} menaquinone) and maximum specific growth rates (μ_{max}=0.31 h^{-1} or 0.46 h^{-1} for batch cultivations) or dilution rates (D=0.07 h^{-1} for chemostat cultivations).
‡ Specific rates of substrate uptake (v) and metabolite production (π) were calculated as (s-aF)·D/X, where a is the concentration (mmol l^{-1}) in the effluent (which is identical to the concentration within the bioreactor), aF is the concentration (mmol l^{-1}) in the feed, D is the dilution rate (h^{-1}) and X is the cell dry weight (CDW, g l^{-1}). For batch growth the average specific rates during the first 8 h of cultivations where shown. Positive (+) or negative (-) sign indicates, respectively, production or consumption during the growth.
Bold values indicate significant changes in substrate and metabolite concentrations (C; mmol l^{-1}), compared with values measured in un-inoculated mCDM. A change ≥ 3.0 standard errors calculated in un-inoculated mCDM replicates was considerate as significant change.
$Y_{X/S}$= biomass yield (biomass yield, g l^{-1}, relative to total sugar consumed, mmol l^{-1}; g mmol^{-1})
$Y_{P/S}$= lactic acid yield (lactic acid produced, mmol/l, relative to total sugar consumed, mmol l^{-1})
% excess pyruvate = percentage of pyruvate not reduced to lactate and available for conversion in other products (i.e. acetic acid, formate, succinate, ethanol; calculated as mmol l^{-1} of pyruvate, derived from consumed mmol l^{-1} of glucose, subtracted of produced mmol l^{-1} of lactic acid)

❖ The mutant strain exhibited higher biomass production compared to the wild type (wt) C17, especially in presence of air, heme and menaquinone, confirming its oxygen-tolerant phenotype (Zotta *et al.* 2013b).
❖ The highest rates of glucose consumption were measured in AN-batch cells of mutant and wt, while the lowest substrate uptake was detected in RS cells of C17-m58.
❖ The AN-batch cells of wt C17 used exclusively a homolactic pathway ($Y_{P/S}$ = 98.3%), while lower lactate yields were observed in the other growth conditions and in the mutant strain, suggesting a reconversion of lactate into pyruvate by lactate dehydrogenase (LDH) activity or a reduced conversion of pyruvate into lactate (LDH).
❖ Residual pyruvate (**Table 1**) was converted into different products on the basis of the active metabolic pathway. Significant amounts of formate and acetate were found in AN steady-state cultures of wt and mutant strains.
❖ AN-batch cells of C17-m58, on the contrary, exhibited consumption of acetate, probably converted in acetyl-P by ACK and than in acetil-CoA by phosphate acetyltransferase (PTA; KEGG pathway).
❖ Low amounts of succinate were also found in AN broths of batch C17 and steady-state ones of mutant, possibly from pyruvate-malate-fumarate pathway (catalised by malate dehydrogenase- fumarate hydratase- fumarate reductase/ succinate dehydrogenase system; KEGG pathway).
❖ In RS supernatants of both wt and mutant strains fumarate and succinate were not detected, suggesting the possible activation of aerobic pathway POX-ACK.

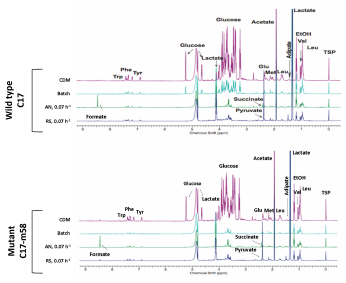

Fig. 1: ^{1}H-NMR spectra of substrate consumed and metabolites produced by batch and steady-state (D=0.070 h^{-1}) cells of *Lactobacillus plantarum* C17 (wild type, wt) and its mutant C17-m58, grown under anaerobic (AN; nitrogen 0.1 vol vol^{-1} min^{-1}) and respiratory promoting conditions (RS; air 0.2 vol vol^{-1} min^{-1}, with 2.5 μg ml^{-1} hemin and 1 μg ml^{-1} menaquinone supplementation). Spectra were generated with Chenomx NMR Suite 7.6 software.

❖ High concentrations of pyruvate and low amounts of acetate were measured in RS supernatants, indicating a reduced functionality of POX *in vivo* at 35°C (the optimal growth temperature for *L. plantarum* C17).
❖ Measurements of enzymatic activities at both 25 °C and 37 °C confirmed the ^{1}H-NMR results, since no POX activity was detected in RS cells of wt and mutant strains at 37°C (**Fig. 2**); however, at 25 °C POX was slightly higher in C17-m58 and lower concentrations of pyruvate were found in RS supernatants of mutant, but the reason it remain unclear.
❖ Small amounts of adipate were also found in RS broths, probably because of lipid oxidation by O_2 or H_2O_2.
❖ Amino acids consumption was higher in chemostat cultures (both AN and RS) of wt C17 compared to batch cultures, while in the mutant strain the specific uptake depended on the type of amino acid, although most of them were principally consumed in AN (batch and chemostat) cultivation.

Enzymatic activities

The activities of POX, NOX and NPR (the enzymes involved in oxygen and H_2O_2 degradation) were shown in **Fig. 2**.
✓ POX was detected only in presence of oxygen.
✓ NOX and NPR were not strictly related to the aerobic and respiratory growth, and coherently with the previous data of Zotta *et al.* (2013a), were also present in AN conditions. Contrarily to POX, these enzymes were not affected by the temperature of the assay and significant activities were also measured at 37 °C.
✓ Catalase was detected only in RS conditions (24.3 μkatal g^{-1} biomass in wt C17; 10.9 μkatal g^{-1} biomass in mutant C17-m58) when hemin was added to the substrate, confirming the heme-dependent nature of this enzyme in *L. plantarum* C17 (Guidone *et al.* 2013; Zotta *et al.* 2013a, 2013b).
✓ Even if significant amounts of catalase and NPR were synthesized in RS cells, H_2O_2 (a product of POX activity) was found in the RS supernatants of wt (0.016 mmol l^{-1} g^{-1} biomass) and mutant (0.021 mmol l^{-1} g^{-1} biomass) strains, confirming the production of the toxic compound in the aerobic growth.

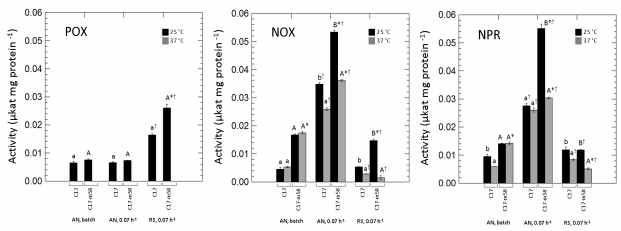

Fig. 2: Activities of pyruvate oxidase (POX), NADH oxidase (NOX) and NADH peroxidase (NPR) in batch and steady-state (D=0.070 h^{-1}) cells of *Lactobacillus plantarum* C17 (wt) and its mutant C17-m58, grown under anaerobic (AN; nitrogen 0.1 vol vol^{-1} min^{-1}) and respiratory promoting conditions (RS; air 0.2 vol vol^{-1} min^{-1}, with 2.5 μg ml^{-1} hemin and 1 μg ml^{-1} menaquinone supplementation). Lowercase (a, b) and uppercase (A, B) letters on plot bars indicate significant differences (Tukey's HSD, $p < 0.005$) in enzymatic activities, respectively, of wt C17 and mutant C17-m58 within the assay temperature (25 °C, black bars, and 37 °C, grey bars) and growth condition; the asterisk (*) indicates significant differences ($p < 0.005$) in enzymatic activities between wt and mutant strains within the same assay temperature (25 °C, black bars, and 37 °C, grey bars) and growth condition; the cross (†) indicates significant differences ($p < 0.005$) in enzymatic activities of chemostat cultures of wt C17 or mutant C17-m58 compared with batch cultivation. Differences in enzymatic activities between AN and RS steady-state cells (D=0.070 h^{-1}) were always significant. Standard error bars of two independent inactivation experiments are shown.

Stress tolerance

Oxidative stress

❖ Respiratory growth significantly ($p < 0.005$) affected the resistance to H_2O_2. Both wt and mutant strains were highly tolerant (100% survivors) of low (from 0.86 to 3.43 mmol l^{-1}) H_2O_2 concentrations, but completely inactivated at 440 mmol l^{-1} of H_2O_2.
❖ Coherently with the high values of catalase, RS cells of *L. plantarum* C17 tolerated up to 110 mmol l^{-1} H_2O_2; however, a satisfactory robustness was also found in AN cultures because of NPR activity (Fig. 2).
❖ The mutant was generally more resistant than the wt strain, even if the number and the ratio of survivors among AN and RS cultures strongly varied with the increase of H_2O_2 concentration: in presence of moderate (from 6.87 to 27.5 mmol l^{-1}) levels of H_2O_2, in fact, the continuous cultures of C17-m58 showed the highest tolerance of oxidative stress, but in the harshest conditions (higher than 55 mmol l^{-1} of H_2O_2) the batch cultures, surprisingly, exhibited the greatest robustness.

Heat stress

❖ Respiratory growth significantly ($p < 0.005$) impaired the survival to heat stress, decreasing the time to reach 3-log cycle reduction (t3D) in both wt (t3D=7.14 min) and mutant (t3D=3.90 min) strains.
❖ Anaerobic cells of C17 and C17-m58 had similar level of survival in batch and chemostat cultivations, while the mutant showed the lowest robustness to heat treatments in all growth conditions.

CONCLUSIONS

• The mutant strain C17-m58 had higher capability to shift towards aerobic and respiratory metabolism compared to wt strain C17 because of greater POX activity and lower pyruvate concentration found in respiratory supernatants
• The understanding of stress response behaviour in the mutant strain, however, remains complex because of random selection process (Zotta *et al.* 2013b,) which may have altered different genes.
• Regulation and activation of aerobic and respiratory metabolism in *L. plantarum* need further investigation.

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