Investigation of factors affecting aerobic and respiratory growth in *Lactobacillus casei* N87

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INTRODUCTION

Some lactic acid bacteria (LAB) are able to use oxygen during aerobic or respiratory (presence of heme and menaquinone in the substrate) cultivation, with significant effects on the metabolism and stress response properties (increase in biomass, activation of an electron transport chain, ETC; production of antioxidant enzymes). However, in presence of oxygen, LAB may accumulate reactive oxygen species (ROS including hydrogen peroxide, superoxide anions and hydroxyl radicals), which may damage proteins, nucleic acids and lipids leading to cell death. Studies on the aerobic and respiratory growth are principally related to *Lactobacillus plantarum* and *Lactococcus lactis* (Pedersen et al. 2012; Zotta et al. 2013), while only one report has been published on strains belonging to the *L. casei* group (Zotta et al. 2014).

AIM of work: to investigate the effect of aeration and heme and menaquinone on the growth, stress response and antioxidant capability of *L. casei* N87, selected for its noticeable oxygen-tolerant phenotype (Zotta et al. 2014).

MATERIALS AND METHODS

Growth, production of metabolites and oxygen uptake: Batch fermentations were carried out in modified WMB pH 6.5 (Zotta et al. 2012) under **anaerobic** (nitrogen 0.1 vol/vol/min, **AN**), **aerobic** (30% and 60% of dissolved oxygen, dO_2; **AE30**, **AE60**) and **respiratory** (supplementation with 2.5 µg/mL hemin and 1 µg/mL menaquinone; 30% and 60% of dO_2; **RS30**, **RS60**) conditions, at 37°C for 24 h. O_2 consumption was evaluated by monitoring the time (h) of resazurin discoloration according to Ricciardi et al. (2014), while residual glucose and production of lactic, acetic and citric acids were spectrophotometrically measured by using enzymatic kits.

Enzymatic activities: Activities of pyruvate oxidase (POX), NADH oxidase (NOX), NADH peroxidase (NPR), catalase and production of H_2O_2 were measured as reported in Zotta et al. (2013).

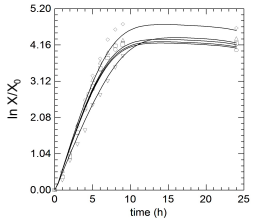
Stress tolerance and antioxidant capability: Tolerance of oxidative stresses was performed exposing exponential (E) and stationary (S) cells to ROS generating compounds (50 mM H_2O_2, 300 mM pyrogallol, 5 mM menadione) for 30 min at 37°C; cells survival was evaluated by plate counts on WMB agar. Hydroxyl- and DPPH-radicals scavenging activity were measured in E and S cells according to Wang et al. (2009).

Gene expression and changes in proteins profile: Transcription of *pox* (encoding for pyruvate oxidase; aerobic pathway) and *cydAB* (encoding for cytochrome oxidase subunit I and II; electron transport chain, ETC) was estimated during growth by qRT-PCR, while changes in protein profiles were evaluated by 1D and 2D electrophoresis.

Growth under anaerobic, aerobic and respiratory conditions

Growth condition	µ max (h^{-1})	lag (h)	R^2	max X ($g\ L^{-1}$)
AN	0.64±0.01	0.37±0.01	0.996±0.01	4.06±0.25
AE 30	0.65±0.01	0.36±0.03	0.997±0.01	3.15±0.07
AE 60	0.48±0.00	0.36±0.02	0.996±0.00	2.56±0.20
RS 30	0.65±0.01	0.37±0.01	0.996±0.01	4.24±0.01
RS 60	0.68±0.01	0.35±0.02	0.998±0.01	5.28±0.07

Growth condition: AN, anaerobic growth condition; AE30, aerobic growth condition with 30% of dissolved oxygen, dO_2; AE60, aerobic growth condition with 60% of dO_2; RS30, respiratory growth condition, 30% of dO_2 and supplementation with 2.5 µg/mL hemin and 1 µg/mL menaquinone; RS60, respiratory growth condition, 60% of dO_2 and supplementation 2.5 µg/mL hemin and 1 µg/mL menaquinone.
μ_{max}= maximum specific growth rate, estimate±standard error
lag: lag phase, estimate ± standard error
max X: maximum cell dry weight (CDW, g/L)



Symbols: circle, AN; up triangle, AE30; down triangle, AE60; square, RS30; diamond, RS60.

• Kinetics of growth were estimated with the dynamic model of Baranyi and Roberts (1994) using DMFit v. 2.0 (Baranyi and Le Marc, 1996). Respiration increased biomass yield compared to anaerobic and aerobic growth (because of extra ATP generation by respiration chain; Pedersen et al. 2012) but (with exception of aerobiosis with 60% dO_2) did not significantly affect the growth rate. When heme and menaquinone were not supplemented, the high levels of dO_2 (AE60 cultivation) strongly impaired the biomass production.
• The highest oxygen uptake was measured in the late exponential respiratory cells, suggesting a boost of oxygen consumption by cytochrome oxidase activity (activation of ET chain). As expected, anaerobic cultures were unable to consume oxygen.

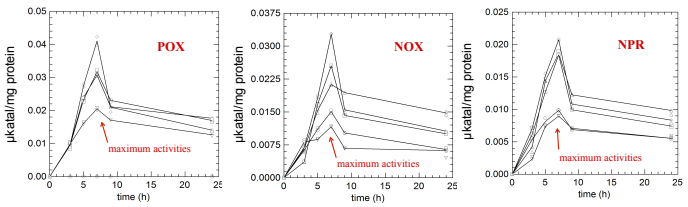
Table 2: Consumption of substrates and production of metabolites

Growth condition	Growth phase	GluX Or (S-S0)/X	$Y_{x/s}$ (biomass)	$Y_{p/s}$ (lactic acid)	Acetic acid (mM)	% excess pyruvate	H_2O_2 (mM)
AN	E	3.26±0.26	0.05±0.00	0.59±0.00		40.96±0.16	
	ES	2.53±0.00	0.07±0.00	0.85±0.00		15.04±0.05	
	S	3.11±0.00	0.06±0.00	0.98±0.01		2.22±0.52	
AE 30	E	4.71±0.04	0.04±0.00	0.58±0.00		42.01±0.06	
	ES	3.46±0.02	0.05±0.00	0.66±0.00		33.81±0.16	
	S	3.33±0.00	0.05±0.00	0.75±0.01	0.93±0.00	25.02±0.22	0.03±0.00
AE 60	E	6.03±0.33	0.03±0.00	0.55±0.01		45.45±0.56	
	ES	4.12±0.02	0.04±0.00	0.72±0.00		28.04±0.21	
	S	3.95±0.00	0.05±0.00	0.82±0.00	0.85±0.00	18.21±0.21	0.03±0.01
RS 30	E	2.19±0.10	0.06±0.00	0.58±0.01	0.91±0.02	41.58±0.88	
	ES	2.32±0.01	0.08±0.00	0.67±0.01	4.22±0.07	32.75±0.55	
	S	2.62±0.00	0.07±0.00	0.66±0.01	10.24±0.16	33.84±0.30	0.02±0.00
RS 60	E	2.15±0.146	0.08±0.01	0.67±0.01	1.47±0.01	32.87±0.52	
	ES	1.979±0.01	0.09±0.00	0.64±0.03	6.25±0.14	35.51±0.16	
	S	2.212±0.00	0.08±0.00	0.62±0.00	13.40±0.41	37.59±0.07	0.02±0.00

Growth phase: E, exponential phase; ES, early stationary phase; S, stationary phase.
GluX: glucose consumed, mmol, relative to biomass production, g of CDW (cell dry weight.
$Y_{x/s}$: biomass yield (biomass yield, g of CDW, relative to total sugar consumed, mmol).
$Y_{p/s}$: lactic acid yield (lactic acid produced, mmol, relative to total sugar consumed, mmol)
% excess pyruvate = percentage of pyruvate generated by glycolysis not reduced to lactate.

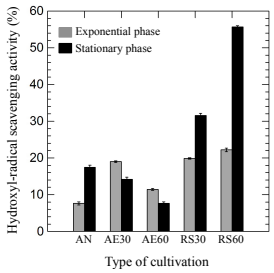
Lactic acid was the only product of anaerobic cultivation, while acetic acid (produced by oxidation of pyruvate into acetate by pyruvate oxidase-acetate kinase pathway) was measured in respiratory and aerobic supernatants. Citrate was not added to the substrate and no further production was detected in AN and RS supernatants.

Enzymes related to the oxygen metabolism



Symbols: circle, AN; up triangle, AE30; down triangle, AE60; square, RS30; diamond, RS60.

• POX, NOX and NPR activities were higher in respiratory cells and lower in aerobically grown cells in presence of 60% dO_2, probably because of inhibition of enzyme synthesis by oxygen and H_2O_2 accumulation.
• The highest activities for all enzymes were measured at the end of exponential phases (7 h incubation).
• Contrarily to NADH-dependent oxidases, POX was not detected in anaerobic cells, confirming the role of oxygen in POX regulation and indicating that NOX and NPR are not exclusively associated to the aerobic/respiratory metabolism.
• Catalase activity, noticeable in exponential cultures of all conditions (from 18 µkatal/mg protein in anaerobioc cells to 40 µkatal/mg protein in respiratory cells growing with 60% dO_2), decreased in stationary phases when consistent amounts of H_2O_2 (mainly in AE60 supernatants) were detected. These data confirmed the presence of a possible non-heme catalase activity in *L. casei* N87 (Zotta et al. 2014).

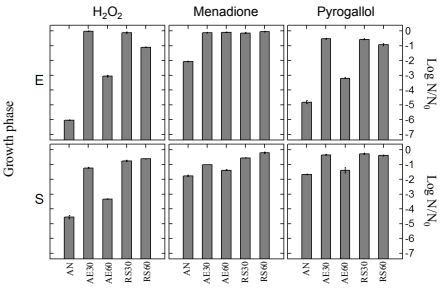


Scavenging of DPPH- and hydroxyl-free radicals

- Growth conditions did not affect the capability to remove DPPH-radicals, while a wide variability was observed in the hydroxyl-radical scavenging activity.
- **Respiratory cultivation** (specially in presence of 60% dO_2) significantly ($p<0.05$) improved the radical degrading capability of stationary phase cells.
- **Aerobiosis** (both with 30% and 60% dO_2) dramatically reduced the ability to scavenge hydroxyl-radicals in stationary phase, probably because of toxic accumulation of oxygen and lower activities of antioxidant enzymes in cells growing in aerobiosis without supplementation.

Response to oxidative stresses

• Cells grown under aerobic (30% dO_2) and respiratory (with both 30% and 60% dO_2) conditions showed the highest tolerance of H_2O_2 and pyrogallol in both E and S growth phase
• Survival of cells grown in anaerobiosis and aerobiosis with 60% dO_2 significantly ($p<0.05$) impaired the tolerance of oxidative stresses, probably because of low catalase and NRP activities, resulting in oxygen, ROS and H_2O_2 accumulation
• In all growth conditions tolerance of **menadione** was satisfactorily high



Damaged and viable but non cultivable cells

Significant numbers (up to 10% of the total population) of damaged (measured on acidified WMA pH 5.5) and viable but non cultivable (VBNC) cells (measured on WMA pH 6.5 containing 0.05% w/v cysteine as recovery agent) were found in stationary AE60 cultures, confirming the noxious effect of O_2 and H_2O_2 accumulation, which may cause sub-lethal damage in addition to death.

Gene expression and changes in protein pattern

• **qRT-PCR:** confirmed the expression of *pox* only in presence of oxygen, already in the exponential growth phase and increasing towards stationary growth phase. Levels of *pox* were higher when dO_2 was maintained at 30%. On the contrary, *cydAB* was expressed in all conditions, suggesting that synthesis of cytochrome bd quinol oxidase is not strictly related to the presence of oxygen. However, its functionality (i.e. activation and assembly with heme group) needs further investigation.
• **Proteome** differed principally as function of growth phase, although aerobic and respiratory cultivations resulted in increased number of detectable protein spots compared to anaerobic growth. Protein identification are in progress.

CONCLUSIONS

➢ Respiration increased biomass yield and functionality of *L. casei* N87 compared with anaerobic and aerobic conditions.
➢ If respiratory pathways (synthesis of ET chain and utilization of oxygen as electron acceptor) are not activated, a toxic accumulation of oxygen and ROS occurs with negative effect on the enzyme activities, stress tolerance and antioxidant capability.
➢ Since the exploitation of oxygen-tolerant phenotypes may be useful for the development and production of starter and/or probiotic cultures, further studies are needed to elucidate the regulation and mechanism of aerobic and respiratory growth.

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