Title

Biochemical analysis of respiratory metabolism in the heterofermentative *Lactobacillus spicheri* and *Lactobacillus reuteri*

Running title

Respiration in heterofermentative lactobacilli

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Abstract

Aims: This study evaluated the aerobic and respiratory metabolism in *Lactobacillus reuteri* and *Lactobacillus spicheri*, two heterofermentative species used in sourdough fermentation.

Methods and Results: *In silico* genome analysis, production of metabolites and gene expression of pyruvate oxidase, pyruvate dehydrogenase and cytochrome oxidase were assessed in anaerobic and aerobic cultures of *L. reuteri* and *L. spicheri*. Respiring homofermentative *L. casei* N87 and *L. rhamnosus* N132 were used for comparison.

Aerobiosis and respiration increased the biomass production of heterofermentative strains compared to anaerobic cultivation. Respiration led to acetoin production by *L. rhamnosus* and *L. casei*, but not in heterofermentative strains, in which lactate and acetate were the major end-products. *L. spicheri* LP38 showed the highest oxygen uptake. Pyruvate oxidase, respiratory cytochromes, NADH-oxidase and NADH-peroxidase were present in the genome of *L. spicheri* LP38. Both *L. spicheri* LP38 and *L. rhamnosus* N132 overexpressed *pox* in aerobic cultures, while *cydA* was up-regulated only when heme was supplied; *pdh* was repressed during aerobic growth.

Conclusions: Aerobic and respiratory growth provided physiological and metabolic advantages also in heterofermentative lactobacilli.

Significance and Impact of the Study: The exploitation of oxygen-tolerant phenotypes of *L. spicheri* may be useful for the development of improved starter cultures.

Keywords: *Lactobacillus spicheri*; *Lactobacillus reuteri*; genome analysis; respiration; metabolism

Introduction

Lactic acid bacteria (LAB) are facultative aerobic or anaerobic organisms in the order *Lactobacillales* that derive metabolic energy from fermentable carbohydrates by substrate level phosphorylation (Holzapfel and Wood, 2014). The availability of genome sequence data substantiated earlier reports that many LAB are conditionally respiring (Duwat *et al.* 2001, Pedersen *et al.* 2012). LAB are unable to synthesize hemin and many LAB are auxotrophic for menaquinone; however, when both cofactors are available during aerobic growth, NADH dehydrogenase, in conjunction with respiratory cytochromes, exports protons and supports ATP generation through F_0F_1ATPase activity (Broijmans *et al.* 2007; Pedersen *et al.* 2012).

Respiratory metabolism has been elucidated mainly in homofermentative LAB, which metabolise hexoses via the Emden Meyerhoff Pathway with pyruvate as the key intermediate. Investigations of respiratory metabolism are available for *Lactococcus lactis* (Duwat *et al.* 2001, Gaudu *et al.* 2002) and for the members of the *Lactobacillus plantarum* group (Brojimans *et al.* 2009; Guidone *et al.* 2013; Zotta *et al.* 2013; Zotta *et al.* 2014a) and the *Lactobacillus casei* group (Zotta *et al.* 2014b; Ianniello *et al.* 2015). In homofermentative LAB, the presence of oxygen shifts pyruvate metabolism from lactate formation to acetate and acetoin production (Duwat *et al.* 2001; Goffin *et al.* 2006). Re-routing of pyruvate metabolism to acetoin prevents acidification of the medium, while acetate formation through pyruvate oxidase and acetate kinase activities supports substrate level phosphorylation (Gaudu *et al.* 2003; Lorquet *et al.* 2004; Goffin *et al.* 2006). Pyruvate conversion by pyruvate dehydrogenase activity provides an alternative route to acetate formation (Quatravaux *et al.* 2006; McLeod *et al.* 2010). In respiratory metabolism the production of cytochrome bd oxidase, the final component of the minimal electron transport chain in LAB, contributes to additional energy supply (through extra ATP generation) and depletion of intracellular oxygen. Consequently, respiration substantially increases growth rates and final cell densities of LAB (Duwat *et al.* 2001; Guidone *et al.* 2013; Ianniello *et al.* 2015).

Aerobic growth of LAB typically accumulates hydrogen peroxide as product of NADH oxidases and NADH peroxidises (Kandler, 1983; Rezaiki *et al.* 2004). Respiratory metabolism, however, prevents H_2O_2 accumulation through heme-dependent catalase activity (Watanabe *et al.* 2012; Guidone *et al.* 2013) and increases the resistance of LAB to oxidative stress when compared to anaerobic and aerobic cultures (Duwat *et al.* 2001; Watanabe *et al.* 2012; Zotta *et al.* 2014a). An increased resistance to oxidative stress also improves dry survival of LAB (Carvalho *et al.* 2004); therefore, respiratory metabolism is exploited in the production of starter cultures or probiotic cultures to increase the fermentor yield, and to enhance the storage life of dried cultures (Pedersen *et al.* 2012).

Heterofermentative LAB metabolise hexoses and pentoses via the phosphoketolase pathway with acetyl phosphate as the key branching point of metabolism (Kandler, 1983; Gänzle *et al.* 2007). Aerobic metabolism in heterofermentative LAB supports co-factor regeneration via NADH oxidases and peroxidases and shifts metabolism to acetate as a major metabolite (Kandler 1983; Gänzle *et al.* 2007; Jänsch *et al.* 2011; Kang *et al.* 2013). Genomic analyses demonstrate that heterofermentative LAB also have the capacity for respiration (Brooijmans *et al.* 2009; Pedersen *et al.* 2012) but only a few studies provide experimental evidence for respiration (Jääskeläinen *et al.* 2013). Respiration in *Leuconostoc gasicomitatum* improved growth rates and biomass accumulation, but the investigation on metabolic shifts in this organism was limited to volatile compounds (Jääskeläinen *et al.* 2013). It was therefore the aim of this work to characterize respiratory growth and metabolism in strains of the heterofermentative species *Lactobacillus spicheri* and *Lactobacillus reuteri*, representatives of two different phylogenetic groups in the genus *Lactobacillus*. *L. reuteri* and *L. spicheri* occur in artisanal and industrial sourdoughs (Meroth *et al.* 2004; Valcheva *et al.* 2005; Su *et al.* 2012), contributing to the acidification and production of flavor compounds. *L. reuteri* also is a gut symbiont in humans and animals (Frese *et al.* 2011) and some strains of *L. reuteri* are used as probiotic cultures (Savino *et al.* 2007). The study and exploitation of aerobic and respiratory growth in *L. reuteri* and *L. spicheri* may be useful to develop

starter and/or probiotic cultures with enhanced technological properties, and improved survival during culture preparation. This study complemented metabolic analyses by assessment of oxidative stress resistance, the *in silico* analysis of respiratory pathways and the quantification of gene expression. Strains of *L. casei* and *L. rhamnosus* served as reference (Ianniello *et al.* 2015).

Materials and methods

Strains and media

The homofermentative strains *L. casei* N87 and *Lactobacillus rhamnosus* N132 (Iacumin *et al.* 2015) and the heterofermentative *L. reuteri* LTH2584 (Böcker *et al.* 1995), FUA3168 (Sekwati-Monang and Gänzle, 2011), LTH5448 (Schwab and Gänzle, 2006), DSM20016 as well as *L. spicheri* FUA3125, FUA3486 and LP38 were used in this study. Strains were maintained as frozen stocks in glycerol (30% v v^{-1}, final concentration) in the Food Microbiology culture collection of the University of Alberta, Edmonton, Canada and routinely propagated (1% v v^{-1}) at 34 °C in modified MRS (mMRS) broth (Stolz *et al.* 1995a) containing the following ingredients per litre: 10 g maltose, 5 g glucose, 5 g fructose, 10 g peptone, 5 g yeast extract, 4 g K_2HPO_4, 2.6 g KH_2PO_4, 3 g NH_4Cl, 0.5 g L-cysteine, 1 g Tween 80, 0.05 g $MnSO_4$ monohydrate, 0.2 g $MgSO_4$ heptahydrate and 10 g malt extract, pH 6.8.

Screening for aerobic and respiratory growth

Strains were cultivated with 1% inoculum for 16 h at 34 °C in four different conditions: i) anaerobiosis, static incubation in 50 ml screw-cap tubes filled with mMRS broth supplemented with 1 mg l^{-1} menaquinone, ii) heme-supplemented anaerobiosis, static incubation in 50 ml screw-cap tubes filled with mMRS broth supplemented with 1 mg l^{-1} menaquinone and 2.5 mg l^{-1} hemin, iii) aerobiosis, aerated cultures in 250 ml flasks that were filled with 50 ml of mMRS supplemented with 1 mg l^{-1} menaquinone and agitated on a rotary shaker at 250 rpm and iv) heme-supplemented

aerobiosis, aerated cultures in 250 ml flasks that were filled with 50 ml of mMRS supplemented with 1 mg l^{-1} menaquinone and 2.5 mg l^{-1} hemin, and agitated on a rotary shaker at 250 rpm. The last condition supported respiratory growth. After 16 h of incubation, the optical density at 650 nm, the pH values (Thermo Scientific, Wilmington, MA, USA; model9106BNWP) and the oxygen uptake (resazurin assay; Ricciardi *et al.* 2014) were measured. Two biological and two technical replicates were analysed at each growth condition.

Analysis of metabolites by High Performance Liquid Chromatography (HPLC)

Cells from cultures were removed by centrifugation and supernatants were supplemented with perchloric acid to a final concentration of 7% (v v^{-1}) to remove proteins. Solids were removed by centrifugation at 7500 rcf followed by filtration through a 0.2 µm filter (Schleicher&Schuell, Dassel, Germany). Sugars and metabolites were quantified using an RI detector after separation on an Aminex HPX 87H column (Biorad, Mississauga, ON, Canada). The samples were eluted at 70 °C with 0.4 ml min^{-1} of 5 mmol l^{-1} H_2SO_4. Fructose, maltose, glucose, lactic acid, acetic acid, ethanol, acetoin and butanediol were used as external standards.

***In silico* analysis of genes involved in aerobic and respiratory metabolism**

DNA for genome sequencing was isolated from overnight cultures of *L. spicheri* LP38 by using the Wizard® Genomic DNA Purification Kit (Promega, Madisson, Wisconsin, USA). The quality and quantity of each sample was assessed using gel electrophoresis and a NanoDrop® 2000c spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). Genomic DNA was sequenced using Illumina HiSeq2000 system with an insert size of 300 bp by Axeq Technologies (Seoul, South Korea). The quality of the 100-bp paired-end reads was assessed using the FastQC tool (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) and low quality reads were filtered by Quake (Kelley *et al.* 2010). Sequence assembly was obtained using ABySS 1.3.4 (Simpson *et al.*

2009). The genome was annotated automatically by the RAST server and submitted to Genebank with the accession No. JZCR00000000.

Genes coding for lactate dehydrogenase (*ldh*), pyruvate oxidase (*pox*), pyruvate dehydrogenase (*pdh*), phosphotransacetylase (*pta*), acetate kinase (*ack*), NADH oxidase (*nox*), NADH peroxidase (*npr*), synthesis and transport of respiratory cytochromes (*cydABCD*) and thioredoxin reductase (*trx*) from *L. casei* BL23 and *L. rhamnosus* ATCC 8530 were used as query sequences to identify homologues in the whole genome shotgun data of *L. reuteri* LTH5448 and LTH2584 (bioprojectPRJNA248653, accession NoGCA_000758185.1 and GCA_000712555.1, respectively) and *L. spicheri* LP38 (this study). Unidirectional (genes vs genomes) sequence similarity was detected using the default cut-off parameters for E-value and % of identity of the NCBI BLAST tool.

Effect of anaerobic and aerobic conditions on the growth and metabolite production

L. spicheri LP38 was selected as heterofermentative respiration-competent strain because of growth performance and capability to consume O_2; the homofermentative *L. rhamnosus* N132 was used as reference strain (Zotta *et al.* 2014b). Both strains were cultivated at 34 °C with 1% inoculum under static or agitated conditions, respectively, with addition of hemin, or not, as described above. Cultures were sampled every 60 min to measure the OD_{650} and their growth curves were fitted to the dynamic model of Baranyi and Roberts (1994) using DMFit v. 2.0 program. Samples taken after 5, 8,11, 24, 30 and 35 h of cultivation were used to quantify sugars, metabolites and oxygen uptake as described above. H_2O_2 was quantified as described by Zotta *et al.* (2014b).

Oxidative stress tolerance

Tolerance of oxidative stress was evaluated in exponentially growing and stationary cultures. Exponentially growing cultures were harvested at an OD_{650} = 1.0; stationary cells were harvested after 24 h of incubation and adjusted to an OD_{650} of 1.0. The oxidative stress tolerance of

exponential and stationary phase cells was assessed by exposure to 50 mmol l^{-1} H_2O_2 for 30 min at 34 °C. Surviving cells were enumerated by pour plating in mMRS agar, followed by incubation at 34 °C for 48 h under anaerobic conditions.

RNA extraction and complementary DNA synthesis

RNA was isolated from early-exponential cells (OD_{650}=0.6) of *L. spicheri* LP38 and *L. rhamnosus* N132 grown under static or agitated conditions, supplemented with heme, or not, as described above, by using the RNeasy Mini kit (Qiagen, Ontario, Toronto, Canada). RNA was quantified with a NanoDrop® 2000c spectrophotometer (Thermo Scientific, Wilmington, MA, USA) and DNA was digested with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) according to the manufacturer's instructions. RNA was isolated from three independent cultures. One µg of RNA was used as a template for cDNA synthesis. RNA was mixed with 200 ng of random primers (Promega, Madison, WI, USA), 10 mmol l^{-1} of dNTP mix (Invitrogen™, Burlington, Ontario, Canada) and nuclease-free water (Ambion, Streetsville, Ontario, Canada) to a final volume of 13 µl and incubated at 65 °C for 5 min. After cooling on ice, 4 µl of 5x first strand buffer, 100 mmol l^{-1} of DTT, 40 units μl^{-1} of RNase OUT™ Recombinant RNase Inhibitor (Invitrogen™, Burlington, Ontario, Canada) and 200 units μl^{-1} of SuperScript™ III RT (Invitrogen™, Burlington, Ontario, Canada) were added to the mixture and the reaction blend was incubated for 10 min at 25 °C, 50 min at 50 °C and 15 min at 70 °C. The cDNA samples were stored at -20 °C.

Quantification of relative gene expression using quantitative PCR (qPCR)

Expression of *cydA* (encoding for subunit I of cytochrome oxidase), *pox* (encoding for pyruvate oxidase) and *pdh* (encoding for pyruvate dehydrogenase) genes was quantified with qPCR using cDNA as template. Primers were designed using Primer Express software 3.0 (Applied Biosystems, Concord, Ontario, Canada) (Table 1). PCR was carried out using a SYBR Green master mix (Qiagen, Toronto, Ontario, Canada) in a 7500 fast real-time PCR instrument (Applied Biosystems,

Concord, Ontario, Canada). The amplification program was 95 °C for 5 min, 40 cycles of 95 °C for 30 s, and 60 °C for 30 min. The calculation of relative gene expression was carried out according to Pfaffl (2001), using *pho* encoding for the phosphoketolase and *fba* encoding for fructose-bisphosphate aldolase as reference genes for *L. spicheri* and *L. rhamnosus*, respectively. Gene expression was calculated relative to the expression during static growth in media without supplementation of heme (reference condition). PCR reactions included reactions with DNase I-treated RNA and the reaction mix without template DNA as negative controls. Analysis of gene expression was performed in two technical replicates for each of the triplicate biological repeats.

Statistical analysis

All statistical analyses and graphs were performed using SigmaPlot 12.0 for Windows (Systat Software Inc., Richmond, CA, USA).

Results

Initial screening for growth performances, oxygen uptake and metabolite production

To identify heterofermentative lactobacilli with the capability for respiration, the growth performances, oxygen uptake, sugar consumption, and metabolite production of *Lactobacillus reuteri* and *L. spicheri* strains were quantified (Table 2). *L. rhamnosus* and *L. casei* were used as references (Table S1). *L. reuteri* LTH2584 was unable to growth in presence of oxygen but the cell yield of all other strains was increased by aerobic incubation. Aerobic growth increased glucose consumption by all strains of *L. spicheri* but not by *L. reuteri*. Aerobic incubation shifted the metabolism from production of lactate and ethanol as major metabolites towards the production of lactate and acetate. Acetoin was produced by *L. rhamnosus* and *L. casei* but not by heterofermentative lactobacilli (Table 2, Table S1 and data not shown). Aerobic cultures of *L. casei* N87 and *L. rhamnosus* N132 produced more acetoin and less lactate when media were

supplemented with hemin. In heme-supplemented media, the aerobic cultures of *L. spicheri* FUA3486 and *L. spicheri* LP38 produced more acetate. Oxygen consumption was observed in the heme-supplemented aerobic cultures of the two homofermentative lactobacilli, *L. reuteri* LTH5448, and *L. spicheri* FUA3486 and LP38. Taken together, the biomass yield, oxygen uptake, and the metabolite patterns of *L. reuteri* LTH5448 and *L. spicheri* FUA3168 and LP38, indicate the typical traits of a potential respiratory phenotype (Brojimans *et al.* 2009).

Effect of aerobic conditions on the growth, oxygen uptake, metabolite production and gene expression of *L. spicheri* LP38 and *L. rhamnosus* N132

On the basis of the initial screening, *L. spicheri* LP38 and *L. rhamnosus* N132 were selected to compare the effect of anaerobiosis, heme-supplemented anaerobiosis, aerobiosis and heme-supplemented aerobiosis (respiration) on the metabolic pathways of homofermentative and heterofermentative lactobacilli. Growth parameters are listed in Table 3. Aerobiosis and respiration increased the maximum specific growth rates (μ_{max}) of *L. spicheri* compared to un-supplemented and heme-supplemented anaerobiosis, while the μ_{max} of *L. rhamnosus* was similar in all growth conditions. Moreover, aerobic and respiratory growth reduced the lag phase and increased the final cell density of both strains when compared to both anaerobic conditions. Respiration increased the biomass production of *L. spicheri* compared with aerobic incubation without heme.

Kinetics of substrate consumption and metabolite production are shown in Figure S1 and Figure 1, respectively. *L. rhamnosus* utilized glucose and fructose but not maltose; aerobic incubation slightly accelerated substrate consumption (Figure S1). *L. spicheri* consumed all sugars simultaneously. Aerobic cultivation accelerated glucose and maltose consumption but decreased the rate of fructose consumption, in keeping with the use of glucose and maltose as carbon sources and the use of fructose as electron acceptor (Figure S1). *L. rhamnosus* and *L. spicheri* produced mainly lactate when grown anaerobically. Aerobic cultivation of *L. rhamnosus* reduced lactate formation and resulted in formation of substantial amounts of acetate and acetoin; this effect was most pronounced

in respiratory growing cells. Aerobic cultivation of *L. spicheri* eliminated ethanol production in favour of acetate but also increased lactate formation when compared to anaerobic cultures. Remarkably, acetate was the most abundant metabolite in respiratory cultures of *L. spicheri*. H_2O_2 was produced by respiratory cultures of both strains and aerobic culture of *L. spicheri*.

Oxidative stress tolerance

The resistance to H_2O_2 was similar for both strains (Figure 2). Aerobic cultivations substantially increased the tolerance of oxidative stress when compared to anaerobic cultures; however, the highest resistance to oxidative stress was observed in stationary phase cultures grown in respiratory condition.

In silico* analysis of aerobic and respiratory pathways in *L. reuteri* and *L. spicheri,* and expression of *cydA, pox* and *pdh

To confirm the presence and expression of genes coding for respiratory metabolism in *L. spicheri* LP38, the whole genome shotgun sequence was obtained and assembled to 25 contigs (Table 4). The genome size was 2,855,247 bp with a GC content of 55.4% (Table 4), 6 rRNA operons and a total of 2576 predicted open reading frames. Carbohydrate metabolism and transport, and energy production and conversion accounted for 5.7 and 2.8%, respectively, of the predicted proteins (Table 4). The *in silico* analysis of metabolism in *L. spicheri* LP38 revealed the presence of respiratory cytochromes, NADH oxidase and peroxidase, and the presence of pyruvate oxidase. However, genes for α-acetolactate synthase, butanediol dehydrogenase and pyruvate formate lyase were absent (Figure 3). In contrast to *L. spicheri* LP38, the genome of *L. reuteri* LTH5448 harbors genes coding for production of acetoin but not pyruvate oxidase (Figure 3). The expression of *cydA* coding for subunit I of cytochrome oxidase, *pox* coding for pyruvate oxidase and *pdh* coding for pyruvate dehydrogenase was quantified in *L. spicheri* and *L. rhamnosus* relative to the cultures of the same strains, respectively, growing anerobically (Table 5). Both *L. spicheri* and *L. rhamnosus*

overexpressed *pox* in presence of oxygen, and hemin addition (promoting respiration) increased *pox* expression in *L. rhamnosus* but not in *L. spicheri*. Overexpression of *cydA* was observed only in respiratory cultures. The expression of *pdh* was significantly ($p<0.01$) repressed in aerobic and respiratory cells of *L. spicheri* and *L. rhamnosus*, compared to anaerobic cultivations (Table 5).

Discussion

The shift towards aerobic and respiratory growth has been largely investigated in homofermentative LAB (Pedersen *et al.* 2012), only a few studies report on the respiratory growth and metabolism of heterofermentative LAB (Jääskeläinen *et al.* 2013). This study compared the effect of aerobic and respiratory cultivation on the growth, metabolism and stress resistance of homo- and heterofermentative lactobacilli. Metabolic and biochemical studies were complemented by *in silico* analyses of metabolism and the quantification of key genes that are involved in aerobic and respiratory growth.

In homofermentative lactobacilli, glucose is the preferred carbon source, fructose is used as carbon source but not converted to mannitol, and maltose metabolism is subject to carbon catabolite repression (Monedero *et al.* 2001; Gänzle *et al.* 2007). In heterofermentative lactobacilli, maltose is preferentially metabolised over glucose and fructose is used as electron acceptor rather than as carbon source (Stolz *et al.* 1995a; Gänzle, 2007, Paramithiotis *et al.* 2007). Our data for *L. spicheri* demonstrate that the availability of oxygen reduced fructose consumption because co-factor regeneration by NADH oxidase and peroxidase activities eliminates the need for co-factor reduction by mannitol dehydrogenase. In the heterofermentative *L. panis*, the use of oxygen as electron acceptor is preferred over glycerol reduction to propanediol (Kang *et al.* 2013). The availability of oxygen or other electron acceptors shifts metabolism of heterofermentative lactobacilli from ethanol production to acetate as main product of acetyl-phosphate (Stolz *et al.* 1995a and 1995b; Kang *et al.* 2013). Remarkably, some strains of *L. reuteri* did not grow under aerobic conditions. Because the *in silico* analysis of metabolic genes in *L. reuteri* LTH2584 revealed no differences to *L. reuteri*

LTH5448 (Figure 3 and data not shown), the tolerance of oxidative stress (Turner *et al.* 1999) rather than the ability to use oxygen as electron acceptor may account for this phenotype.

In homofermentative lactic metabolism, pyruvate is metabolised by the alternative enzymes lactate dehydrogenase, pyruvate formate lyase, pyruvate oxidase, or α-acetolactate synthase (Axelsson, 2003). Lactate production is preferred under aerobic or anaerobic conditions when the supply of substrates is abundant (Axelsson, 2003; Lorquet *et al.* 2004). Oxygen supports the production of acetate from lactate during the stationary phase of growth but lactate remains the major metabolite (Quatravaux *et al.* 2006; Pedersen *et al.* 2008). Respiration shifts metabolism to acetoin as the major product of metabolism in *Lc. lactis* (Pedersen *et al.* 2008). In keeping with these prior observations with *Lc. lactis*, respiration in *L. rhamnosus* N132 increased substrate consumption and re-directed metabolism towards production of acetate and acetoin at the expense of lactate.

Acetate formation from pyruvate at aerobic conditions may be mediated by pyruvate dehydrogenase (PDH) or by pyruvate oxidase (POX). Pyruvate dehydrogenase contributed to acetate formation by *Lc. lactis* during growth under substrate limitation and microaerophilic conditions (Jensen *et al.* 2001) and a *pdhA* deficient mutant of *Streptococcus mutans* displayed a decreased resistance to acid conditions (Korithoski *et al.* 2008). The characterization of a *pox* deficient mutant of *L. johnsonii*, however, suggested that pyruvate dehydrogenase supplies acetyl-CoA and TPP to support anabolic metabolism, and is complemented or replaced by pyruvate oxidase at aerobic conditions (Hertzberger *et al.* 2013). This study demonstrated that *pox* is overexpressed in *L. rhamnosus* under aerobic and respiratory conditions, while the expression of *pdh* is repressed, further indicating that pyruvate oxidase mediates acetate production in presence of oxygen (Goffin *et al.* 2006; Quatravaux *et al.* 2006).

In heterofermentative lactobacilli, pyruvate is almost invariably converted to lactate (Gänzle *et al.* 2007). Pyruvate formate lyase activity is absent in heterofermentative LAB and acetoin formation has been observed in *Leuconostoc* spp. and *Oenococcus* spp. but not in heterofermentative lactobacilli (Olguín *et al.* 2009; Jääskeläinen *et al.* 2013). Accordingly, the metabolic response of *L.*

spicheri and *L. reuteri* differed substantially from the response observed in *L. rhamnosus* and *Lc. lactis* (this study, Duwat *et al.* 2001; Pederson *et al.* 2008; Gaudu *et al.* 2009). Pyruvate conversion to acetoin was not observed in *L. spicheri* LP38 or in *L. reuteri* LTH5448 although the metabolic pathway for acetoin formation is present in *L. reuteri* LTH5448 (Table 1, Figures 1 and 3). Acetoin formation in homofermentative LAB does not contribute to ATP generation but prevents acidification of the growth medium and hence allows more quantitative substrate utilization (Tsau *et al.* 1992; Gaudu *et al.* 2009). The lack of acetoin formation by heterofermentative lactobacilli may indicate that rapid resource utilization and ATP generation allows quantitatively more utilization of substrates and the formation of products that do not acidify the growth substrate.

In heterofermentative lactobacilli, pyruvate is converted to lactate even if oxygen is available as alternative electron acceptor (Stolz *et al.* 1995a and 1995b; Kang *et al.* 2013). We observed production of approximately equimolar concentrations of lactate and acetate during aerobic growth of *L. reuteri* and *L. spicheri*, confirming the dominant role of lactate dehydrogenase in pyruvate metabolism (Table 2 and Figure 1). *L. spicheri* also produced lactate and acetate in equimolar amounts during exponential growth under aerobic and respiratory conditions; however, in respiratory cultures (Stolz *et al.* 1995a; Kang *et al.* 2013; Figure 2) lactate was consumed during the stationary phase of growth while acetate levels continued to increase (Figure 2). In combination with the overexpression of pyruvate oxidase in *L. spicheri*, this result suggests pyruvate conversion to acetate via pyruvate oxidase activity.

In presence of oxygen LAB accumulate reactive oxygen species, which oxidise proteins, nucleic acids and lipids, leading to cell death (Watanabe *et al.* 2012). *L. spicheri* accumulated hydrogen peroxide under aerobic conditions independent by hemin supplementation, while H_2O_2 production by *L. rhamnosus* was observed only in the supernatants of aerobic cultures. The shift towards aerobic and respiration growth, compared to anaerobic cultivation, increased the oxidative stress tolerance in both strains. An increased tolerance of oxidative stress in *L. plantarum* was attributed to the expression of a heme-dependent catalase (Zotta *et al.* 2014a). However, genes coding for

heme-dependent catalase (Knauf *et al.* 1992) are absent in *L. spicheri* and *L. rhamnosus*. Tolerance of oxidative conditions may be attributable to other factors, e.g. overexpression of flavin oxidases, glutathione reductase, cysteine uptake, or thioredoxin reductase activities (Turner *et al.* 1999; Zotta *et al.* 2014a).

In conclusion, this is the first study that to demonstrate the effect of respiratory metabolism on growth and biomass yield, metabolism, and oxidative stress tolerance in heterofermentative lactobacilli. The major metabolic differences between heterofermentative and homofermentative lactobacilli also extend to metabolism under conditions supporting respiration. *L. spicheri* LP38, showing a potential respiratory phenotype, may be selected for further investigations on the regulation and activation of respiratory pathways in heterofermentative lactobacilli.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Legend of Figures

Figure 1: Metabolite production by *Lactobacillus spicheri* LP38 (left panels A, C, E, and G) and *Lactobacillus rhamnosus* N132 (right panels B, D, F, and H). Cells were grown under anaerobic (open symbols) or aerobic conditions (black symbols) in mMRS supplemented with 1 mg l^{-1} menaquinone (●, ○); cultures were additionally supplemented with 2.5 mg l^{-1} hemin (▲, Δ). Shown are the concentrations of lactate (Panels A, B); acetate (Panels C and D); H_2O_2, (Panels E and F); ethanol formation by *L. spicheri* (Panel G) and acetoin formation by *L. rhamnosus* (Panel H). Data are shown as means ± standard deviations of duplicate independent experiments.

Figure 2: Resistance of cells of *Lactobacillus spicheri* LP38 (black bars) and *Lactobacillus rhamnosus* N132 (gray bars) to oxidative stress imposed by exposure to 50 mmol l^{-1} H_2O_2 for 30 min at 34 °C. Panel A: Exponential phase cells. Panel B: Stationary phase cells. Cells were grown under anaerobic or anaerobic conditions in mMRS supplemented with 1 mg l^{-1} menaquinone; cultures were additionally supplemented with 2.5 mg l^{-1} hemin indicated.

Figure 3: *In silico* analysis of pyruvate metabolism and respiratory enzymes in *Lactobacillus spicheri* LP38 (●, ○) and *Lactobacillus reuteri* LTH5448 (■, □). Genes that were present or absent in *L. reuteri* LTH5448 were also present or absent, respectively, in *L. reuteri* LTH2584. Black symbols indicate presence of the gene encoding for the enzyme, open symbols mean the absence of the gene. Enzymes are indicated by numbers as follows:

[1], NADH oxidase; [2], NADH peroxidase;

[3], α-acetolactate synthase; [4], α-acetolactate decarboxylase; [5], butanediol dehydrogenase;

[6], lactate dehydrogenase;

[7], pyruvate formate lyase; [8], pyruvate dehydrogenase; [9], acetaldehyde dehydrogenase; [10], alcohol dehydrogenase;

[11], pyruvate oxidase; [12], phospho-transacetylase; [13], acetate kinase;

[14], respiratory cytochromes cydAB; [15], type II NADH dehydrogenase complex.

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563 **Table 1:** qPCR primers used in this study

Strain	Gene	Primer name	Sequence
Lactobacillus spicheri LP38	*pho*	*pho*-Lbspi-Forward	5'-GGAATACTTGCCAGCCGAT-3'
	pho	*pho*-Lbspi-Reverse	5'-AGTCGATGTAGCCTAACCCG-3'
	cydA	*cydA*-Lbspi-Forward	5'-TGCGTTACCTGCTGAACAAC-3'
	cydA	*cydA*-Lbspi-Reverse	5'-CTTGTTGGCCTGGTTCATCC-3'
	pox	*pox*-Lbspi-Forward	5'-AGGTGGTTCTTCTTGGCGTA-3'
	pox	*pox*-Lbspi-Reverse	5'-CGCCTTTAACCACATCCAGG-3'
	pdh	*pdh*-Lbspi-Forward	5'-ACAAGGAACGCAGGTCAATG-3'
	pdh	*pdh*-Lbspi-Reverse	5'-ACGACAAGTACACGGTTCCT-3'
Lactobacillus rhamnosus N132	*fba*	*fba*-Lbrha-Forward	5'-GTGGGCCAGCTTAACGATTT-3'
	fba	*fba*-Lbrha-Reverse	5'-AAACCATGATCGAAGCTGCC-3'
	cydA	*cydA*-Lbrha-Forward	5'-AGCTCGTTTTCAGTTTGCGA-3'
	cydA	*cydA*-Lbrha-Reverse	5'-AGAAAATCTTGCCCCAGAACT-3'
	pox	*pox*-Lbrha-Forward	5'-TCGGGTTTTCATTCATTTCCTGA-3'
	pox	*pox*-Lbrha-Reverse	5'-ACGTTTGGGTCAGCAGGT-3'
	pdh	*pdh*-Lbrha-Forward	5'-CGATGCGCTTGATGTTGAGT-3'
	pdh	*pdh*-Lbrha-Reverse	5'-ATGCCTGATTCTGCCAATGG-3'

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Table 2: Growth parameters, sugar consumption and metabolite production of *Lactobacillus reuteri* and *Lactobacillus spicheri* strains during the growth in aerobic and anaerobic conditions

Strain	Growth condition	Final optical density (OD_{650})	Final pH	O_2 uptake (min)	Sugar consumption			Metabolite production		
					Glucose (mmol l^{-1})	Maltose (mmol l^{-1})	Fructose (mmol l^{-1})	Lactic acid (mmol l^{-1})	Acetic acid (mmol l^{-1})	Ethanol (mmol l^{-1})
	ANM	6.4±0.1	3.7±0.0	0	13.5±0.6	26.1±0.0	26.6±0.1	83.5±1.2	14.1±0.2	24.5±0.1
L. reuteri	ANHM	7.4±0.0	3.6±0.0	0	12.9±0.2	26.7±0.1	26.5±0.0	83.2±0.7	12.9±0.8	25.7±0.1
LTH5448	AEM	8.0±0.1	3.5±0.0	0	11.7±0.6	24.2±0.1	24.5±0.1	75.0±1.3	65.1±0.2	0
	RS	8.7±0.1	3.5±0.0	172±0.0	13.0±0.3	24.1±0.1	24.5±0.4	74.1±0.5	65.3±0.2	0
	ANM	4.1±0.0	3.8±0.1	0	4.5±0.1	15.7±0.2	27.1±0.0	76.8±0.6	13.1±0.3	24.9±0.2
L. reuteri	ANHM	4.4±0.0	3.8±0.0	0	4.4±0.1	15.2±0.5	27±0.0	74.1±0.4	12.7±0.3	25.6±0.0
LTH2584	AEM	0.2±0.0	6.1±0.0	0	3.5±0.1	0.8±0.1	0	5.3±0.0	0	0
	RS	0.1±0.0	6.1±0.0	0	3.8±0.3	1.2±0.1	0	5.3±0.0	0	0
	ANM	7.5±0.0	3.6±0.0	0	12.3±0.5	26.9±0.1	27.5±0.0	83.8±0.9	11.3±0.3	27.8±0.0
L. reuteri	ANHM	7.6±0.1	3.6±0.0	0	10.5±0.1	25.9±0.0	27.5±0.0	96.5±0.2	12.1±0.1	27.6±0.2
FUA3168	AEM	7.8±0.0	3.7±0.0	0	6.3±0.0	25.4±0.0	27.5±0.0	75.2±0.1	49.7±2.0	0
	RS	8.3±0.1	3.6±0.0	0	5.6±0.0	24.8±0.0	27.5±0.0	73.3±0.2	48.3±0.4	0
	ANM	7.1±0.0	3.7±0.0	0	12.5±0.0	26.8±0.3	27.5±0.0	83.5±0.2	13.4±0.3	26.1±2.0
L. reuteri	ANHM	7.7±0.1	3.7±0.0	0	12.7±0.1	26.7±0.2	27.5±0.0	83.7±0.1	13.9±0.4	27.5±0.5
DSM20016	AEM	7.8±0.0	3.6±0.0	0	6.2±0.4	25.0±0.0	26.3±0.0	62.0±0.8	61.0±0.2	7.3±0.4
	RS	8.4±0.1	3.6±0.0	0	5.0±0.4	25.0±0.1	26.3±0.0	61.3±0.6	58.1±0.3	4.7±0.2
	ANM	5.4±0.0	3.8±0.0	0	6.6±0.2	26.1±0.2	27.5±0.0	84.7±0.6	11.7±0.3	27.9±019
L. spicheri	ANHM	5.5±0.1	3.8±0.0	0	4.2±0.2	26.7±0.1	27.5±0.0	82.9±0.5	11.7±0.2	28.3±0.1
FUA3486	AEM	6.0±0.1	3.7±0.0	0	13.1±0.2	21.3±0.1	25.6±0.2	86.1±0.3	37.0±0.3	0
	RS	6.8±0.1	3.7±0.0	170±0.0	12.3±0.1	21.6±0.0	25.6±0.0	86.3±0.1	45.7±0.2	0
	ANM	3.8±0.1	3.9±0.0	0	3.7±0.2	14.7±0.0	26.1±0.1	57.2±0.8	10.2±0.9	29.3±0.0
L. spicheri	ANHM	3.6±0.0	4.0±0.0	0	3.9±0.2	13.7±0.5	26.3±0.1	55.1±1.0	10.6±0.3	28.2±0.1
FUA3125	AEM	8.12±0.0	3.6±0.0	0	13±0.1	23±0.0	25.9±0.0	85.3±0.0	70.6±0.0	0
	RS	7.7±0.1	3.6±0.0	0	12.6±0.4	23.1±0.0	25.7±0.0	84.1±0.4	60.8±0.8	0
	ANM	2.8±0.0	4.1±0.0	0	4.3±0.3	10.2±0.2	22.2±0.1	53.9±0.9	6.7±0.1	28.0±0.1
L. spicheri	ANHM	3.2±0.0	3.9±0.0	0	4.0±0.4	10.2±0.2	22.1±0.1	48.7±0.3	6.4±0.0	30.6±1.4
LP38	AEM	6.1±0.0	3.8±0.1	0	12.7±0.1	23.4±0.1	22.2±0.2	84.6±0.1	67.6±0.2	0
	RS	6.2±0.0	3.7±0.0	148±0.0	13.0±0.2	23.4±0.1	22.1±0.2	86.8±0.4	72.3±0.3	0

Growth condition: ANM, anaerobiosis (static growth, supplementation with 1 μg ml^{-1} menaquinone); ANHM, heme-supplemented anaerobiosis (static cultivation, supplementation with 2.5 μg ml^{-1} hemin and 1 μg ml^{-1} menaquinone); AEM, aerobiosis (aerated growth, supplementation with1 μg ml^{-1} menaquinone); RS, respiration (aerated growth, supplementation with 2.5 μg ml^{-1} hemin and 1 μg ml^{-1} menaquinone). O_2 uptake: rate of resazurin colour change

Table 3: Parameters of growth kinetics of *Lactobacillus spicheri* LP38 and *Lactobacillus rhamnosus* N132

Strains	Growth condition	μ max (h^{-1})	lag (h)	Max X (g l^{-1})	Max cfu ml^{-1}	Final pH
Lactobacillus spicheri LP38	ANM	0.46±0.01 a)	1.60±0.02 a)	3.08±0.04 a)	8.67 x10^{8} a)	4.00±0.01 b)
	ANHM	0.42±0.01 a)	1.37±0.02 a)	3.30±0.01 b)	8.88 x10^{8} a)	3.94±0.02 b)
	AEM	0.64±0.02 b)	0.81±0.02 b)	3.74±0.01 c)	3.67 x 10^{9} b)	3.67±0.03 a)
	RS	0.64±0.01 b)	0.65±0.02 b)	4.02±0.02 d)	5.03 x 10^{9} c)	3.64±0.02 a)
Lactobacillus rhamnosus N132	ANM	0.44±0.01 a)	3.14±0.01 a)	2.89±0.01 a)	9.74 x 10^{8} a)	3.89±0.01 a)
	ANHM	0.44±0.01 a)	2.98±0.01 a)	2.97±0.02 a)	1.07 x 10^{9} b)	3.79±0.01 a)
	AEM	0.43±0.01 a)	1.32±0.02 b)	3.83±0.03 b)	4.39 x 10^{9} c)	3.79±0.02 a)
	RS	0.44±0.01 a)	1.49±0.01 b)	3.92±0.02 b)	4.63 x 10^{9} d)	3.80±0.02 a)

Growth condition: ANM, anaerobiosis (static growth, supplementation with 1 mg l^{-1} menaquinone); ANHM, heme-supplemented anaerobiosis (static cultivation, supplementation with 2.5 mg l^{-1} hemin and 1 mg l^{-1} menaquinone); AEM, aerobiosis (aerated growth, supplementation with1 mg l^{-1} menaquinone); RS, respiration (aerated growth, supplementation with 2.5 mg l^{-1} hemin and 1 mg l^{-1} menaquinone).
μmax= maximum specific growth rate, estimate value ± standard error
lag: lag phase duration, estimated value ± standard error
Max X: maximum biomass production
Values for the same strain in the same column are significantly different if they do not share a common superscript (Tukey's test, $P<0.01$)

Table 4: General features of the whole shotgun genome of *L. spicheri* LP38

Attribute	**Value**
Genome size (bp)	2,855,247
Contigs Number	25
N50 (bp)	228,460
L50	5
GC content (%)	55.4
rRNA operons	6
tRNA genes	66
Protein-coding genes	2576
Protein coding genes with function prediction	1893
Protein coding genes assigned to COG database	1938

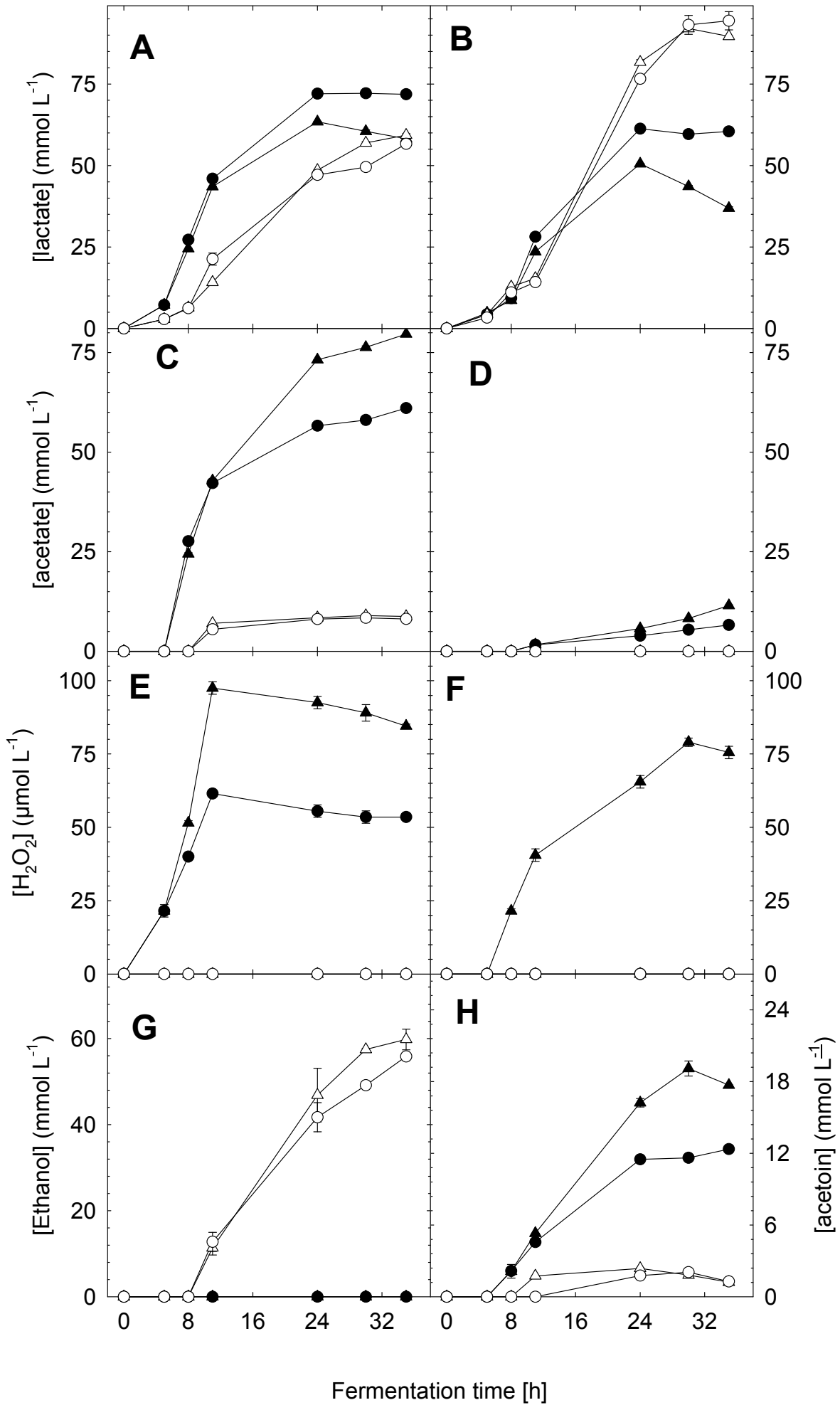
Functional category and number of genes

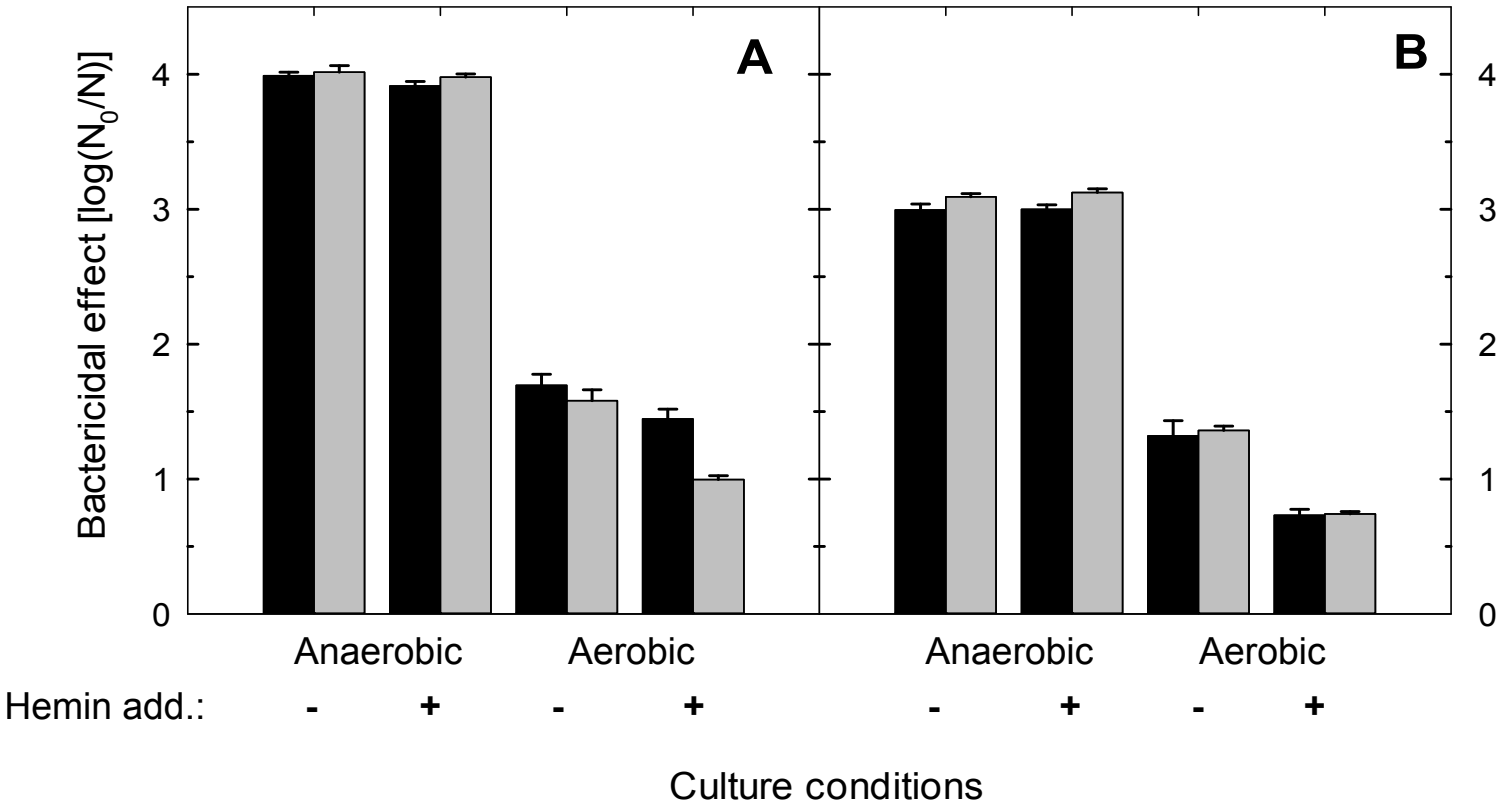
Transcription	203	Lipid metabolism	57
Replication and repair	149	Post-translational modification, protein turnover, chaperone functions	53
Carbohydrate metabolism and transport	146	Signal Transduction	46
Translation	139	Defense mechanisms	46
Cell wall/membrane/envelop biogenesis	138	Coenzyme metabolism	43
Amino Acid metabolism and transport	114	Cell cycle control	28
Inorganic ion transport and metabolism	86	Intracellular trafficking and secretion	16
Nucleotide metabolism and transport	78	Secondary Structure	12
Energy production and conversion	71	Cell motility	5

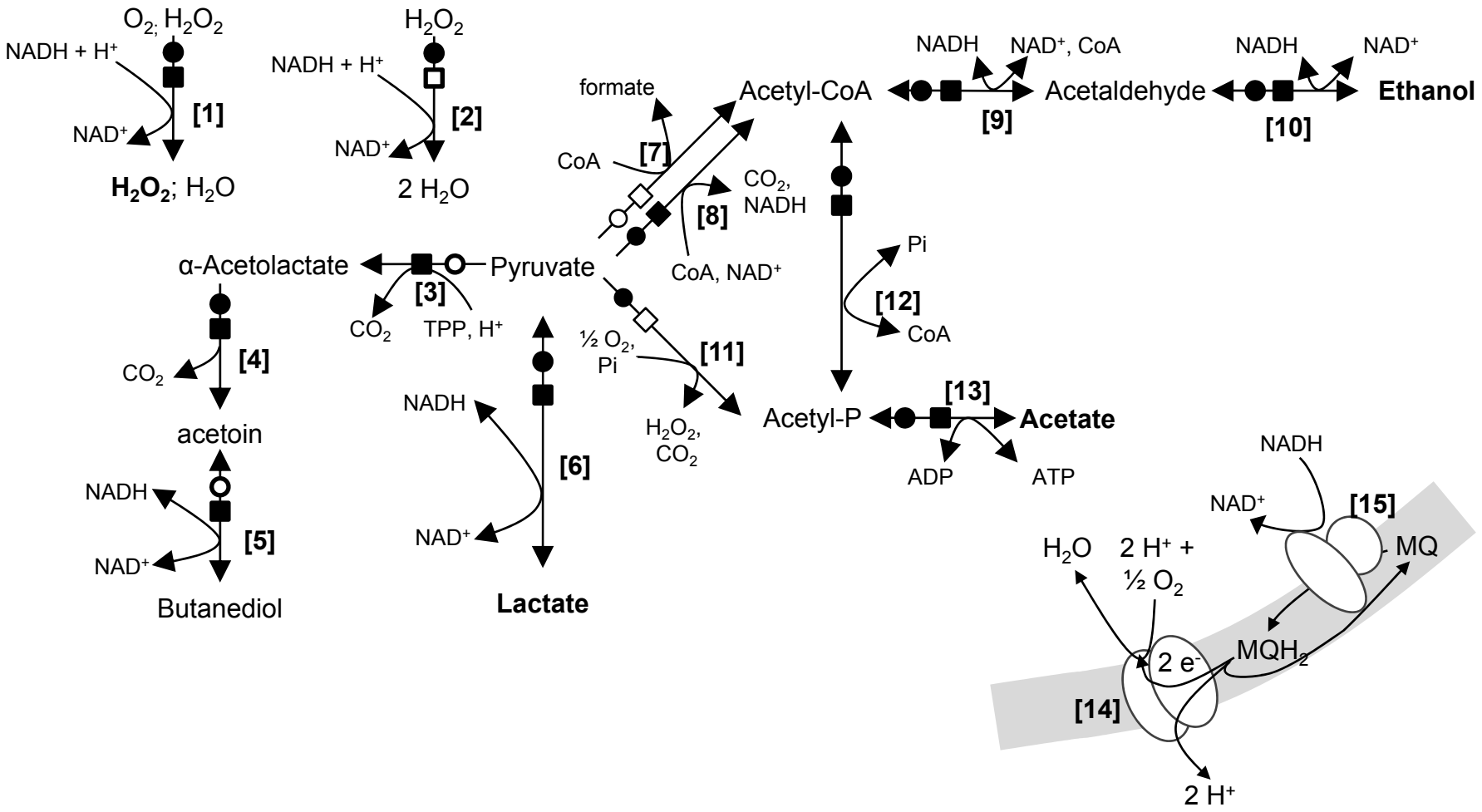
Table 5: Influence of heme supplementation and aeration on expression of *cydA*, *pdh* and *pox* genes in *L. spicheri* LP38 and *L. rhamnosus* N132. Gene expression was calculated relative to anaerobic cultures without heme supplementation as reference conditions

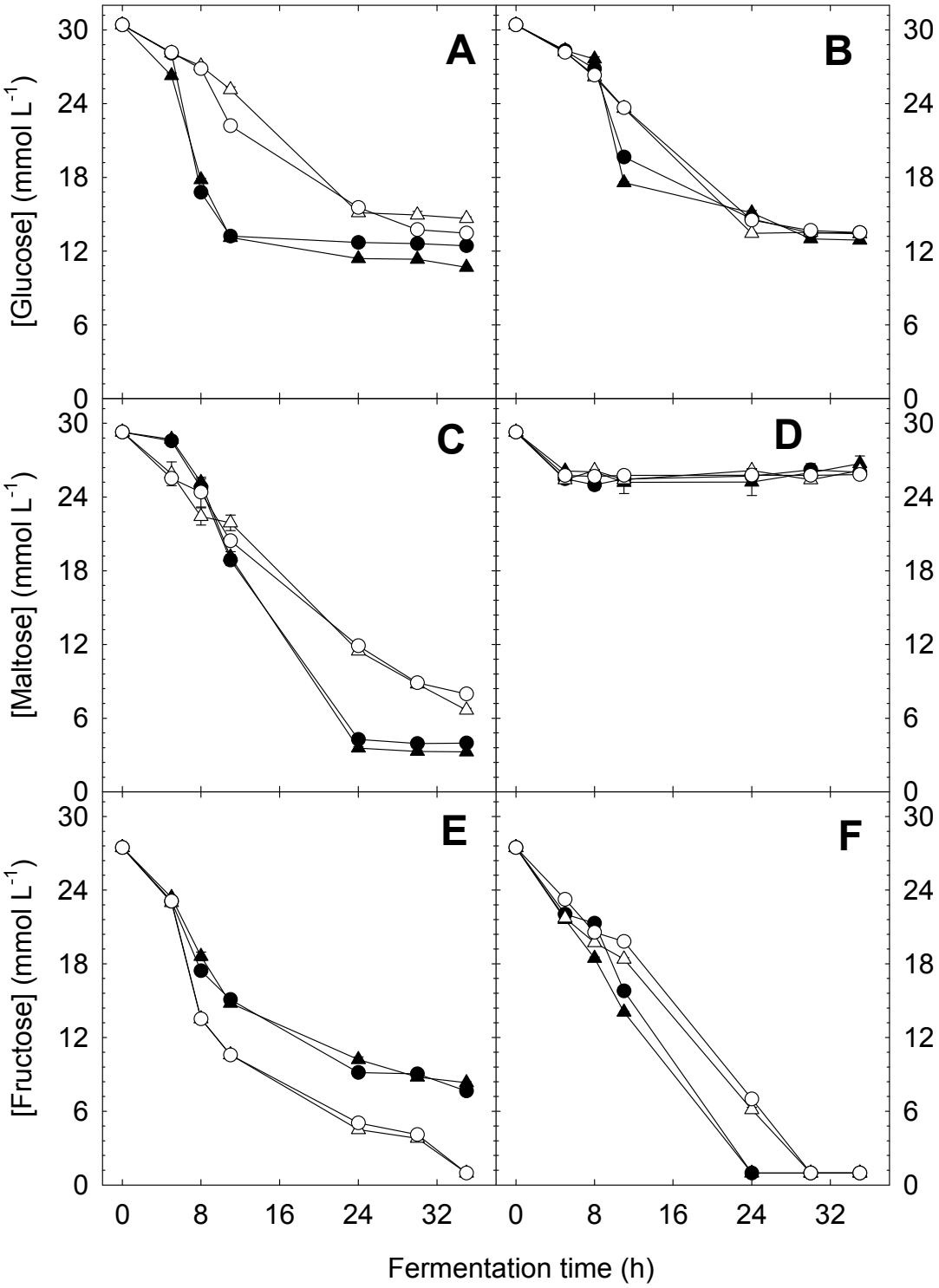
	L. spicheri [2)]			*L. rhamnosus*[2)]		
	ANHM[1)]	AEM	RS	ANHM	AEM	RS
cydA	1.00±0.12[a)]	1.00±0.02[a)]	7.30±0.12[b)]	1.00±0.19[a)]	2.24±0.31[a)]	31.1±2.48[b)]
pdh	0.72±0.22[a)]	0.04±0.00[b)]	0.04±0.00[b)]	3.50±0.07[a)]	0.43±0.06[b)]	0.55±0.08[b)]
pox	0.74±0.11[a)]	5.10±0.22[b)]	4.02±0.24[c)]	1.22±0.27[a)]	11.7±0.28[b)]	13.2±1.03[c)]

[1)] Growth conditions: ANHM, heme-supplemented anaerobiosis (static cultivation, supplementation with 2.5 μg ml^{-1} hemin and 1 μg ml^{-1} menaquinone); AEM, aerobiosis (aerated growth, supplementation with1 μg ml^{-1} menaquinone); RS, respiration (aerated growth, supplementation with 2.5 μg ml^{-1} hemin and 1 μg ml^{-1} menaquinone).
[2)] Relative gene expression in *L. spicheri* and *L. rhamnosus* was calculated using genes coding for phosphoketolase and fructose-bisphosphate aldolase, respectively, as housekeeping genes. Data are shown as means ± standard deviation of three independent replicates.
Values in the same row for the same strain are significantly (Tukey's test, $P<0.01$) different if they do not share a common superscript.


A
B
C
D
E
F
G
H
[lactate] (mmol L-1)
[acetate] (mmol L-1)
[H2O2] (µmol L-1)
[Ethanol] (mmol L-1)
[acetoin] (mmol L-1)
0
25
50
75
100
20
40
60
6
12
18
24
0
8
16
24
32
Fermentation time [h]


A
B
Bactericidal effect [log(N0/N)]
0
1
2
3
4
Anaerobic
Aerobic
Anaerobic
Aerobic
Hemin add.:
-
+
-
+
-
+
-
+
Culture conditions


O2; H2O2
NADH + H+
[1]
NAD+
H2O2; H2O
H2O2
NADH + H+
[2]
NAD+
2 H2O
formate
CoA
[7]
CO2, NADH
[8]
CoA, NAD+
Acetyl-CoA
NADH
NAD+, CoA
[9]
Acetaldehyde
NADH
NAD+
[10]
Ethanol
α-Acetolactate
[3]
CO2
TPP, H+
Pyruvate
Pi
[12]
CoA
CO2
[4]
acetoin
½ O2, Pi
[11]
H2O2, CO2
Acetyl-P
[13]
Acetate
ADP
ATP
NADH
[6]
NAD+
Lactate
NADH
[5]
NAD+
Butanediol
NADH
NAD+
[15]
MQ
H2O
2 H+ + ½ O2
MQH2
2 e-
[14]
2 H+


A
B
C
D
E
F
[Glucose] (mmol L-1)
[Maltose] (mmol L-1)
[Fructose] (mmol L-1)
0
6
12
18
24
30
0
8
16
24
32
Fermentation time (h)