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Transcriptome analysis of *Lactococcus lactis* subsp. *lactis* during milk acidification as affected by dissolved oxygen and the redox potential

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ABSTRACT

Performance of *Lactococcus lactis* as a starter culture in dairy fermentations depends on the levels of dissolved oxygen and the redox state of milk. In this study the microarray analysis was used to investigate the global gene expression of *L. lactis* subsp. *lactis* DSM20481^T during milk acidification as affected by oxygen depletion and the decrease of redox potential. Fermentations were carried out at different initial levels of dissolved oxygen (dO_2) obtained by milk sparging with oxygen (high dO_2, 63%) or nitrogen (low dO_2, 6%). Bacterial exposure to high initial oxygen resulted in overexpression of genes involved in detoxification of reactive oxygen species (ROS), oxidation–reduction processes, biosynthesis of trehalose and down-regulation of genes involved in purine nucleotide biosynthesis, indicating that several factors, among them trehalose and GTP, were implicated in bacterial adaptation to oxidative stress. Generally, transcriptional changes were more pronounced during fermentation of oxygen sparged milk. Genes up-regulated in response to oxygen depletion were implicated in biosynthesis and transport of pyrimidine nucleotides, branched chain amino acids and in arginine catabolic pathways; whereas genes involved in salvage of nucleotides and cysteine pathways were repressed. Expression pattern of genes involved in pyruvate metabolism indicated shifts towards mixed acid fermentation after oxygen depletion with production of specific end-products, depending on milk treatment. Differential expression of genes, involved in amino acid and pyruvate pathways, suggested that initial oxygen might influence the release of flavor compounds and, thereby, flavor development in dairy fermentations. The knowledge of molecular responses involved in adaptation of *L. lactis* to the shifts of redox state and pH during milk fermentations is important for the dairy industry to ensure better control of cheese production.

1. Introduction

Lactococcus lactis is commonly used as a starter culture for the manufacture of cheese and other dairy products. The metabolic activity of *L. lactis* determines pH, texture, flavor and other organoleptic characteristics of cheeses (Smit et al., 2005). The starter cultures are exposed to various physicochemical stresses throughout cheese production, including acid, oxidative and osmotic stresses which affect their metabolic activity and, thereby, may influence the cheese quality (Bachmann et al., 2010; Raynaud et al., 2005). An important parameter affecting performance of starter cultures during milk fermentation is an oxidation–reduction (redox) potential (Abraham et al., 2013; Jeanson et al., 2009; McSweeney et al., 2010). The redox potential characterizes the ability of a system to accept (reduce) or donate (oxidize) electrons. It primarily depends on the levels of dissolved oxygen in milk along with the other factors such as lactate/pyruvate ratio, ascorbate content, the thiol groups and metal ions. There is an indication that redox potential might have an impact on cheese aroma by modification of amino acid catabolism in *L. lactis* (Kieronczyk et al., 2006). It was shown recently that oxidative conditions in yogurt affect aroma through altering the metabolic pathways in *Lactobacillus bulgaricus* and *Streptococcus thermophilus* towards production of acetaldehyde, dimethyl sulfide and ketones (Martin et al., 2011). The microarray-based transcriptome profiling has been widely applied to study gene expression in *L. lactis* as affected by environmental stresses, such as, starvation and acid stress (Cretenet et al., 2014; Pedersen et al., 2008; Raynaud et al., 2005; Xie et al., 2004). Pedersen et al. (2008) assessed expression changes in *L. lactis* subsp. *lactis* during aerobic fermentations as compared to the static growth in nonaerated synthetic medium. The study revealed a number of stress response genes essential for aerobic fermentation, however, the impact of redox potential was not considered. Only one study has been published so far in which transcriptional responses in *L. lactis* subsp. *cremoris* MG1363 were investigated in relation to

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acidification kinetic and the redox potential (Cretenet et al., 2014). Contrast to the current research fermentations by MG1363 were performed in the laboratory medium and mostly focused on oxidative stress responses.

We showed recently that initial levels of dissolved oxygen had an effect on acidification kinetics and the ability of *L. lactis* strains to decrease the redox potential in milk (Larsen et al., 2015). In this study, global gene expression of *L. lactis* subsp. *lactis* DSM20481^T grown in milk was further investigated with the use of the DNA microarrays. The objectives of the study were to define genetic factors related to the dynamic changes of oxygen and redox potential during milk acidification and to reveal the possible impact of initial oxygen on expression profiling in *L. lactis*. For this purpose transcriptomic analysis was performed for the cultures grown at low and high initial oxygen obtained by milk sparging with oxygen and nitrogen, respectively.

2. Materials and methods

2.1. Bacterial strain and growth conditions

Lactococcus lactis subsp. *lactis* DSM20481^T (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany) was maintained in M17 medium (Oxoid A/S, Denmark) supplemented with 0.5% lactose (LM17) in 20% (vol/vol) glycerol at −80 °C. Before the experiments the strain was grown overnight in full fat UHT milk (3.5% fat) supplemented with 0.2% tryptone as the strain was non-proteolytic. Reagents were purchased from Merck (Germany) and Sigma-Aldrich (Denmark) unless otherwise stated.

2.2. Milk fermentation and sample collection for microarray analysis

Inoculum for fermentations was prepared by inoculation of UHT (ultra-high temperature pasteurized) milk with 1% (vol/vol) overnight culture of *L. lactis* subsp. *lactis* DSM20481^T and incubation for 3 h at 30 °C to the exponential growth phase. Inoculum was added at concentration of 10^6 CFU/ml into fermenters (Duran bottles GL80, Duran Group, Germany) containing 700 ml milk (added 0.2% tryptone, pH 6.5) preheated to 30 °C, with either: (i) low initial level of dissolved oxygen (6% dO_2, corresponding to 30% air saturation) obtained by milk sparging with N_2, or (ii) high initial level of dissolved oxygen (63% dO_2, corresponding to 300% air saturation) obtained by oxygen sparging. The level of oxygen in untreated milk was 12%, corresponding to 57% air saturation. Fermentations were performed at 30 °C, with stirring at 250 rpm (2Mag magnetic stirrer, Germany) in water-bath (Comfort Heto Master Shake). Parameters monitored during fermentation (DaqLogger FX112-4-2, Yokogawa, Japan) were dissolved oxygen concentration (Visiferm DO120, Hamilton Bonaduz, Switzerland), redox potential (E_{h7}) and pH (Easyferm Plus Rx VP120, Hamilton Bonaduz, Switzerland). Calibration of the sensors and conversion of redox measurements into conventional redox potential at pH 7 (E_{h7}) was performed as described before (Abraham et al., 2013; Tachon et al., 2010).

In total five samples of *L. lactis* subsp. *lactis* DSM20481^T culture were withdrawn from each fermenter with low (L) or high (H) initial oxygen as follows: (i) 1 h after fermentation start (reference samples L_R and H_R); (ii) 2 h after fermentation start before the drop of E_{h7} (L1 and H1), (iii) at a time of oxygen depletion (L2 and H2); (iv) 20 min after oxygen depletion (L3 and H3), (v) 2 h after oxygen depletion at minimum E_{h7} (L4 and H4). Samples were frozen immediately in liquid nitrogen and stored at −80 °C. Fermentations were performed in three biological replicates.

2.3. Isolation of total bacterial RNA

Frozen cell cultures of 5 ml were added 10 ml RNAprotect Bacteria Reagent (Qiagen, Denmark) and treated for 30 s in an ultrasound-bath (Branson 2210, Branson Ultrasonics, USA). Five milliliters sodium citrate (1 M) and 1.95 ml buffered saline solution (0.145 M sodium chloride, 0.016 M sodium β-glycerophosphate and 0.1% Tween 80) were added to 15 ml cell suspensions and the mixture was incubated at room temperature for 5 min. The cells were harvested by centrifugation (4000 × *g* for 12 min) and washed in 0.9% (*w*/*v*) sodium chloride. Total bacterial RNA was isolated using RNeasy minikit (Qiagen, Denmark), according to the manufacturer's instructions. Briefly, cells were lysed in 200 μl TE-buffer (Life Technologies, Denmark) containing 15 mg/ml lysozyme, 15 μl proteinase K (20 mg/ml) and 2 μl mutanolysin (25 U/μl) at 37 °C for 10 min. On-column DNase treatment was performed using DNaseI (Qiagen, Denmark). RNA was eluted with 100 μl RNase free water and kept at −80 °C. The quality of RNA and the absence of residual DNA were evaluated using the Agilent RNA 6000 Nano Kit on the Bioanalyzer 2100 (Agilent Technologies, Denmark). The RNA samples of high integrity (RIN > 8) and 23S/16S rRNA ratios of 1.9–2.1 were used for the cDNA synthesis.

2.4. The microarray-platform, cDNA synthesis, hybridization and pre-analysis of the microarrays

Four arrays were produced using cDNA obtained from the test samples versus reference samples to evaluate transcriptional changes during each fermentation condition, i.e., low oxygen ($L1/L_R$, $L2/L_R$, $L3/L_R$ and $L4/L_R$) and high oxygen ($H1/H_R$, $H2/H_R$, $H3/H_R$ and $H4/H_R$). Additionally, one array was constructed using cDNA from reference samples at high versus low oxygen (H_R/L_R) to determine transcriptional responses related to initial oxygen content. Design of oligonucleotides for microarrays and array-spotting was performed at Chr. Hansen A/S (Hørsholm, Denmark). The microarrays contained in total 2208 oligonucleotides, including the coding sequences of *L. lactis* subsp. *lactis* IL1403 genome, selected coding sequences for phage bIL170, the lactose plasmid and plasmid-encoded proteinase genes (*prtM* and *prtP*). cDNA synthesis, processing and microarray analysis were performed as described by Pedersen et al. (2008). Briefly, the cDNA synthesis and labeling with Cy3 (reference) or Cy5 (test) was done using 10 μg of total RNA and CyScribe post-labeling kit (Amersham Biosciences, Denmark) according to the manufacturer's protocol. The arrays were scanned using a GenePix 4100 A scanner with GenePix Pro 6.0 software (Axon Instruments, Inc., Union City, CA). The GenePix Pro software was used to convert the spot-signals of the arrays into intensity values. The values of the spot intensity (median) minus the background were used for both Cy5 and Cy3. Subtracting the background from very low signals resulted in negative values. The negative values were replaced with <no data> before the data was imported into Acuity 4.0 Microarray Informatics Software (Axon Instruments Inc., New York, USA). The data were ratio-normalized using the default settings. The $\log_2$(ratio) between the channel signals [$\log_2$(Cy5/Cy3)] was calculated. Criteria for gene selection were: (i) at least two of the four replicate spots were found, (ii) the intensity value in "median − background" was above 200 in one channel, and (iii) the $\log_2(\text{ratio}) \geq |2|$ and the standard deviation was <0.8. The mean of the $\log_2$(ratio) of replicate spots was given as the working variable. Genes with more than 2 fold change in expression ($p < 0.05$) were considered as differentially expressed (DE). Functional analysis of genes was based on the classification made by Bolotin et al. (2001).

2.5. Real-time quantitative PCR (qPCR)

Expression levels of selected genes were analyzed by qPCR to validate the microarray results. Selected genes were *sodA*, *noxE*, *yeeA*, *pgmB*, *yedE* and *yedF* differentially expressed in *L. lactis* subsp. *lactis* DSM20481^T during milk fermentation as determined by microarrays. Expression levels of *rluD*, *topA* and *rpoD* were used for qPCR data normalization. Expression stability of the house-keeping genes across the sampling points was confirmed by the BestKeeper software (Pfaffl et al., 2004). Gene specific primers for qPCR (Table S1, Supplementary