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Published in:
Proteomics

Link to article, DOI:
10.1002/pmic.201600178

Publication date:
2017

Document Version
Peer reviewed version

Comparative proteomics of oxidative stress response of *Lactobacillus acidophilus* NCFM reveals effects on DNA repair and cysteine *de novo* synthesis

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[§]*We dedicate this work to the late Susanne Jacobsen*

Running title: *Lactobacillus acidophilus* NCFM response to oxidative stress

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Abbreviations used:

ABM; Antibiotic biosynthesis monooxygenase

cfu; Colony forming units

GAPDH; Glyceraldehyde-3-p dehydrogenase

GIT; Gastrointestinal tract

LAB; Lactic acid bacteria

LABSEM; Semi-synthetic media for lactic acid bacteria

PK; Pyruvate kinase

R5P; ribose-5-phosphate

PRPP; 5-phospho-α-d-ribosyl-1-pyrophosphate

PRPPS; Ribose-p pyrophosphokinase

ROS; Reactive oxygen species

Keywords:

2DE-MS / ClpP-ATP-dependent protease-peptidase / Cysteine synthase / Glyceraldehyde-3 P dehydrogenase / Hydrogen peroxide

Word count: 6242

Abstract

Probiotic cultures encounter oxidative conditions during manufacturing, yet protein abundance changes induced by such stress have not been characterized for some of the most common probiotics and starters. This comparative proteomics investigation focuses on the response by *Lactobacillus acidophilus* NCFM to H_2O_2, simulating an oxidative environment. Bacterial growth was monitored by BioScreen and batch cultures were harvested at exponential phase for protein profiling of stress responses by 2D gel-based comparative proteomics. Proteins identified in 19 of 21 spots changing in abundance due to H_2O_2 were typically related to carbohydrate and energy metabolism, cysteine biosynthesis, and stress. In particular, increased cysteine synthase activity may accumulate a cysteine pool relevant for protein stability, enzyme catalysis and the disulfide-reducing pathway. The stress response further included elevated abundance of biomolecules reducing damage such as enzymes from DNA repair pathways and metabolic enzymes with active site cysteine residues. By contrast, a protein-refolding chaperone showed reduced abundance, possibly reflecting severe oxidative protein destruction that was not overcome by refolding. The proteome analysis provides novel insight into resistance mechanisms in lactic acid bacteria against reactive oxygen species and constitutes a valuable starting point for improving industrial processes, food design or strain engineering preserving microorganism viability.

Statement of significance

Lactic Acid Bacteria (LAB) are widely used as starter cultures in food fermentation and as probiotics and it is important to secure a high titer of viable cells. During manufacturing processes, however, bacteria go through various stresses, one notably being exposure to oxygen and reactive oxygen species (ROS). *Lactobacillus acidophilus* contributes beneficial effects on human health, which warranted its wide application as probiotic. In the gastrointestinal tract, probiotics encounter oxidative stress from oxygen gradients and the immune system, possibly reducing viable cell counts below the recommended daily intake. Here, analyses of proteome changes induced by hydrogen peroxide shed light on the molecular response to oxidative stressors and represent a first step towards strain amelioration.

1 Introduction

Lactic acid bacteria (LAB) constitute a heterogeneous group of prokaryotes colonizing different habitats: human and animal bodies, plants, and food [1]. Since LAB are unable to synthesize heme, they lack catalases as well as other oxygen defense enzymes and possess low oxygen tolerance thus being defined as aerotolerant anaerobes [2]. This implicates sensitivity to reactive oxygen species (ROS); superoxide anion radical (O_2^-); hydroxyl radical ($OH^{\cdot}$); and hydrogen peroxide (H_2O_2). Oxidative stress reflects imbalance between generation of and ability to detoxify ROS or repair resulting damages. Some, but not all, LAB use manganese superoxide dismutase, pseudocatalase and peroxidase or manganese (an O_2^- scavenger) to convert ROS to harmless compounds [3, 4]. However, this is not the sole strategy, indeed some LAB devote a large part of their genome to counteracting oxygen stress [5]. Protection mechanisms worth noting include i) increased activity of oxygen consuming routes [6], ii) maintenance of a reducing intracellular environment through disulfide-reducing pathways [7, 8], iii) protection of sensitive thiols by metal ions [9], and as a last resort iv) DNA repair to overcome oxidative damage of the genome [10].

LAB are crucial in food fermentations and contribute to taste and texture of food products [11]. They inhibit food spoilage and pathogenic bacteria by lowering pH through lactic acid formation and by producing bacteriocins [12]. Thanks to these properties, LAB are used as starters and as biocontrol agents [13]. Furthermore, several LAB are marketed as probiotics, with a range of health benefits [14, 15, 16, 17, 18].

Lactobacillus acidophilus is among the most widely used LAB species in yogurt and fermented milk products [19]. The probiotic functionality of *L. acidophilus* is well documented *in vitro* and *in vivo* including attenuation of lactose intolerance, reduction of cholesterol level,

immunomodulation *via* stimulation of host cytokines and immunoglobulin A (IgA) expression, exclusion of pathogens, and alleviation of cold-like symptoms in children [20, 21]. *L. acidophilus* grows optimally at 37 – 42°C [21] suitable for application in food industry, but belongs to the least oxygen tolerant LAB because it lacks superoxide dismutase and has low levels of manganese that constitutes the major antioxidant defense in heme-deficient strains [11]. These features strongly reduce the performance of *L. acidophilus* during harsh industrial processing.

Food starters and probiotics have to cope with oxidative stress at manufacturing stages from fermentation to freeze- and spray-drying used in production of high-density probiotic powder as end-product for the market, and during storage [22]. Several studies recommended a daily probiotics intake of $10^8 – 10^9$ colony-forming units (cfu) and food with health claims on probiotics requires at least $10^6 – 10^7$ cfu of probiotic bacteria per gram (FAO/WHO, 2001). However, ambient storage temperature and exposure to oxygen might diminish the viable cell count below the recommended limit [23]. Thus, various commercial products contained as little as ~10^3 cfu/mL at the end of shelf life, while starting with 10^7 cfu/mL [24]. Additionally, survival of probiotics during gastrointestinal tract (GIT) transit depends on ability to sense and respond to steep oxygen gradients [22]. Altogether preserving a high number of viable cells is an important challenge for industrial products.

L. acidophilus NCFM is commercially available since 1972 in the United States in dairy products and dietary supplements. It does not encode a superoxide dismutase, but possesses genes associated with oxygen consuming routes, disulfide-reducing pathways (a thioredoxin system and glutathione reductase) and DNA repair [25]. Reports on levels of relevant enzyme and protein forms are lacking, however, the present comparative proteome analysis discloses important molecular aspects of the response of *L. acidophilus* NCFM to oxidative stress, a paradigmatic

condition for microaerophilic LAB physiology. The ultimate goal is to gain insights facilitating control, improvement and optimization of bacterial behavior in industrial starter and probiotics production.

2 Materials and methods

2.1 Bacterial strain and growth conditions

Growth of *L. acidophilus* NCFM (NCFM 150B, FloraFIT® Probiotics; DuPont, USA Inc., Madison, WI) without agitation at 37°C in 50 mL cultures in preheated LABSEM supplemented with 1% glucose [26] was monitored by OD_{600} (Ultrospec 2100pro, Amersham Biosciences) and pH (Panpeha pH-indicator strips range 0 – 14, Sigma-Aldrich) measurements every 3 h.

2.2 BioScreen

The BioScreen instrument (Labsystems BioScreen C, Bie & Berntsen A/S) allows simultaneous incubation, shaking and OD measurement of up to 200 samples. Cultures at early exponential phase (OD_{600} ~0.2) were inoculated in fresh preheated LABSEM containing 0.0 – 1.2 mM H_2O_2 [27] and pipetted (300 µL) into wells. OD_{600} was monitored every 30 min at 37 ^{0}C for 48 h. Three biological replicates each in three technical replicates were analyzed at each condition.

2.3 Oxidative stress

Batch cultures (50 mL) in LABSEM without H_2O_2 were harvested (3200 x g, room temperature, 10 min; Centrifuge CR3i, Jouan) at early exponential phase (OD_{600} ~0.2; t ~12 h) and sub-cultured in fresh preheated LABSEM (50 mL) ~~added none~~containing no, 0.4, 0.8 or 1.2 mM H_2O_2 according to [28]. The experiment was performed in four biological replicates.

2.4 Protein sample preparation

Cells were harvested as above at late exponential phase (OD_{600} ~0.7, t ~35 h), washed with 0.9% NaCl, disrupted by manual grinding with a small amount of acid-washed glass beads (<100 µm diameter, Sigma-Aldrich) using a rounded glass Pasteur pipette, 60 µL of sample buffer (28 mM Tris-HCl, 22 mM Tris-base pH 8.5, 100 mM DTT) was added to the sample and heated (100 ^{0}C, 2 min). ~~added 60 µL sample buffer (28 mM Tris-HCl, 22 mM Tris-base pH 8.5, 100 mM DTT) and heated (100 ^{0}C, 2 min).~~ After 5 min at room temperature, 240 µL rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT) was added. The mixture was vortexed, centrifuged (10000 x g, 10 min) and the supernatant collected [29]. Protein concentration was determined using the Bradford Protein Assay Kit (Thermo Scientific) and BSA as standard. Proteins were precipitated by addition of four volumes of ice-cold acetone to ~100 µg protein ~~A volume containing ~100 µg protein was added four volumes of ice-cold acetone, kept~~ (overnight, –20 °C) [30] and centrifuged (14000 x g, 25 min, 4 °C). Protein pellets were air-dried and dissolved in 350 µL rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG-buffer pH 3 – 10, 0.3% DTT, Orange G) immediately prior to 2DE.

2.5 2DE

Rehydrated samples (100 µg protein in 350 µL rehydration buffer) were loaded on 18 cm dry-strips (pH 3 – 10; GE Healthcare) for IEF (IPGphor; GE Healthcare) at 20^{0}C, 50 µA/strip until ~67 kVh [29]. Strips were then soaked with 2 x 5 mL equilibration buffer (6 M urea, 30% glycerol, 50 mM Tris-HCl pH 8.8, 2% w/v SDS, 0.01% bromophenol blue) containing 1% DTT and 2.5% iodoacetamide, respectively (15 min each). The second dimension (12.5% SDS-PAGE, 26×20 cm gel size; Ettan™ DALT*six* Electrophoresis unit; GE Life Sciences) was run overnight at 1 W/gel.

Gels were stained overnight by CBB G250 [31], destained, and scanned (Scan maker 9800XL, TM1600, Microtek9).

2.6 Image analysis

Differentially abundant proteins were revealed using SameSpots software (TotalLab). Interference due to different CBB G250 batches and external conditions affecting spot volume calculation was minimized by comparing gels run together. Fold-changes (SameSpots) were averaged for individual spots and standard deviations calculated of four biological and three technical replicates of stressed and control samples. Data reliability was checked using Student's t-test (Microsoft Excel). Due to low abundance of some proteins displaying changes, high p-values in the range 0.05 – 0.09 were also considered, while even higher p-values were just informed. Spots changing at least 1.3-fold between control and 1.2 mM H_2O_2 were analysed by MS.

2.7 In-gel digestion and protein identification by mass spectrometry

Differentially abundant spots were excised from a master gel and stored (Eppendorf™ tubes, –20 °C) until use. Gel pieces were washed with 40% ethanol (100 µL) at 50 °C with shaking until colorless and the ethanol was decanted. ACN (30 µL) was added to shrink the gel (10 min) and removed. Gel pieces were air-dried leaving lids open in a LAF bench (10 min), added 5 µL 12.5 ng/µL trypsin (Promega) in 25 mM NH_4HCO_3, kept on ice (45 min), added 10 µL NH_4HCO_3 and incubated (overnight, 37 °C). Supernatants were transferred to fresh tubes and stored at –20 °C until MS. Digests (1 µL) were loaded on a MALDI AnchorChip target (Bruker-Daltonics), air-dried (20 min), added 1 µL matrix solution (0.5 µg/µL CHCA in 90% ACN, 0.1% TFA), air-dried and washed with 2 µL 0.5% TFA. Tryptic peptides of β-lactoglobulin were used for calibration. MS spectra were obtained (Ultraflex II MALDI-TOF MS mass spectrometer; Bruker-Daltonics)

in auto-mode using Flex Control v.3.0 (Bruker-Daltonics). Peaks were identified after calibrating the Flex Analysis v3.0 software (Bruker-Daltonics) to exclude peaks of autodigested trypsin and keratin. Spectra were searched in NCBI nr database for bacteria (NCBI nr 20140323; 38032689 sequences; 13525028931 residues) using MASCOT 2.0 (http://www.matrixscience.com) integrated in Biotools v3.2 (Bruker-Daltonics). Search parameters were monoisotopic; peptide mass tolerance ±50 ppm; maximum of one missed cleavage; fixed carbamidomethylation of cysteine; partial oxidation of methionine. Identification by PMF was confirmed with a MASCOT score of 86 ($p \leq 0.05$) and a minimum six of matched peptides [29].

3 Results

3.1 BioScreen

Growth of *L. acidophilus* NCFM with 0.0 – 1.2 mM H_2O_2 present was analyzed [7, 27] using BioScreen, which indicated a critical level of 0.4 mM H_2O_2 causing stress without being lethal. The growth was retarded as reflected by the longer lag phase and deferred start of exponential phase. The slope of the exponential phase decreased with 0.4 mM H_2O_2 and growth stopped at $H_2O_2 > 0.4$ mM (Fig. 1).

3.2 Growth curves and pH profiles

The time-course of bacterial growth can provide insight into influence of environmental conditions. In standard medium (LABSEM, pH 5.5 – 6.0) *L. acidophilus* NCFM enters exponential phase after ~10 h and stationary phase at ~24 h. pH of the culture decreases (pH 4.0 – 4.5) due to lactic acid production. Initially, the effect of 0.4 mM H_2O_2 was tested as this was the highest concentration allowing growth in BioScreen (Fig 1). Following a protocol of Serata et al. [32], 0.4

mM H_2O_2 was added 7 h after the inoculum, without *L. acidophilus* NCFM displaying appreciable stress (data not shown). Previously 0.6 – 1.2 mM H_2O_2 was found to decrease, but not completely arrest *L. acidophilus* growth [27]. Therefore 0.8 mM or 1.2 mM H_2O_2 was added at the start of the exponential phase, using the Bruno-Bàrcena protocol [27]. The growth was slightly more slowed at 1.2 than 0.8 mM H_2O_2 (Fig. 2A).

3.3 Growth curves and pH profiles after sub-culturing

Cells were transferred to fresh medium supplemented with H_2O_2 (OD_{600} ~0.2, 12 – 15 h, see Methods) *L. acidophilus* NCFM entered an adapting lag phase (Fig. 2B) and synthesized molecules to cope with the stress. The lag phase in 1.2 mM was longer than in 0.8 mM H_2O_2 and the corresponding pH profiles differed importantly (Fig. 2B; Supporting Fig. S1), also considering the pH was measured by using indicator paper.". Notably re-adaptation after sub-culture is well-known to cause a second lag prior to the manifested decrease in pH [33]. Because more significant changes in protein abundance were assumed for 1.2 mM H_2O_2, only this culture was subjected to differential proteome analysis.

3.4 2DE-based comparative proteome analyses

Stressed and control cultures were harvested in late exponential phase (OD_{600} ~0.7). In total 507 spots were detected by 2DE of the intracellular proteins. Comparison of stressed and control cultures revealed 21 differentially abundant spots (Fig. 3; Table 1) using a threshold of 1.3 fold relative abundance change of spots selected for MS analysis [30]. The resulting PMFs searched against NCBI identified 19 unique proteins from *L. acidophilus* NCFM with a high MASCOT score, except for ribose-p-pyrophosphokinase (spot 18) that gave a PMF score of 77 (Table 1, Supporting Table S1). The proteins are classified into functional categories i) energy metabolism;

ii) nucleic acids; iii) general stress; and iv) oxidative stress, discussed below. Protein abundancy changes were supported by semi-quantitative RT-PCR (Supporting Methods S1) analysis of expression of four selected genes (*lba0698* encoding GAPDH, *lba0957* encoding pyruvate kinase (PK), *lba0497* encoding antibiotic biosynthesis monooxygenase, and *lba1248* encoding heat shock protein GrpE), using expression of 16S rDNA (*lba2001*) as internal control (Supporting Fig. S2) [29].

4 Discussion

Stress responses play a key role in cellular adaptation of all organisms to changes in the environment. The best known biological stress reaction is synthesis of chaperones and proteases which counter accumulation of aberrant proteins [34]. As LAB evolved on Earth before oxygen appeared in the atmosphere [5], accommodating oxidative stress has high priority. Bacteria may use non-enzymatic (Mn^{2+}, ascorbate, tocopherols, glutathione) and enzymatic (thioredoxin and thioredoxin-reductase, catalase, NADH oxidases, NADH peroxidases, superoxide dismutase) systems for defense against ROS. LAB genomes however, mostly do not encode enzymes eliminating ROS, which adversely affect cell fitness by attacking proteins, lipids and nucleic acids, and represent a major cause of cell death [35].

Reports specifically addressing oxidative stress defense in probiotic prokaryotes are scarce and only concern *Bifidobacterium longum* [36] and *Lactobacillus sakei* [37]. A larger number of papers (mainly review articles) refer to concerted physiological responses to general stressors (heat, cold, carbon starvation, osmotic, oxidative and acidic stress) both in LAB [6, 35, 38] and in Bifidobacteria [39]. Only one report, however addressed behavior of *L. acidophilus* in an oxidative

environment [40] showing increased NADH oxidase and NADH peroxidase together with H_2O_2 decomposing ability upon exposure to 21% oxygen.

In the present study 1.2 mM H_2O_2 was not lethal *L. acidophilus* NCFM but prolonged lag phase latency which together with a shallower exponential growth compared to control cultures, underlined that time is needed to recover from oxidative damage. Lower H_2O_2 (> 0.4 mM) was harmful to *L. acidophilus* NCFM in the BioScreen experiment, probably reflecting increased exposure to oxygen due to small wells and larger surface in the culture plates which augmented H_2O_2 sensitivity.

Remarkably, several identified enzymes with increased abundance in 1.2 mM H_2O_2-treated *L. acidophilus* NCFM e.g. GAPDH and PK have active-site cysteine essential for catalysis~~,~~. As thiol groups are very sensitive to oxidative stress [22], we speculate *de novo* synthesis of these enzymes and cysteine synthase is triggered to overcome oxidative damage. Moreover, as 2DE allows monitoring multiple protein forms, oxidative stress could be observed to cause increased and reduced abundance for different protein species derived from the same gene, as described below.

4.1 Energy metabolism

GAPDH of 42 kDa (spots 10 and 12,) increased >+1.3-fold in relative abundance, while GAPDH of 27 kDa (spot 27) significantly decreased (−1.75; $p < 0.01$). Notably RT-PCR of the GAPDH~~K~~ gene (*lba0698*, Supporting Fig. S2) showed essentially no overall change under oxidative stress compared to control, whereas the 2DE analysis revealed specific GAPDH forms to be either increased or decreased in abundancy. Previously, proteomics showed two isozymes in *Lactobacillus spp*., GAPDH II (41 kDa), and GAPDH I (38 kDa) exhibiting different expression profiles, but only synthesis of GAPDH II was repressed by amino acids in the culture medium

[41]. The three *L. acidophilus* NCFM GAPDH forms are encoded by a single gene (Table 1) supporting stress affects post-translational modifications (PTMs). GAPDH is an evolutionary well-conserved moonlighting protein playing various cellular roles [42, 43]. Jungblut et al. [44] and Schluter et al. [45] discussed several GAPDH PTM forms and hypothesized that they exert different physiological functions. In *Eukarya*, for example GAPDH nitrosylated by environmental NO is involved in control of apoptosis and not in energy metabolism [45]. Additionally, GAPDH acts as a redox sensor, and after appropriate PTM, as mediator of DNA repair in response to oxidative stress [46]. The increased relative abundance in H_2O_2 of both 42 kDa GAPDH and DNA repairing enzymes (see below) in *L. acidophilus* NCFM supports that this GAPDH species in bacteria may be a redox sensor eliciting DNA repair mechanisms. GAPDH was also target of oxidative modifications in microorganisms due to the active site cysteine [47]. Thus, higher copy number of GAPDH could secure functions during oxidative stress. Increased abundance of GAPDH under different stresses, such as high selenium exposure, has been demonstrated in other LAB [48].

Two protein species of PK in H_2O_2 occur with increased abundance: spot 72 changing +1.42 fold, and spot 32 change +1.32-fold, although the analysis of the latter was less reliable (Table 1). They are products of the same gene. Different molecular mass (68.25 and 68.75) and pI (5.41 and 5.31) values support they differ in PTM (Fig. 3; Table 1). PK is the last enzyme of the Embden-Meyerhof pathway and plays a central role in the metabolism of microaerophilic LAB. It catalyzes formation of pyruvate and produces ATP by phosphate transfer from phosphoenolpyruvate to ADP. Higher amounts of PK, as in the present study, leads to more ATP by substrate level phosphorylation, improving handling of enhanced energy requirement linked to stress. PK has three functional cysteines susceptible to oxidization, Cys^{358} essential for catalysis, and Cys^{31} and Cys^{424} involved

in subunit interface interaction [49]. Overexpression of PK can compensate for various inactivating reactions and elevated pyruvate production in *Pseudomonas fluorescens* was part of adaptation to H_2O_2 stress [50]. Moreover, the abundance change of PK was confirmed by slight increase in *lba0957* gene expression observed by RT-PCR (Supporting Fig. S2).

4.2 Nucleic acids

Ribonucleoside triphosphate reductase (spot 3; +1.3 fold) was identified with high MASCOT score and 36% sequence coverage. It catalyzes reduction of ribonucleotides to deoxyribonucleotides providing all dNTPs for DNA synthesis and repair and is a target of concerted modulations at molecular and cellular levels [51]. Ribonucleoside triphosphate reductase is coupled with a thioredoxin supplying reducing power for its reaction [52, 53], and the increase in abundance generates building blocks to repair damaged DNA. In *E. coli,* ~~abundance increased of~~ only one of two ribonucleoside triphosphate reductase isoforms increased in abundance during oxidative stress, especially in strains lacking other antioxidant systems [54]. Similarly, in the present investigation one spot (spot 3) increased with good significance ($p < 0.05$), suggesting an analogous regulation in *L. acidophilus* NCFM.

Ribose-p pyrophosphokinase (PRPPS) is present in two forms (spots 13 and 18) derived from different genes (LBA0131 and LBA0224, respectively). According to KEGG they are isozymes having the same specificity (EC 2.7.6.1). They differ significantly both in their molecular mass (40.25 and 35.75) and in pI (6.67 and 6.97). One of them (spot 18) showed increased abundance (+1.37-fold) during oxidative stress caused by H_2O_2. Ribose-p pyrophosphokinase catalyzes transfer of diphosphate from ATP to ribose-5-phosphate (R5P) generating 5-phospho-α-d-ribosyl-1-pyrophosphate (PRPP) involved in nucleotide biosynthesis. PRPPS also has a role in

biosynthesis of histidine, tryptophan and pyridine nucleotide coenzymes [55] of which especially NADH is involved in regeneration of antioxidant enzymes. In rats, oxidative modifications of PRPPS affect enzyme activity and hence PRPP availability [57]. *L. acidophilus* NCFM needs PRPP for repair of ROS-damaged nucleic acids and *de novo* synthesis of DNA.

4.3 General stress proteins

L. acidophilus NCFM heat shock protein GrpE (spot 28) related to DnaK [57, 58] possessing chaperone functions decreased in abundance (−1.30) by H_2O_2 exposure, and expression of its gene (*lba1248*) was decreased dramatically (−35 fold; Supporting Fig. S2). In all living organisms, cellular stress responses are concerted to avoid harmful conditions, adjust reversible modifications and eliminate irreversibly destroyed molecules. To fulfill these purposes, different stress proteins are synthesized as classified according to their main function: i) modifying/protecting enzymes (*e.g.* regulating lipid/protein ratio or unsaturated/saturated lipids ratio in membranes, during stress), ii) refolding proteins (chaperones) or iii) hydrolytic enzymes (proteases including chaperone-proteases degrading destroyed cellular structures). Generally, when damage is light, refolding chaperons are able to restore functional proteins, whereas under harsher conditions degradation by proteolytic enzymes prevails. The present results indicate that part of the chaperone function is limited by oxidative stress in *L. acidophilus* NCFM, whereas the ClpP-ATP-dependent protease-peptidase subunit (spot 21) increased +1.38 fold (good MASCOT score; Table 1). This suggests H_2O_2 caused irreversible protein damage and that ClpP hydrolyzes unfolded or misfolded proteins and recycle amino acids for *de novo* protein synthesis.

4.4 Oxidative stress-related proteins

Cysteine synthase increased during oxidative stress (spot 34; +1.32 fold; p = 0.09) amplifies the cysteine pool and hence availability of cysteine-containing enzymes (e.g. GAPDH and PK) as well as disulfide bonds important for protein stability. This finding agrees with cysteine synthase reported to protect *Staphylococcus aureus* against H_2O_2-induced stress [60]. Secondly, cysteines are at the similar active sites in thioredoxin and glutaredoxin, the most universally used antioxidant systems in living organisms [25, 32, 59], and undergo intramolecular disulfide bond formation in the presence of oxidizing agents. *L. acidophilus* NCFM contains these two members of the disulfide-reducing pathway. Increased abundance of cysteine synthase may be a first step to boost the disulfide-reducing pathway.

Antibiotic biosynthesis monooxygenase (ABM) (spot 52) showing −1.61 fold abundance change ($p < 0.05$) and dramatic decrease in gene expression (*lba0497*, –4.5 fold; Supporting Fig. S2) is a non-heme monooxygenase having a ferredoxin-like fold and catalyzing oxidation by activation of molecular oxygen to the hydroxyl-radical reacting with substrate [61]. In many monooxygenases, e.g. phenol-hydroxylase, uncoupling in the catalytic cycle forms ROS [60] and decreased ABM in H_2O_2-treated *L. acidophilus* NCFM reduce the overall accumulation of free radicals in the environment already rich in ROS.

5 Concluding remarks

Molecular aspects connected with growth of *L. acidophilus* NCFM in an oxidizing environment were disclosed using 2DE-based comparative proteomics. The main metabolic responses to H_2O_2 stress consisted in enhancement of energy metabolism and nucleic acid repair. With regard to general stress responses, proteolytic degradation seems prevalent over refolding (chaperones), probably because H_2O_2 severely damages proteins. Furthermore, specific oxidative-stress related

enzymes were detected, among which cysteine synthase deserves attention due to the role of cysteine residues in protein stability, catalytic sites, and disulfide-reducing pathways in overcoming oxygen stress.

The findings provide new insights into mechanisms of *L. acidophilus* NCFM oxidative stress resistance, anticipated to favor survival in industrial processes. Knowing how levels of proteins and metabolic pathways are regulated during oxidative stress are helpful for i) screening for tolerant strains by gene bio-typing, ii) understanding whether cells are fully adapted and able to survive or, conversely, are stressed and will be suboptimal in the process, and iii) optimizing growth conditions and media to improve fitness during culture. Finally, as suggested [22], identification of crucial stress-related proteins can reveal candidates for manipulation at the gene level to gain stress resistance.

The present global identification of H_2O_2-induced protein abundance changes in *L. acidophilus* NCFM sheds new light on LAB response to oxidative stress. This represents a starting point for future investigations and integration of transcriptome and metabolome analyses. From a practical stand-point, acclimatization procedures can trigger induction of adaptive responses that can increase bacterial tolerance to stress which *per se* can be useful to improve strains for harsh industrial conditions.

Acknowledgements

Karina Jansen and Anne Blicher are thanked for technical assistance and Regina Åris Schürmann for use of the BioScreen instrument. The Center for Advanced Food Studies (LMC) contributed to the acquisition of the mass spectrometer. This work was supported by the Danish Strategic Research Council's Program Committee on Health, Food and Welfare. Elia Calderini and Julia Villarroel benefitted from an Erasmus agreement to perform their master projects at DTU. Hasan Ufuk Celebioglu is grateful to the Republic of Turkey, Ministry of National Education for a PhD scholarship.

Conflict of interest

The authors declare no conflict of interest.

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Captions to figures

Figure 1. BioScreen of *Lactobacillus acidophilus* NCFM (see Methods). Growth under stress: 0.2 (–□–), 0.4 (–△–), 0.6 (–x–), 0.8 (–*–), 1 (–○–), 1.2 (–+–) mM H_2O_2 and control (0 M H_2O_2, –◊–) are displayed.

Figure 2. Growth (solid lines) and pH (broken lines) curves of *Lactobacillus acidophilus* NCFM under control (–◊– and --x--) and stress conditions: 0.8 (–□– and --*--) and 1.2 (–△– and --○--) mM H_2O_2 (A) and of a representative colony of *L. acidophilus* NCFM after a sub-culturing step (B). The arrows show the harvest time of cells for proteome analysis.

Figure 3. Comparison of 2DE spot patterns of intracellular soluble proteomes. **A.** Representative 2DE images of intracellular soluble proteins of *L. acidophilus* NCFM treated with 1.2 mM H_2O_2. **B.** Selected spots illustrating differential relative abundance when treated with oxidative stress. Mean values of fold changes are indicated.

Table 1. Mass spectrometric identification of proteins of *Lactobacillus acidophilus* NCFM with changed abundance in 1.2 mM H_2O_2. pI and MW are obtained by using the algorithm in SameSpots (TotalLab). Fold changes with standard deviations (S.D) are mean values from four biological replicates.

Spot N°	Fold Change ±S.D.	p value	Accession	Protein name	MW/pI theoretical	MW/pI measured	PMF score	E-value	Sequence coverage %
2	+1.34±0.64	0.3	YP_193550	p-enolpyruvate-protein p-transferase PTSI	63.89/4.79	83.5/5.08	160	2.50E-09	44%
3	+1.30±0.14	0.02	YP_192977	ribonucleoside triphosphate reductase	83.98/5.62	87.5/6.08	220	2.5E-15	36%
10	+1.45±0.25	0.09	YP_193604	glyceraldehyde-3-p dehydrogenase	36.64/5.92	43/5.98	195	7.80E-13	52%
11	+1.55±0.55	0.1	YP_193604	glyceraldehyde-3-p dehydrogenase	36.64/5.92	43.25/6.15	227	4.90E-16	61%
12	+1.30±0.20	0.05	YP_193604	glyceraldehyde-3-p dehydrogenase	36.64/5.92	42/6.51	232	1.6E-16	67%
13	+1.34±0.59	0.3	YP_193063	ribose-p pyrokinase	36.71/5.81	40.25/6.67	125	7.8E-06	29%
14	+1.42±0.72	0.3	YP_193604	glyceraldehyde-3-p dehydrogenase	36.64/5.92	42.25/6.72	206	6.2E-14	58%
18	+1.37±0.30	0.09	YP_193150	ribose-p-pyrophosphokinase	35.68/6.01	35.75/6.97	77	0.26	28%
21	+1.38±0.35	0.1	WP_003547145	ATP-dependent protease peptidase subunit	18.76/5.21	22/5.79	102	0.0016	44%
26	–1.43±0.31	0.1	YP_194367	transcriptional elongation factor	17.31/4.54	28.75/4.38	93	0.013	58%

27	−1.75±0.12	0.01	YP_193604	glyceraldehyde-3-p dehydrogenase	36.64/5.92	27.25/5.89	186	6.2E-12	45%
28	−1.30±0.04	0.09	YP_194112	heat shock protein GrpE	22.04/5.79	27/6.29	94	0.0099	41%
32	+1.32±0.36	0.17	YP_193840	pyruvate kinase	63.14/5.23	68.25/5.41	222	1.2E-15	38%
34	+1.32±0.26	0.09	YP_194102	cysteine synthase	32.66/6.99	35/8.19	164	9.8E-10	62%
35	+1.31±0.37	0.19	YP_194428	phenylalanyl-tRNA synthetase subunit beta	23.73/4.75	33.75/4.92	132	1.6E-06	72%
			YP_194361	N-acetylglucosamine kinase	32.9/4.76		101	0.002	42%
39	+1.56±0.9	0.3	YP_193706	cell division protein FtsZ	48.12/4.55	66.5/4.34	140	2.50E-07	48%
52	−1.61±0.22	0.04	WP_011254170	antibiotic biosynthesis monooxygenase	24.69/4.85	36.25/4.99	141	2E-07	46%
							91		30%
64	−1.47±0.17	0.2	YP_194106	adenine phosphoribosyltransferase	19.34/6.1	30/6.72	147	4.9E-08	60%
72	1.42±0.31	0.07	YP_193840	pyruvate kinase	63.14/5.23	68.75/5.31	217	4.9E-15	38%