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Impact of aerobic and respirative life-style on *Lactobacillus casei* N87 proteome

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ABSTRACT

Lactic acid bacteria (LAB) are used as starter, adjunct and/or probiotic cultures in fermented foods. Several species are recognized as oxygen-tolerant anaerobes, and aerobic and respiratory cultivations may provide them with physiological and technological benefits.

In this light, mechanisms involved in the adaptation to aerobic and respiratory (supplementation with heme and menaquinone) growth conditions of the O_2-tolerant strain *Lactobacillus casei* N87 were investigated by proteomics. In fact, in this bacterial strain, respiration induced an increase in biomass yield and robustness to oxidative, long-term starvation and freeze-drying stresses, while high concentrations of dissolved O_2 (dO_2 60%) negatively affected its growth and cell survival.

Proteomic results well paralleled with physiological and metabolic features and clearly showed that aerobic life-style led to a higher abundance of several proteins involved in carbohydrate metabolism and stress response mechanisms and, concurrently, impaired the biosynthesis of proteins involved in nucleic acid formation and translation processes, thus providing evidence at molecular level of the significant damage to *L.casei* N87 fitness. On the contrary, the activation of respiratory pathways due to heme and menaquinone supplementation, led to a decreased amount of chaperones and other stress related proteins. These findings confirmed that respiration reduced oxidative stress condition, allowing to positively modulate the central carbohydrate and energy metabolism and improve growth and stress tolerance features.

Results of this study could be potentially functional to develop competitive adjunct and probiotic cultures effectively focused on the improvement of quality of fermented foods and the promotion of human health.

1. Introduction

Lactic acid bacteria (LAB) are used as starter, adjunct and/or probiotic cultures to improve quality of fermented foods and promote human health. LAB are recognized as oxygen-tolerant anaerobes that use fermentative pathways for biomass and energy production. Interestingly, several LAB strains are able to grow in presence of oxygen, activating alternative pathways to conventional anaerobic metabolism (Lechardeur et al., 2011; Pedersen et al., 2012; Zotta et al., 2017).

Aerobic growth significantly changes the central carbon metabolism rerouting pyruvate away from lactate or lactate and ethanol in homofermentative and heterofermentative strains, respectively, and other metabolites (i.e. acetate, acetoin, diacetyl) may be accumulated due to the activation of O_2-dependent pathways (Zotta et al., 2017).

Moreover, in some strains, the aerobic cultivation carried out in presence of exogenous heme and menaquinone may induce the activation of a minimal respiratory chain consisting of an electron donor (NADH dehydrogenase), a quinone electron shuttle (menaquinone) and a terminal oxidase (heme-binding cytochrome *bd* oxidase, CydAB) (Pedersen et al., 2012; Zotta et al., 2017). The shift from anaerobic fermentative metabolism to aerobic respiration may result in several physiological advantages, including increase in biomass yield and robustness to oxidative, long-term starvation and freeze-drying stresses

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(Zotta et al., 2017). The activation of respiratory chain, in fact, may contribute to energy gain (extra ATP generation) and depletion of intracellular O_2 (used as final electron acceptor), promoting growth rates and final cell densities. Besides, the increased activity of O_2-removing enzymes and reactive oxygen species (ROS)-scavenging enzymes in oxygen-tolerant and respiratory phenotypes improves survival when exposed to radical and non-radical ROS (Maresca et al., 2018; Zotta et al., 2017, 2018).

The capability to activate a minimal electron transport chain (ETC) has been largely investigated in homofermentative LAB species, including the dairy-associated *Lactococcus lactis*, and the more versatile strains of the *Lactobacillus casei* and *Lactobacillus plantarum* groups (Pedersen et al., 2012; Zotta et al., 2017). More recently, the capability to grow under aerobic and respirative life-style has been demonstrated also in some heterofermentative LAB (Zotta et al., 2017, 2018). These studies have principally addressed the effect of unsupplemented aerobiosis and aerobic respiration on the growth performances and robustness of LAB, highlighting significant differences with cells grown anaerobically. The activation and regulation of O_2-mediated pathways have been also investigated at transcriptional levels, defining factors that affected the expression level of genes encoding for key enzymes of aerobic and respirative metabolisms and the oxidative stress tolerance (Ricciardi et al., 2018; Zotta et al., 2017). High-throughput omics strategies have been applied in a few studies to gain a global picture of the molecular basis of adaptation to aerobiosis and/or respiration conditions in LAB strains. In particular, the impact of aeration and heme-activated respiration on *Lc. lactis* cells was studied at transcriptomic level by Pedersen et al. (2008). Stevens et al. (2008) and Eikmeyer et al. (2015) analyzed, respectively, the O_2-responsive transcriptome of *L. plantarum* and *L. buchneri* grown under unsupplemented aerobic conditions, while Larsen et al. (2016) evaluated the effect of different O_2 concentrations on milk fermentation and transcriptome profile of *Lc. lactis*.

As to proteomics, Vido et al. (2004) examined changes of *Lc. lactis* MG1363 proteome grown under static, unsupplemented aerated and heme-supplemented aerated conditions (non-buffered medium and uncontrolled conditions), suggesting that respirative-promoting growth remarkably affected the synthesis of proteins related to carbohydrate and nitrogen metabolism. Successively, Mazzeo et al. (2012) investigated the effect of oxygen (uncontrolled levels) and the role of carbon catabolite control protein A (CcpA) on the proteome of *L. plantarum* WCFS1, confirming that aerated conditions and CcpA-dependent regulation led to significant changes in *L. plantarum* metabolism.

Among O_2-tolerant LAB, the strain *L. casei* N87 has been characterized for its capability to grow under aerobic and respiratory promoting conditions at physiological (e.g. increased biomass production and stress robustness in respiratory promoting conditions; capability to consume oxygen), transcriptional (e.g. expression of genes involved in the activation of aerobic and respirative pathways) and genomic (genome sequencing to assess the occurrence of genes involved in aerobic and respiratory metabolism, oxygen consumption and ROS degradation) levels (Ianniello et al., 2015, 2016; Ricciardi et al., 2018; Zotta et al., 2014, 2016a, 2016b) However, although the benefits of aerobic respiration have been previously proven for this strain (increased biomass and stress robustness, production of secondary metabolites and antioxidant enzymes), Ianniello et al. (2016) demonstrated that the oxygen levels strongly affect the fitness and stress survival of *L. casei* N87 and confirmed that supplementation with heme and menaquinone might alleviate the oxidative damages due to boosted aerated conditions.

The present proteomic study was aimed to elucidate the mechanisms involved in the adaptation to aerobic and respiratory growths of the respiration-competent *L. casei* N87, thus providing molecular bases to the physiological evidence already reported (Ianniello et al., 2016). The effect of incubation atmosphere (anaerobic and controlled aerobic conditions) and/or supplementation with heme and menaquinone on the proteome of *L. casei* N87 was analyzed in exponential and stationary phase cells. To the best of our knowledge, this represents the first proteomic study investigating aerobiosis and respiration (under controlled growth conditions) life-style in LAB.

As *L. casei* N87 grown under respiration has been advantageously tested to improve the production of some fermented foods (Reale et al., 2016a, 2016b), the understanding of adaptation mechanisms to aerated and respirative conditions may be useful to increase the robustness and competitiveness of respirative phenotypes in several foods and human health related-applications.

2. Materials and methods

2.1. Strain and culture conditions

L. casei N87, used in this study, was isolated from infant faces (Iacumin et al., 2015; Zotta et al., 2014). The strain was maintained as freeze-dried stock in reconstituted 11% (w/v) Skim Milk containing 0.1% (w/v) ascorbic acid, in the culture collection of the Laboratory of Industrial Microbiology, University of Basilicata, and was routinely propagated in Weissella Medium Broth, pH 6.8 (WMB; Zotta et al., 2012), for 16 h at 37 °C.

For proteomic analyses, *L. casei* N87 was cultivated (3 L bioreactor; Applikon, Schiedam, the Netherlands) in mWMB broth, at pH 6.5 and 37 °C, under controlled anaerobic (AN; nitrogen flow at 0.1 vol/vol/min), aerobic (AE; air flow to reach 60% of dissolved oxygen, dO_2) and respiratory (RS; 60% dO_2, supplementation of mWMB with 2.5 μg/mL hemin and 1 μg/mL menaquinone) conditions as described in Ianniello et al. (2016). Hemin and menaquinone (Sigma-Aldrich, St. Louis, MO, USA) solutions used for respiratory growth were prepared, respectively, in 0.05 mM NaOH and in 99% ethanol; the same volumes of solvents (NaOH and ethanol) were added to mWMB used for anaerobic and unsupplemented aerobic cultivations. Dissolved oxygen concentration was measured using a polarographic electrode (Applisens, Applikon) and was automatically controlled (set point 60%; *ez*Control controller, Applikon) by varying air flow and stirrer speed as described in Ianniello et al. (2016). pH set-point and foaming were automatically controlled by adding sterile 4 eq/L NaOH and sterile 5% (v/v) Antifoam A solution, respectively. Growth kinetics during batch cultivations were investigated in detail and modelled in Ianniello et al. (2016). However, relevant data on the main growth parameters are reported in Supplementary Table 1.

For protein extraction, cell suspensions were aseptically withdrawn in exponential (Exp; optical density at 650 nm, OD_{650}, of 1.0, 5 h of incubation) and stationary (Stat; 24 h of incubation) phases, harvested by centrifugation (10,000 × *g* for 5 min at 4 °C), washed twice with sterile 50 mM Tris-HCl pH 7.5 and frozen at −80 °C until use. Two biological replicates of each growth condition were carried out.

2.2. Protein extraction

Bacterial cells were thawed, standardized at OD_{650} of 100 in a lysis buffer (Tris-HCl 50 mM, lysozyme 2 mg/mL, mutanolysin 50 U, pH 7.5) containing the protease inhibitor cocktail (GE Healthcare, Uppsala, Sweden), and destroyed mechanically. In detail, 400 μL of cell suspension were added of glass beads (0.15–0.21 mm diameter, Sigma-Aldrich), vortexed for 3 min, incubated for 2 h at 37 °C, vortexed for 3 min and sonicated for 5 min on ice with an ultrasonic homogenizer (100 watts power; 100% amplitude, 0.8 cycle; Labsonic M, Sartorius, Göttingen, Germany) using a microtip probe of 0.5 mm diameter. Supernatants were recovered by centrifugation (16,000 × *g* for 30 min at 4 °C) and protein concentration was measured with a Bradford-based protein assay kit (Bio-Rad, Hercules, CA, USA).

2.3. Two-dimensional gel electrophoresis (2-DE) of extracted proteins

Extracted proteins (800 μg) were precipitated using a methanol/chloroform protocol (Wessel and Flugge, 1984), solubilized in the rehydration buffer (8 M Urea, 2% v/v CHAPS, 50 mM dithiothreitol (DTT), 2% v/v IPG buffer 3–10, 0.002% w/v bromophenol blue) and loaded on 24-cm IPG strips pH 4–7 (GE Healthcare). Isoelectric focusing (IEF) was carried out at 20 °C and at total 65 kVh using an Ettan IPGphor apparatus (GE Healthcare).

After focusing, IPG strips were equilibrated for 25 min in the equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, 0.002% w/v bromophenol blue) containing 65 mM DTT and for further 25 min in the equilibration buffer containing 70 mM iodoacetamide.

Equilibrated IPG strips were sealed on the top of 1.5 mm thick polyacrylamide gels (12.5% T, 3.3% C) and the second dimension gel electrophoresis (SDS-PAGE) was carried out on a vertical Ettan DALTsix Electrophoresis System (GE Healthcare) at 15 °C and constant current of 18 mA/gel. Gels were stained for 2 h with the Bio-Safe colloidal Coomassie Blue G-250 (Bio-Rad) and digitalized using a GS-800 calibrated densitometer (Bio-Rad). Two independent technical replicates were performed for each sample, so that four different gels were run for each growth condition.

2.4. Image analysis and spot quantification

Gel images were analyzed with Image-Master 2D Platinum v.6 software (GE Healthcare) as described by Lippolis et al. (2011). Briefly, spot detection was carried out using the optimized setting values for spot volume, spot area and saliency, determined by applying real-time filters in order to minimize the detection of artifacts. After spot detection, manual spot editing was carried out to remove artifacts that escaped the filtering process. Four replicate gels (two biological replicates run in duplicate) for each growth condition (AN-Exp, AN-Stat, AE-Exp, AE-Stat, RS-Exp, RS-Stat) were used to create the six match sets (one for each growth condition). Normalized spot volume (% vol, i.e. digitized staining intensity integrated over the area of the individual spot divided by the sum of volume of all spots in the gel and multiplied by 100) was used as variable for statistical analyses and spot quantification.

Analysis of variance (ANOVA) with Bonferroni post-hoc correction was used to perform pairwise comparisons of normalized spot volumes in all growth conditions (AN, AE, RS) and phases (Exp, Stat). A statistical filter ($p < 0.05$, ANOVA p-value cut-off) in at least one of the compared conditions was applied to detect spots showing significantly different volumes. Changes in protein abundance (fold change, FC) were calculated as the ratio between the average normalized spot volumes measured in the two compared conditions for up-regulated proteins and as the negative reciprocal values for down-regulated proteins. Spots showing a fold change $\geq \pm 2$ (see notes to Table 1) when comparing AE/AN, RS/AN and RS/AE in at least one growth phase were excised and proteins contained in these spots were identified (Supplementary Fig. 1, Supplementary Table 2). The fold change of proteins contained in more than one spot was calculated summing the average normalized spot volume of all spots containing the same protein. Spots showing differences in average normalized volumes exclusively due to the effect of growth phase were not further analyzed, as this aspect was not considered of interest for this study.

2.5. Protein identification and functional classification

Spots of interest were excised from 2-DE gels and in-gel tryptic digestion was carried out as already described (Tosco et al., 2005). Protein identification was achieved by peptide mass fingerprint (PMF) strategy and nanoliquid chromatography coupled to tandem mass spectrometric experiments (nanoESI-LC-MS/MS). Details of protein identification are reported in Supplementary Table 3. As to the PMF strategy, tryptic peptide mixtures (1 μL) were loaded on the mass spectrometer sample plate, mixed with the matrix (1 μL of α cyano 4 hydroxycinnamic acid solution (10 mg/mL in 0.1% trifluoroacetic acid/50% acetonitrile) containing angiotensin (25 fmol/μL) and adrenocorticotropic hormone (ACTH) fragment 18–39 (125 fmol/μL)) and analyzed on a MALDI-TOF-MS Voyager DE PRO mass spectrometer (ABSciex, Foster City, CA, USA) operating in positive-ion reflectron mode. Mass spectra were calibrated using as internal standards the monoisotopic peaks of angiotensin (m/z 931.5154) and ACTH (m/z 2465.1989) and data were processed using the DataExplorer 5.1 software (ABSciex). Protein identification was achieved based on the mass spectral data using the Mascot Wizard tool for searches against the NCBInr database (http://www.matrixscience.com/). Parameters for all searches were as follows: all entries as taxonomic category, trypsin as enzyme, carbamidomethyl as fixed modification for cysteine residues, oxidation as variable modification for methionine residues, up to one missed cleavage and up to 50 ppm as mass tolerance.

As to the nanoESI-LC-MS/MS experiments, tryptic peptide mixtures were analyzed using a Q-Exactive™ mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) interfaced with an UltiMate 3000 RSLCnano LC system (Thermo Fisher Scientific). Peptide mixtures were concentrated and desalted on a trapping pre-column (Acclaim PepMap C18, 300 μm × 5 mm nanoViper, 5 μm, 100 Å, Thermo Fisher Scientific), using 0.05% formic acid and 2% acetonitrile at a flow rate of 10 μL/min. The peptide separation was performed at 35 °C using a C18 column (Acclaim Easy Spray PepMap RSLC C18, 75 μm × 15 cm nanoViper, 3 μm, 100 Å, Thermo Fisher Scientific), using as eluent A 0.1% formic acid and as eluent B 80% acetonitrile in 0.08% formic acid and a linear gradient from 4 to 50% B over 30 min, hold for 6 min, from 50 to 90% B over 1 min, hold for 10 min before column re-equilibration to 4% B.

Mass spectra were acquired in the m/z range 350–1600. Data acquisition was performed in a data dependent mode Full MS/ddMS^2, enabling the acquisition of MS/MS spectra for the ten most intense precursor ions (top ten) and dynamic exclusion of 30 s. Resolution was set to 70.000 for MS spectra acquisition and 17.500 for MS/MS spectra acquisition.

MS data processing for protein identification was performed using ProteomeDiscoverer™ platform (version 2.1.0.81; Thermo Fisher Scientific), interfaced with the Sequest HT Search Engine server (University of Washington, United States) (Washburn et al., 2001). The parameters used for the data-base searches were as follows: *Lactobacillus casei* protein database (taxon 1582, downloaded from NCBInr on May 2018) and a contaminant protein data-base (Contaminantcombinedcontaminant_finalfasta, provided by the manufacturer), trypsin as proteolytic enzyme, up to two missed cleavages, carbamidomethyl as fixed modification for cysteine residues, oxidation of methionine residues and formation of pyro-glutamic acid of N-terminal glutamine residues as dynamic modifications, 20 ppm mass tolerance for precursor ions and 0.02 Da mass tolerance for MS/MS fragments. Results were filtered for high confident peptides and proteins (False Discovery Rate 0.01%).

Identified proteins were classified on the basis of their biological functions using the database of Clusters of Orthologous Groups of Proteins (COGs) (http://clovr.org/docs/clusters-of-orthologous-groups-cogs/) (Tatusov et al., 2000).

Integrated function and protein-protein interactions were explored using the database and web resource STRING v.9.1 (Search Tool for the Retrieval of Interacting Genes/Proteins, http://string-db.org/). Active prediction methods used in our analysis were neighbourhood, co-expression, experiments, gene fusions, gene co-occurrence, databases and text mining, using high confidence value (0.700) (Jensen et al., 2009).

2.6. Statistical analyses

All statistical analyses (analysis of variance, multiple mean comparison, Pearson's correlations) and graphs were performed using Systat 13.0 for Windows (Systat Software Inc., San Jose, CA, USA). The Matrix

Hierarchical Cluster Analysis (normalized data, Euclidean distance, McQuitty's linkage WPGMA method) was obtained with PermutMatrix program v. 1.9.3 (LIRMM, France). Clustering was generated by using the Weighted Pair Group Method with Arithmetic Mean (WPGMA) and Euclidean distance similarity. For proteins present only in one of the two compared conditions (Table 1) an arbitrary FC of ± 10 was used. A z-value transformation was applied to all variables.

3. Results

Molecular mechanisms involved in the adaptation of *L. casei* N87 to unsupplemented aerobiosis (AE; 60% dO_2) and respiratory growth (RS; aerobic growth with 60% dO_2, hemin and menaquinone supplementation) were investigated by proteomics. The above conditions were selected on the basis of results reported by Ianniello et al. (2016) as those providing the greater differences in growth performances and stress survival compared to conventional anaerobic cultivation. Specifically, the unsupplemented aerobiosis significantly impaired the fitness (growth rate, biomass production, robustness) of *L. casei* N87, while respiration resulted in several physiological advantages.

The image analysis of 2-DE maps allowed detecting 359 protein spots. As the aim of this study was to elucidate the effect of oxygen and/or heme and menaquinone supplementation on the proteome of *L. casei* N87, we calculated protein fold changes by comparing AE vs AN (effect of oxygen), RS vs AN (effect of oxygen and supplementation), RS vs AE (effect of supplementation) in both growth phases.

Image analysis of the 2-DE maps obtained from the proteome of *L. casei* N87 grown under the different conditions highlighted fifty-seven spots showing significant differences in their average volume (fold change ≥ ± 2) in at least one of the considered comparisons and mass spectrometric analyses led to the identification of forty-two proteins (Supplementary Fig. 1). However, fold changes ≥ ± 1.5 were also considered to give biologically relevant information on the variation in the amount of proteins contained in these spots under the different experimental conditions. Proteomic results have been summarized in the Venn diagrams reported in Fig. 1 and the identified proteins, reported in Table 1, were included in further functional, statistical and graphical analyses.

Table 1
Regulation of protein abundance in the proteome of *Lactobacillus casei* N87 cultivated in anaerobic, aerobic and respiratory conditions (in exponential and stationary phases) and functional classification of identified proteins according to COG database.

Spot ID	Protein name	MW (kDa)	pI	GenBank accession number[a]	Gene name	Fold change[b]			Fold change[b]		
						Exponential growth phase			Stationary growth phase		
						AE/AN	RS/AN	RS/AE	AE/AN	RS/AN	RS/AE
Carbohydrate transport and metabolism (G)											
99/100/101	Phosphoketolase	89.6	5.41	KLI75291.1	*xpk*	**+4.6**[c]	**+3.1**[c]	-1.5[c]	**+3.2**[c]	**+2.7**[c]	-1.2[c]
291	6-Phosphogluconate dehydrogenase	52.1	5.71	KLI74764.1	*gndA*	**+2.0**	−1.1	**−2.2**	+1.3	+1.3	+1.0
332	6-Phosphogluconolactonase	37.5	5.27	KLI74918.1	*ywcC*	+1.0	+1.0	−1.1	**+2.2**	+1.8	−1.2
427	Citrate lyase subunit beta	31.4	4.98	KLI76738.1	*citE*	**−2.1**	**−2.0**	+1.0	**−2.9**	**−7.9**	**−2.7**
444	Fructose-1,6-bisphosphate aldolase, class II	31.5	5.21	KLI74589.1	*fba*	+1.2	+1.0	−1.2	**+2.1**	+1.4	−1.5
Energy production and conversion (C)											
196	Phosphoenolpyruvate carboxykinase	60.7	5.65	KLI74891.1	*pck*	−1.6	−1.3	+1.3	**AN**	−1.9	**AE**
227	ATP synthase subunit alpha	55.1	5.39	KLI76251.1	*atpA*	+1.7	−1.1	−1.8	**−2.2**	**+2.0**	**+4.4**
237	Pyruvate dehydrogenase complex E3 component	49.0	6.06	KLI76379.1	*pdhD*	**+2.4**	**+2.9**	+1.2	+1.8	**+2.5**	+1.4
248/252/255	FAD-dependent oxidoreductase	48.9	5.05	KLI74720.1	–	**+3.9**[c]	**+4.7**[c]	+1.2[c]	**+4.5**[c]	**+5.4**[c]	+1.2[c]
645	Oxidoreductase	26.1	6.29	KLI77021.1	–	**+3.9**	**AN**	**AE**	**+5.4**	+1.7	**−3.2**
419	L-2-hydroxyisocaproate dehydrogenase	32.4	5.53	KLI75788.1	*hicD3*	**−3.6**	−1.1	**+3.3**	**−3.1**	−1.3	**+2.4**
Amino acid transport and metabolism (E)											
202	Oligoendopeptidase	67.6	5.24	KLI76133.1	*pepF2*	−1.1	+1.0	+1.1	**+2.3**	+1.1	**−2.2**
288	Type I glutamate–ammonia ligase	50.5	5.42	KLI76676.1	*glnA*	+1.1	−1.1	−1.1	**−2.9**	**−2.1**	+1.3
366	Aromatic amino acid aminotransferase	42.0	6.38	KLI75984.1	*araT*	+1.5	**−2.0**	**−2.9**	+1.2	−1.6	**−2.0**
429	Cysteine synthase A	32.6	5.53	KLI74556.1	*cysK1*	+1.7	−1.3	**−2.3**	**+3.3**	+1.8	−1.8
Nucleotide transport and metabolism (F)											
215/267	IMP dehydrogenase	52.5	6.68	KLI75251.1	*guaB*	−1.6[c]	+1.1[c]	+1.7[c]	**−3.5**[c]	−1.9[c]	+1.8[c]
219	GMP synthetase (glutamine-hydrolyzing)	57.9	5.42	KLI76784.1	*guaA*	−1.3	+1.1	+1.4	**−2.8**	−1.2	**+2.4**
644	Bifunctional pyr operon transcriptional regulator/ uracil phosphoribosyltransferase	20.5	6.07	KLI76507.1	*upp*	−1.5	−1.4	+1.1	**−3.9**	**−3.1**	+1.3
Amino acid transport and metabolism (E)/nucleotide transport and metabolism (F)											
64	Carbamoyl phosphate synthase large subunit	116.2	5.26	KLI76502.1	*carB*	−1.5	+1.2	+1.7	**−4.3**	**−2.7**	+1.6
Lipid transport and metabolism (I)											
343	3-Ketoacyl-CoA thiolase	40.6	5.24	KLI74862.1	*thlA*	**AN**	−1.5	**RS**	**AN**	−1.3	**RS**
Cell wall/membrane/envelope biogenesis (M)											
408	dTDP-glucose 4.6 dehydratase	38.6	5.96	KLI76809.1	*rmlB*	−1.5	−1.1	+1.3	**−2.3**	−1.4	+1.6
461	dTDP-4-dehydrorhamnose 3.5-epimerase	21.5	5.39	KLI76810.1	*rmlC*	−1.8	−1.1	+1.7	**AN**	−1.3	**RS**
Secondary metabolites biosynthesis, transport and catabolism (Q)											
214	D-alanine-poly(phosphoribitol) ligase subunit 1	56.3	5.33	KLI74962.1	*dltA*	−1.4	+1.2	+1.7	+1.0	**AN**	**AE**
426/430/642	2.5-Diketo-D-gluconic acid reductase	31.7	6.40	KLI75243.1	–	+1.9[c]	+1.2[c]	−1.6[c]	**+2.7**[c]	+1.7[c]	−1.6[c]
Intracellular trafficking, secretion, and vesicular transport (U)											
95/96	Protein translocase subunit SecA	89.5	5.72	KLI76056.1	*secA*	−1.7[c]	−1.1[c]	+1.5[c]	**−3.5**[c]	**−2.0**[c]	+1.8[c]
Translation, ribosomal structure and biogenesis (J)											
128	Threonine-tRNA ligase	48.6	6.10	KLI74784.1	*thrS*	−1.5	−1.3	+1.2	**−2.1**	−1.4	+1.6
222/224/228	Glutamyl-tRNA amidotransferase	53.4	5.58	KLI76168.1	*gatB*	−1.1[c]	−1.1[c]	−1.0[c]	**−3.7**[c]	+1.1[c]	**+4.2**[c]

(*continued on next page*)

Table 1 (*continued*)

Spot ID	Protein name	MW (kDa)	pI	GenBank accession number[a]	Gene name	Fold change[b]			Fold change[b]		
						Exponential growth phase			Stationary growth phase		
						AE/AN	RS/AN	RS/AE	AE/AN	RS/AN	RS/AE
253	Serine-tRNA ligase	48.6	6.10	KLI76719.1	*serS*	−1.5	−1.0	+1.5	**−3.2**	−1.9	+1.7
258	30S ribosomal protein S1	47.6	5.04	KLI76442.1	*rspA*	−1.8	−1.0	+1.7	**−2.9**	**−2.8**	+1.0
403/405	30S ribosomal protein S2	29.5	5.36	KLI76615.1	*rspB*	**−4.8**[c]	+1.0[c]	**+4.9**[c]	−1.4[c]	**−2.8**[c]	**−2.1**[c]
465	50S ribosomal protein L10	18.2	5.03	KLI77137.1	*rplJ*	**−3.4**	−1.1	**+2.9**	−1.1	−1.4	−1.3
Transcription (K)											
238	Transcription termination/antitermination protein NusA	44.8	4.55	KLI76605.1	*nusA*	+1.1	+1.0	−1.0	**+2.4**	**+2.0**	−1.2
Signal transduction mechanisms (T)											
122	GTP-binding protein TypA	68.0	5.33	KLI76383.1	*bipA*	**−3.1**	−1.4	**+2.3**	+1.1	+1.3	+1.2
Replication, recombination and repair (L)											
126	NAD-dependent DNA ligase LigA	74.1	6.37	KLI76164.1	*ligA*	−1.4	+1.2	+1.6	**−4.4**	**−2.0**	**+2.2**
Posttranslational modification, protein turnover, chaperones (O)											
78	ClpB family protein	96.8	5.58	KLI76420.1	*clpB*	**+2.5**	+1.2	**−2.0**	+1.1	**AN**	**AE**
106/119	Clp protease ClpX	77.1	5.76	KLI74846.1	*clpX*	+1.5[c]	−1.2[c]	−1.8[c]	−1.3[c]	**−2.2**[c]	−1.7[c]
137	Molecular chaperone DnaK	67.5	4.98	KLI76595.1	*dnaK*	+1.6	−1.1	−1.8	**+3.6**	+1.3	**−2.7**
150	Peptidase M13	71.1	5.27	KLI76521.1	*pepO*	**−2.4**	−1.5	+1.6	−1.0	+1.1	+1.1
175/180/184/294	Chaperonin GroL	57.5	5.02	KLI75926.1	*groL*	**+2.0**[c]	+1.2[c]	−1.7[c]	**+2.3**[c]	+1.5[c]	−1.5[c]
187	Trigger factor	49.8	4.53	KLI76404.1	*tig*	+1.2	+1.0	−1.2	**+2.0**	+1.5	−1.4
Function unknown (S)											
466	Alkaline-shock protein	15.7	4.81	KLI75266.1	*asp2*	**−2.6**	**−4.2**	−1.6	**AN**	**−2.8**	**RS**
690	Conserved hypothetical protein	18.3	5.36	KLI75440.1	–	−1.9	**AN**	**AE**	**AN**	**AN**	**UD**

Detailed information for protein spot quantification and protein identification are reported in Supplementary Table 2 and Supplementary Table 3, respectively.

[a] Accession number retrieved from the whole genome shotgun sequencing project of *Lactobacillus casei* N87 (GenBank project LCUN00000000.1).

[b] Fold Changes in protein abundance are reported as the ratio between the average normalized spot volumes measured in the compared growth conditions (AE/AN or RS/AN or RS/AE, in both exponential and stationary growth phase) for up-regulated proteins; the negative reciprocal values are reported for down-regulated proteins. For the spots detected only in one of the two compared conditions, the name of this sample (AN or AE or RS) is reported. UD indicates spots undetectable in both compared conditions. AN: anaerobic growth; AE: aerobic growth; RS: respiratory growth. Bold values indicate a fold change ≥ 2.0 or ≤ -2.0 ($p < 0.05$; ANOVA post-hoc test, Bonferroni protection).

[c] Fold change for proteins contained in more than one spot was calculated summing the average normalized spot volume of all the spots containing the same protein.

3.1. Effect of oxygen on the proteome of L. casei N87

The presence of O_2 (unsupplemented aerobiosis vs anaerobiosis; AE/AN in Table 1) significantly affected the abundance of specific proteins in *L. casei* N87. Thirteen proteins were present in a higher amount and nineteen proteins were present in a lower amount, in exponential phase cells, and thirteen proteins and twenty proteins were more and less abundant, respectively, in stationary phase cells.

Proteomic analyses also showed that twenty-four proteins were differentially abundant under aerobiosis in both growth phases. All of them showed the same trend of abundance (eight proteins were more abundant and fifteen proteins were less abundant), except for ATP F0-F1 synthase subunit alpha whose amount increased in exponential phase and decreased in the stationary one respect to their controls. Seven proteins changed their amount only in exponential phase (three proteins were more abundant and four proteins were less abundant) and nine proteins changed their amount only in stationary phase (five proteins were more abundant and four proteins were less abundant) (Fig. 1).

In both growth phases, the up-regulated proteins principally belonged to COG categories: energy production and conversion (C), carbohydrate transport and metabolism (G) and post-translational modification, protein turnover and chaperones (O). The presence of O_2, instead, negatively affected the abundance of proteins involved in translation, ribosomal structure and biogenesis (J), nucleotide transport and metabolism (F) and cell wall/membrane/envelope biogenesis (M) (Table 1).

Aerobic cultivation significantly reduced the amount of L-2-hydroxyisocaproate dehydrogenase (hicD3) (L-lactate dehydrogenase, as deduced from NCBInr), the key enzyme of fermentative homolactic pathway and induced the synthesis of phosphoketolase and 6-phosphogluconate dehydrogenase, the key enzymes of phosphoketolase and pentose phosphate pathways in LAB, respectively. The pyruvate dehydrogenase complex E3 component, and FAD-dependent oxidoreductase were also up-regulated in aerobically growing cells. The abundance of phosphoenolpyruvate carboxykinase and citrate lyase, which lead to oxaloacetate production from phosphoenolpyruvate (PEP) and citrate, respectively, significantly decreased during aerobic growth. On the contrary, the amount of oxidoreductase (NAD(P)-dependent oxidoreductase as deduced from NCBInr) was strongly increased in aerobically growing cells.

Aerobic cultivation significantly increased the production of ATP-dependent proteases ClpB and ClpX (only in exponential phase) and chaperones DnaK and GroEL, but lowered the amount of alkaline shock protein.

The abundance of the aminoacyl-tRNA synthetases (i.e. glutamyl-tRNA amidotransferase, serine-tRNA ligase, threonine-tRNA ligase) and ribosomal proteins was down-regulated in aerobic conditions. The amount of carbamoyl phosphate synthase (carB), involved in amino acid and nucleotide metabolism, was strongly reduced in stationary cells; concurrently, the abundance of proteins involved in pyrimidine (i.e. uracil phosphoribosyltransferase, upp) and purine (i.e. GMP synthase, guaA; inosine-5′-monophosphate dehydrogenase, guaB) biosynthesis was significantly lower in the same conditions. Aerated

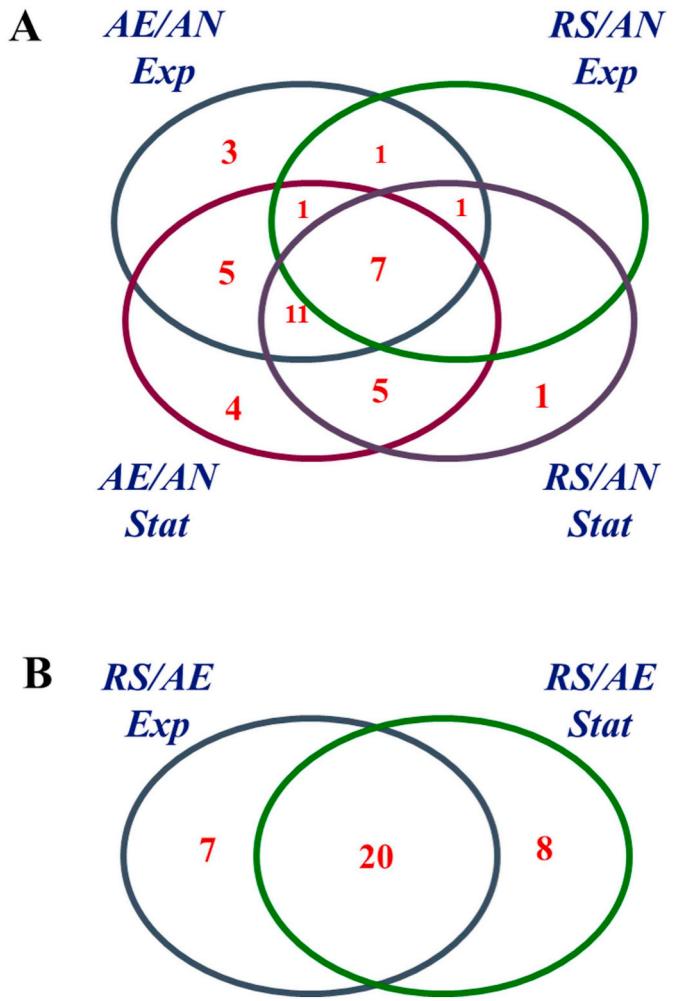

Fig. 1. Venn diagrams summarizing proteomic results. (A) The number of proteins showing significant changes in abundance in the Image Analyses AE/AN and RS/AN under the two growth phases is reported. Three proteins changing their abundance in both Image Analyses AE/AN_Exp and RS/AN_Stat are not included in the diagram (see Table 1). (B) The number of proteins showing significant changes in abundance in the Image Analyses RS/AE under the two growth phases is reported.

growth also decreased the amount of dTDP-glucose 4,6-dehydratase and dTDP-4-dehydrorhamnose 3,5-epimerase, involved in cell wall biosynthesis and EPS production.

3.2. Effect of oxygen and supplementation on the proteome of L. casei N87

Contrarily to the unsupplemented aerobiosis, in exponential phase the abundance of few proteins was modulated by the respiratory conditions (RS/AN in Table 1). In fact, only three proteins were significantly up-regulated, while seven proteins were down-regulated. In stationary phase, the protein pattern was more severely affected as eleven and seventeen proteins were up- and down-regulated, respectively. In particular, seven proteins were differentially abundant in both growth phases, showing the same trend of regulation, while oxidoreductase was less abundant in exponential phase and more abundant in stationary phase, respectively. In addition, twenty proteins changed their amount only in the stationary phase and two proteins, 3-ketoacyl-CoA thiolase and peptidase M13, decreased their relative abundance only in the exponential phase (Fig. 1).

Similarly to aerobic growth, in stationary phase, respiration led to an increased amount of several proteins involved in energy and carbohydrate metabolism and decreased the abundance of some proteins related to nucleotide and amino acid metabolism, and translation, ribosomal structure and biogenesis and that of chaperonin GroEL. Differently to aerobiosis, respiration did not affect the amount of chaperone DnaK but significantly modulated the abundance of the proteases ClpB and ClpX. Regardless of growth phase, the abundance of citrate lyase and alkaline shock protein strongly decreased in respirative cells (Table 1).

3.3. Effect of hemin and menaquinone supplementation on the proteome of L. casei N87

Comparing respiration and unsupplemented aerobiosis (RS/AE in Table 1) more complex effects on *L. casei* N87 proteome were observed. In fact, the number of proteins that were up-regulated or down-regulated in presence of respiratory cofactors was similar in both exponential (twenty-seven proteins) and stationary phase cells (twenty-eight proteins).

In particular, twenty proteins changed their relative abundance in both growth phases and sixteen of them showed the same trend (eight were less abundant and eight were more abundant). As to other proteins, under respiration, ATP F0-F1 synthase subunit alpha and alkaline-shock protein were more abundant in stationary phase and less abundant in exponential phase, while 30S ribosomal protein S2 and D-alanine-poly(phosphoribitol) ligase subunit 1 showed exactly the opposite trend. In addition, seven proteins were differentially abundant only in the exponential phase (three were less represented and four were more represented) and eight proteins were present in a different amount only in the stationary phase (four were less abundant and four were more abundant) (Fig. 1).

The abundance of several proteins involved in energy and carbon metabolism was modulated by hemin and menaquinone supplementation (i.e., phosphoenolpyruvate carboxykinase, citrate lyase, ATP-F0F1 synthetase). The activation of respiratory pathways relieved the negative effect of O_2 on the synthesis of some ribosomal proteins, aminoacyl-tRNA synthetases, carbamoyl phosphate synthase, proteins involved in purine biosynthesis (GMP synthase, IMP dehydrogenase) and cell wall biogenesis. On the contrary, supplementation significantly decreased the abundance of chaperones DnaK and GroEL and proteases ClpB and ClpX.

3.4. Effect of growth phase and conditions on the proteome of L. casei N87

The effect of growth phase and atmosphere of incubation and/or supplementation with hemin and menaquinone on the abundance of identified proteins was analyzed by Pearson's correlation. The scatterplot matrix in Fig. 2 reported the pairwise correlations between the fold changes of protein abundance calculated in two compared conditions. The Pearson's correlation coefficient (r) was reported for each bivariate plot to indicate the strength and type (positive or negative) of correlation. A positive good correlation ($r = 0.691$) was found when protein fold changes of AE/AN_Exp were compared to those of AE/AN_Stat. In fact, as many as twenty-three proteins exhibited the same trend of fold change in both exponential and stationary phases, as already reported. A negative correlation ($r = -0.669$) was found when protein fold changes of AE/AN_Exp were compared to those of RS/AE_Exp, indicating that heme and menaquinone supplementation provided a different regulation in aerobically growing *L. casei* N87. On the contrary, a poor linear correlation was found in the other pairwise comparisons (see also Table 1).

3.5. Correlation heat-map of protein fold changes and protein-protein interaction network

Correlations among protein fold changes were reported in Fig. 3. A Matrix Hierarchical Cluster Analysis was performed on the forty-two proteins (row data) using as variables the FC values calculated in the

compared conditions in both growth phases (column data). Classification generated several clusters differing in the levels of protein abundance. Clustering confirmed that most of proteins involved in nucleotide metabolism (F), ribosomal structure (J) and replication/repair mechanisms (L) were negatively affected by aerated conditions (upper section of heat map) and that supplementation with respirative cofactors may alleviate the toxic of O_2 in aerobically growing cells. On the other hand, a larger group (lower section of graph) of proteins involved in carbohydrate (G) and energy (C) metabolism, and some related to stress response (i.e. chaperons), was positively regulated by O_2. The behavior of other proteins was more complex and affected by interaction of growth conditions and phases.

Protein-protein interactions were analyzed using the web resource STRING and thirty-two out of forty-two identified proteins were connected in the interaction network (Fig. 4). Notably, STRING analysis highlighted three highly interconnected clusters of proteins. The cluster 1 included proteins involved in the carbohydrate and energy metabolism (C and G) and the central node was represented by LDH (hicD3), thus confirming its importance in the cell re-programming under aeration and respiration. Proteins included in the cluster 2 were implicated in amino acid and nucleotide metabolism (E and F). The proteins GuaA and GuaB ideally created a bridge between cluster 2 and cluster 3, which encompassed proteins involved in the protein degradation and folding machinery, ribosome structure and having a role in stress response mechanisms (J and O). Chaperones GroEL and DnaK were the central nodes of this cluster which has several inter-connections to cluster 1. Furthermore, the main metabolic pathways (Carbon and aminoacid metabolism and Aminoacyl-tRNA byosinthesis) affected by the by aerobiosis and/or respirative promoting conditions according to functional classification by KEGG database (Kyoto Encyclopedia of Genes and Genomes; https://www.genome.jp/keg) were highlighted in Supplementary Fig. 2.

4. Discussion

Aerobic and respiratory cultivations may provide several physiological and technological benefits to some LAB (Pedersen et al., 2012; Zotta et al., 2017). Among them, the respiration-competent strain *L. casei* N87 showed an increased biomass yield and stress robustness when cultivated in presence of O_2 and hemin and menaquinone supplementation (i.e. respiratory promoting conditions). *L. casei* N87 was able to grow also in unsupplemented aerated conditions, but high concentrations of dissolved O_2 (dO_2 60%) were detrimental for its growth and cell survival (Ianniello et al., 2016).

Metabolic and functional reorganization of *L. casei* N87 grown under unsupplemented aerobiosis and respiration was investigated in a proteomic perspective. Results showed that cell growth under respiration in exponential phase changed the relative abundance of only ten proteins (RS/AN), mainly involved in the carbohydrate and energy metabolism, while twenty-eight proteins changed their relative abundance in the stationary growth phase. This observation further confirmed that bacterial cells grew initially via fermentation and the metabolic shift occurred in a later growth phase, in limiting condition of sugars and based on the NAD^+/NADH ratio, which allosterically redirected metabolism in favor of enzymes that used NAD or pyruvate as substrates (Arioli et al., 2013; Lechardeur et al., 2011). On the other hand, the number of proteins differentially abundant under aerobiosis in the two growth phases was similar (Fig. 1A), thus suggesting that a metabolic reorganization occurred in the initial growth phase (Fig. 2). Worth noting, bacterial cells activated response mechanisms to respirative conditions by changing the abundance of proteins involved also in the aerobic response (except for D-alanine-poly(phosphoribitol) ligase subunit 1). Nevertheless, a different modulation of the abundance level of these proteins induced by hemin and menaquinone supplementation tightly marked different features of these two physiological conditions (Table 1, Fig. 1B).

The aerobic and respiratory conditions significantly affected the abundance of proteins involved in carbon and energy metabolism in *L. casei* N87. Cultivation in presence of high concentrations of dissolved O_2 (AE/AN) strongly impaired the amount of L-lactate dehydrogenase (L-2-hydroxyisocaproate dehydrogenase), the key enzyme of homolactic fermentative pathway in homofermentative LAB (Zotta et al., 2017). In anaerobic conditions, in fact, the NADH-dependent LDH catalyzes the conversion of pyruvate to lactate, contributing to NAD^+ regeneration and maintenance of redox balance. In presence of oxygen, instead, the activation of NADH-dependent oxidases (NOX) may affect the NADH/NAD^+ ratio, reducing the activity of LDH and re-routing pyruvate away from lactate accumulation; as a consequence, the pyruvate oxidase-acetate kinase (POX-ACK) pathway may be activated to produce acetate and extra ATP via substrate level phosphorylation, promoting the shift from homolactic to mixed-acid fermentation in homofermentative LAB.

The proteomic evidence obtained in this study were in agreement with the phenotypic results obtained by Ianniello et al. (2016) on the same biological system; the activity of LDH and the content of lactate, in fact, were significantly impaired under boosted aerated conditions (AE 60% dO_2). High concentrations of dissolved O_2 may affect gene expression and enzymatic activity also of POX (Quatravaux et al., 2006; Zotta et al., 2017), compromising the activation of aerobic POX-ACK route. As matter of fact, POX and ACK were not detected in this study; this could be due to the tiny amount of these proteins in the analyzed samples and/or to a minor variation in protein abundance (FC $\leq \pm 2$). As already reported for this cell system, gene expression and enzymatic activity of POX were significantly modulated only in respirative growing cells and in unsupplemented aerobiosis with low O_2 concentration (Ianniello et al., 2016); on the contrary, no significant difference was found in aerobiosis with 60% dO_2 when compared to conventional anaerobic growth. However, besides O_2, several other factors (e.g. sugar limitation, carbon catabolite repression, H_2O_2 concentration) may affect the activation and regulation of POX (Mazzeo et al., 2012; Ricciardi et al., 2014; Stevens et al., 2008; Zotta et al., 2013) tuning its functionality in in vivo cell systems.

In this study, O_2 increased the production of phosphoketolase, the key enzyme of phosphoketolase pathway (PKP) in heterofermentative LAB (Gänzle, 2015; Zotta et al., 2017). PKP leads to formation of acetyl-P as a key intermediate, and to the production of lactic acid, CO_2, and acetic acid or ethanol depending on the NADH/NAD^+ ratio. The higher abundance of phosphoketolase suggested a possible shift from homolactic towards mixed-acid fermentation in aerobically growing cultures of *L. casei* N87, when POX-ACK pathway is not activated. Supplementation with hemin and menaquinone did not significantly affect the abundance of phosphoketolase in aerobic cultures (RS vs AE) suggesting that this enzyme is principally regulated by O_2.

Moreover, under aerobic condition, *Lactobacilli* may activate a further acetate-production pathway that involves the pyruvate dehydrogenase complex (PDHc), the phosphotransacetylase (PTA), and the acetate kinase (ACK) (Lopez de Felipe and Gaudu, 2009; Lorquet et al., 2004; Zotta et al., 2017). Many authors reported the enhanced expression of *pdh* genes in different LAB species under aerobiosis (Guo et al., 2017; Lopez de Felipe and Gaudu, 2009; Lorquet et al., 2004). Our data confirmed that the amount of pyruvate dehydrogenase complex E3 component increased both in aerobiosis and respiration in exponential and stationary phases, as also observed in *Lc. lactis* under heme-dependent respiratory conditions (Vido et al., 2004). Heme and menaquinone supplementation (RS vs AE) did not affect its abundance in aerobically growing cells, suggesting that also PDH is mainly regulated by O_2 levels. However, the effect of O_2 on the regulation of PDH remains controversial. In fact, Ianniello et al. (2015) and Larsen et al. (2016) found that *pdh* genes were under-expressed in presence of high dissolved O_2 concentrations in *L. rhamnosus* (aerobic cultivation, uncontrolled dO_2) and *Lc. lactis* (initial dO_2 63%), respectively. The analysis of main metabolites (Ianniello et al., 2016) showed that the acetic

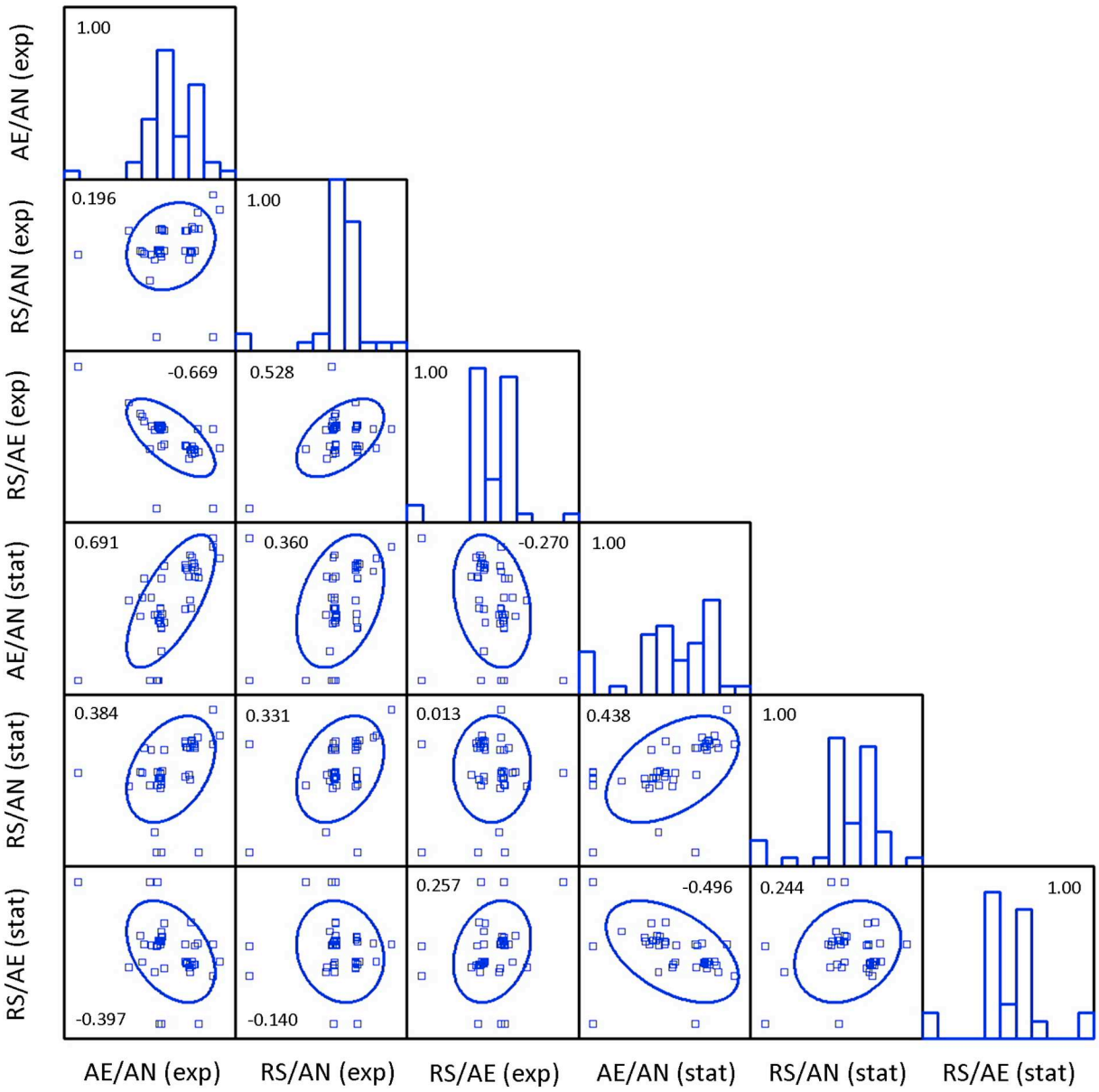

Fig. 2. Pearson's correlation scatterplot matrix of protein fold changes. The pairwise correlations of protein fold changes of all the considered comparisons (AE/AN or RS/AN or RS/AE in Exp and Stat) were reported. Pearson's coefficient is reported for each correlation.

acid was produced by respiratory-growing cells of *L. casei* N87, suggesting that besides O_2 levels also the NADH/NAD^+ ratio affects the direction of metabolic fluxes in *L. casei*. Under respiration, in fact, the NADH may be oxidized to NAD^+ via electron transport chain (ETC), contributing to the flexibility of pyruvate conversion pathways (Zotta et al., 2017).

Supplementation of hemin and menaquinone induced a significant increase of ATP F0-F1 synthase subunit alpha (RS/AE) in stationary phase. ATP synthetase leads to the production of ATP through the extrusion of protons from the cytoplasm driven by the membrane-dependent proton motive force, in turn, generated from the ECT. The activation of ETC in respiration-competent cells contributes to energy gain and reduction of oxidative stress (Zotta et al., 2017) because of extra ATP production and depletion of intracellular oxygen used as electron acceptor in ETC. The higher amount of ATP synthase in *L. casei* N87 grown in presence of O_2, hemin and menaquinone indicated the activation of respiratory chain; this data was confirmed also by transcription of *cydABCD* operon starting from exponential growth phase (Ianniello et al., 2016).

Aerobiosis also affected the abundance level of dTDP-glucose 4,6-dehydratase (Rmlb) and dTDP-4-dehydrorhamnose 3,5-epimerase (Rmlc). These two proteins are involved in dTDP-rhamnose biosynthesis, a sugar nucleotide precursor of capsular exopolysaccharides (EPS) in bacterial cells (Boels et al., 2004; Zivkovic et al., 2015). These results paralleled with previous studies that reported the role of these proteins in the adaptation to heme-dependent respiration in *Lc. lactis* (Vido et al., 2004).

The aerobic growth may induce oxidative stress and cellular damage through the production of reactive oxygen species (ROS); however, as previously observed in this cell system, a significant decrease in the concentration of H_2O_2 in respirative cultures prompted a reduction of cell injuries and improvement in growth efficiency, long-term survival and stress resistance (Ianniello et al., 2016).

In light of this, the molecular chaperones GroEL and DnaK and the ATP-dependent Clp proteases (ClpB, ClpX) were less abundant in aerobic cells grown under respiratory promoting conditions (RS/AE), confirming that supplementation may relieve oxidative damage and reduce the synthesis of proteins involved in stress protection. In *L.*

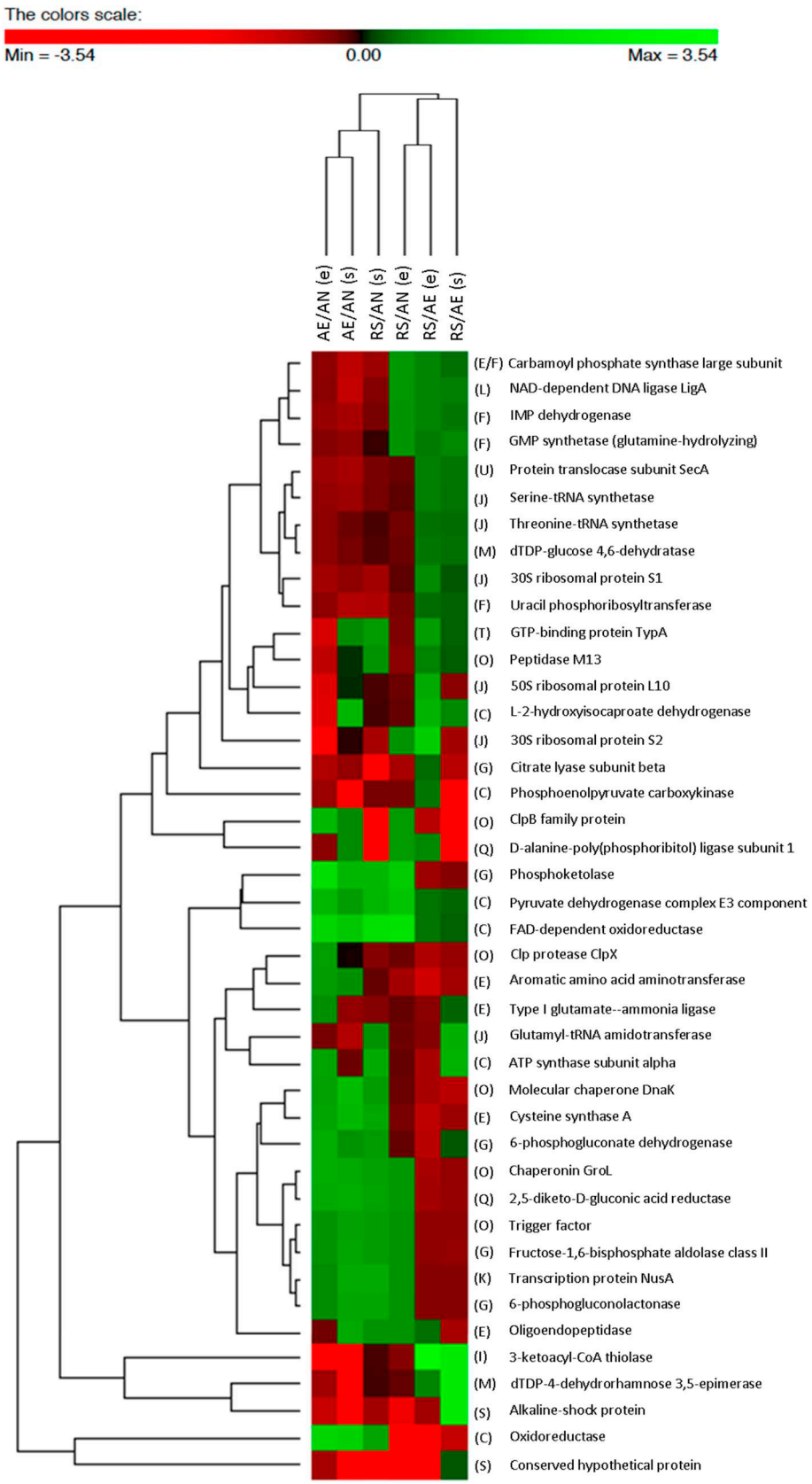

Fig. 3. Matrix Hierarchical Cluster Analysis obtained with the PermutMatrix program v. 1.9. Column dendrogram: protein fold changes measured in two compared conditions (AE/AN or RS/AN or RS/AE) in exponential and stationary growth phases. Row dendrogram: GOG categories and name of identified proteins. Colour scale: from red (negative data, minimum value is −3.54) to green (positive data, maximum values is +3.54), indicates the change from the mean in standard deviation units. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

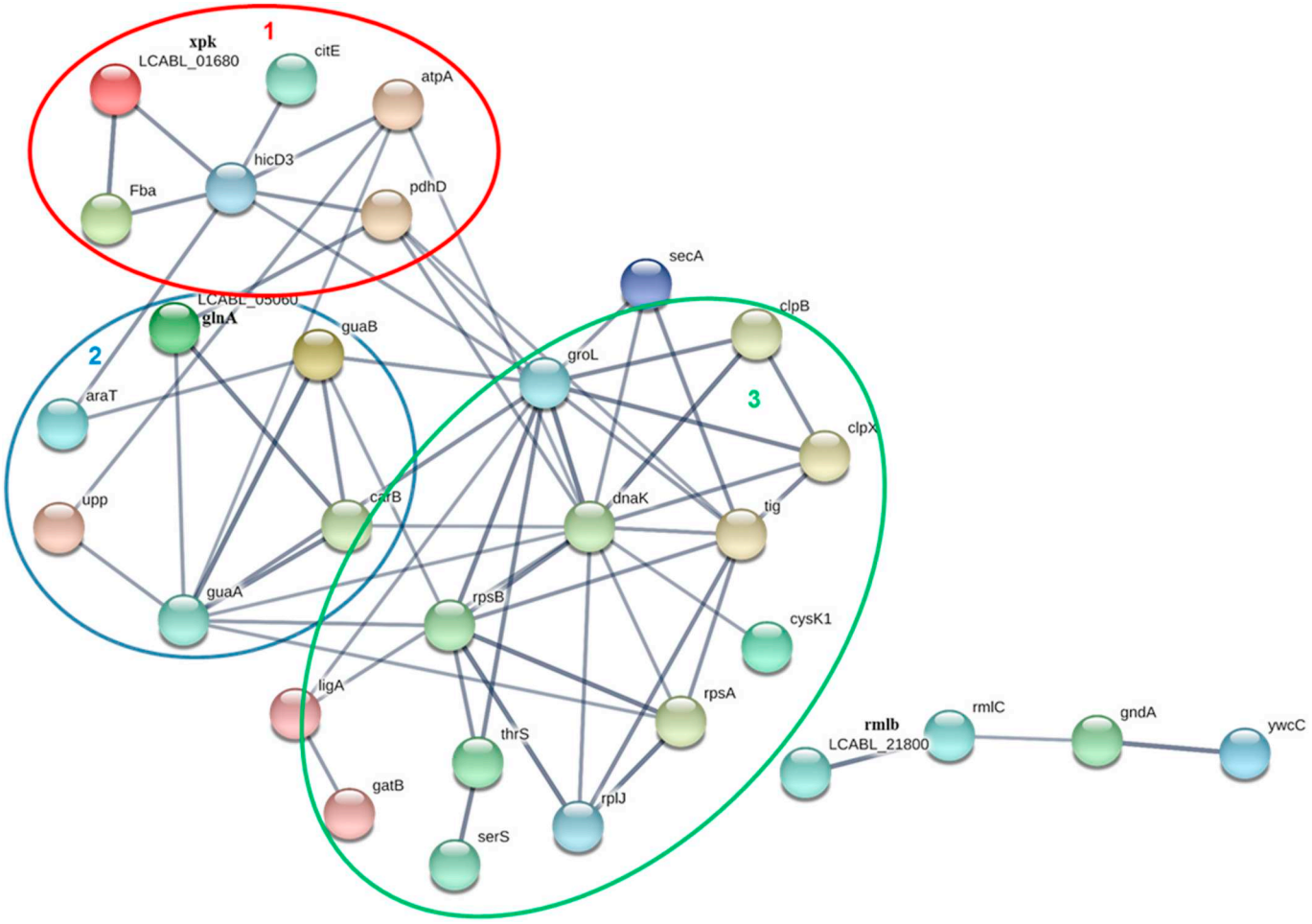

Fig. 4. Protein-protein interaction network of differentially abundant proteins. The network was obtained using EMBL STRING with a confidence cut-off of 0.700. The three main functional modules are circled and numbered.

plantarum, *dnaK* and *groESL* operons were positively controlled by CcpA (Castaldo et al., 2006). Similarly, CcpA could have a pivotal role in modulating stress response mechanisms in *L. casei*, as already reported for *Lc. lactis* (Gaudu et al., 2003). A role in protecting cells against O_2-induced oxidative damage could be also played by cysteine synthase (CysK); this enzyme, involved in cysteine biosynthesis, was over represented under aerobiosis and, only in stationary phase, under respiration. In fact, an increased intracellular level of cysteine may induce the synthesis of the cysteine-containing molecules glutathione and thioredoxin. These molecules, together with cysteine, are responsible for maintaining an intracellular reducing environment and the correct disulfide bonds needed for proper folding and stability of several proteins (Calderini et al., 2017; Lithgow et al., 2004). As for *dnaK* and *groES*L operons, CcpA could be also involved in modulating the expression level of *metC-cysK* genes (Gaudu et al., 2003).

Regardless of heme and menaquinone supplementation, the presence of O_2 negatively affected the abundance of alkaline shock protein in aerobically growing cells of *L. casei* N87. According with these results, unsupplemented aerobiosis impaired the amount of alkaline shock protein also in *L. plantarum* WCFS1 (Mazzeo et al., 2012).

Although several data are present on the aerobic and respiratory metabolism of LAB, the effect of oxygen as well as heme and menaquinone supplementation on the abundance and functionality of proteins involved in nucleic acid biosynthesis and translational processes has been poorly investigated. Proteomic results demonstrated that atmosphere of incubation and supplementation with respiratory cofactors significantly affected the abundance level of proteins related to nucleotide metabolism as well as ribosomal structure and biogenesis. Specifically, aerobic cultivation reduced the production of several aminoacyl-tRNA ligases, thus suggesting that the incorporation of aminoacids into proteins, and, therefore, the translation process could be impaired. Concurrently, the transcription elongation factor NusA, one of the most important transcriptional terminator in prokaryotes, was up-regulated in stationary cells growing aerobically and under respiration, suggesting a decrease in protein synthesis. Aerated conditions also reduced the production of proteins involved in de novo synthesis of purine and pyrimidine. In particular, GMP synthetase (GuaA) and IMP dehydrogenase (GuaB) could be directly involved in stress response mechanisms as the inactivation of these genes in *Lc. lactis* led to mutants exhibiting multi-stress tolerance phenotypes (Rallu et al., 2000).

The supplementation with heme and menaquinone slightly increased the amount of aminoacyl-tRNAs synthetases (Supplementary Fig. 2) and proteins involved in nucleotide metabolism in aerobically growing cells (RS/AE), suggesting that the activation of respirative pathway may alleviate the toxic effect of oxygen on protein translation processes and nucleic acid biosynthesis. Impairment of nucleotide and protein biosynthesis could be detrimental for bacterial cells and, in fact, as previous demonstrated, the high aeration levels significantly impaired biomass production and stress robustness in *L. casei* N87 (Ianniello et al., 2016).

The abundance level of carbamoyl phosphate synthase, involved in production of carbamoyl-P and in pyrimidine and arginine biosynthesis, as well as uracil phosphoribosyltransferase, the key enzyme of uridine-

5′-monofosfate (UMP) biosynthesis, was negatively regulated by O_2. In *L. plantarum*, however, the supplementation of CO_2 (the inorganic C source needed for arginine and pyrimidine pathway) relieved the inhibition of these proteins induced by unsupplemented aerated conditions (Arsène-Ploetze et al., 2006; Bringel et al., 2008; Kilstrup et al., 2005; Stevens et al., 2008).

5. Conclusions

This study provides progress in knowledge of potential molecular mechanisms involved in the aerobic and respiratory metabolism of *L. casei* N87 that coherently parallel with the phenotypic and physiological evidence (growth rate, biomass production, enzymatic activities, stress survival) previously reported by Ianniello et al. (2016). Aerobic cultivation in the presence of high O_2 concentrations, in fact, impaired the synthesis of proteins involved in energy production, stress protection, nucleic acid biosynthesis and translation processes thus confirming, at molecular level, the significant damage to the strain growth and fitness. On the contrary, the activation of respiratory pathways, due to heme and menaquinone supplementation, reduced the detrimental effect of aerated conditions through the positive regulation of central carbohydrate and energy metabolism and preservation of redox balance, resulting in improved growth and stress tolerance features in *L. casei* N87. This proteomic perspective on the oxygen- and respiration-modulated biological functions, could be potentially valuable to develop competitive adjunct and probiotic cultures effectively focused on the quality of fermented foods and on the promotion of human health.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.03.006.

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