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Comparison of changes in the cytosolic proteomes of lactose starved and acid stressed *Lactobacillus rhamnosus* cells

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Abstract
Proteomic analysis was carried out to identify and characterize protein expression in *Lactobacillus rhamnosus* in response to starvation and low pH stress. The cytosolic proteomes of *L. rhamnosus* cells grown in modified MRS broth under control, lactose starvation and acid stress conditions were analysed. Gel-free proteomic analysis using iTRAQ-LC-MS/MS revealed 25 and 27 proteins, produced by starved and acid stressed cells respectively, were differentially expressed in comparison to cells grown in control culture condition. Starved cells produced high amounts of enzymes with functionalities in gluconeogenesis, amino acid metabolism, fatty acid bio-synthesis and sugar metabolisms other than glycolysis. Acid stressed cells showed down-regulations of more proteins than starved cells. The data suggest that *L. rhamnosus* uses two different sets of proteins to cope with each of the stress condition. The results revealed 63% of up-regulated proteins in starved cells were associated with alternative carbon or energy source scavenging activity. Good understanding of the adaptation mechanisms in *Lactobacillus* species during the starvation and other stress conditions is important for successful use of these microorganisms in health and industry.

1. Introduction

Lactose starvation is a common condition faced by lactic acid bacteria during fermentation processes or in applications such as probiotics. One of the key questions about the role of *Lactobacillus* as non-starter lactic acid bacteria (NSLAB) is their ability to survive and grow under nutrient deficient conditions during cheese ripening. Starvation is one of the least investigated stress condition in lactobacilli [1], particularly, where proteomics analyses were employed. One of the early reports by Lorca and De Valdez [2] showed that synthesis of 16 proteins was induced in *Lactobacillus acidophilus* as a result of starvation. Later, Hussain *et al.* [3, 4] reported that adaptation of a NSLAB isolate (*i.e. L. casei* strain GCRL163) under carbohydrate depleted conditions. They used multiple approaches that included viability assessment, metabolites profiling and proteomics changes to explain the survivability and adaptability of the strain in the absence of lactose.

Apart from the above-mentioned investigations, there were no published reports on the proteomics of *Lactobacillus* and carbohydrate starvation between 2001 and 2010.

Since 2011, several studies have emerged reporting starvation responses in the genus *Lactobacillus*. An investigation into the relationship between lactose starvation and glycine, and histidine from dead cells as alternative nutrient sources [6]. The authors used a novel negative, chemically activated, fragmentation/positive (CAF-/CAF+) technique to identify proteins involved in the adaptation of *L. brevis* to nutrient deprivation. Long term starvation impacted on numerous proteins engaged in glucose and amino acid catabolic pathways, glycerolipid metabolic pathways and stress-response mechanisms.

Two more recent reports provided more evidence to support the alternative substrates scavenging theory. Al-Naseri *et al.* [7] showed that lactose starvation suppressed the lactose and galactose catabolic pathways in *L. casei* strain GCRL163. The quantitative proteome analysis of the starved *L. casei* GCRL163 revealed that enzymes associated with the glycolysis/gluconeogenesisy, amino acid synthesis, and pyruvate and citrate metabolism were found in higher abundance. It was also shown that carbohydrate-starved *L. casei* switched to a scavenging mode in the presence of citrate and Tween 80. These adaptive tactics helped the cells to increase survival from weeks to months. Comparative proteomics analysis of one week old cultures showed that 13 proteins were over expressed in lactose-starved *L. casei* GCRL163 cells in comparison to non-starved cells [8]. The up-regulated proteins had functions in protein synthesis, general stress responses and carbohydrate metabolism. Published research work is consistently suggesting that starved *Lactobacillus* cells hunt around for alternative energy sources such as amino acids and pentose sugars.

We report on proteome changes in *L. rhamnosus* ATCC27773 in response to lactose starvation and acid stress. These two conditions are closely associated; depletion of carbohydrate generally results in the accumulation of acidic products, which lowers the pH to sub-optimal levels, i.e. below 5. To our knowledge this is the first investigation employing gel-free proteomics analysis (iTRAQ) to study lactose starvation and the acid stress condition in *L. rhamnosus*. The aim of this research was to understand the physiology of lactose starved cells and compare them with acid stressed cells through proteome changes.

2. Experimental Section

2.1. *Lactobacillus* Strain

The bacterial strain, *L. rhamnosus* ATCC27773 (procured from New Zealand Reference Culture Collection), was stored in 50% MRS-glycerol at -80°C. An overnight culture was prepared by inoculating an appropriate volume of the frozen stock into MRS broth and incubating at 37°C under anaerobic condition.

2.2. Exposure to Stress Conditions

The *L. rhamnosus* ATCC27773 overnight culture was transferred into three different modified MRS broths to expose the cells to different experimental conditions. Modified MRS broth [4] was prepared using 0.3 M phosphate buffer to maintain the required pH during the incubation period. Control cell cultures were grown in MRS broth with 1% lactose and at pH 5.5. For starvation, cells were transferred in MRS broth with 0% lactose and pH 5.5 whereas acid stress was imposed by lowering the pH of the MRS broth (1% lactose) to 4.5. The starting OD_{600nm} of the cultures was adjusted from 0.25 to 0.30. All cultures were incubated at 37°C for 8 h under anaerobic conditions (Anaerobic jars with gas generating kit, Oxoid). OD_{600nm} was measured to monitor the growth of cells under the different conditions. Detailed flow chart of experimental work is given in Fig. 1. Each growth condition was in duplicate and the experiment was repeated at least twice.

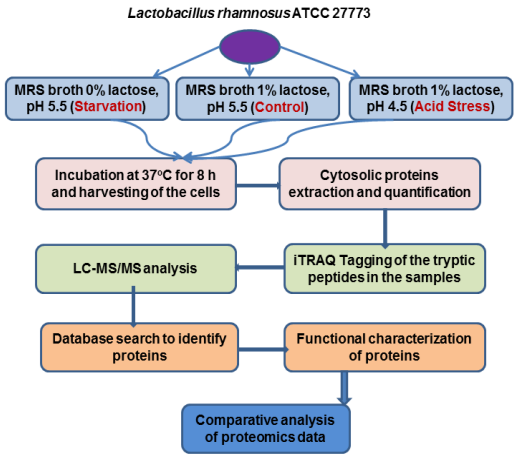

Figure 1. *Experimental flow chart to construct cytosolic proteomes of lactose-starved and acid-stressed L. rhamnosus ATCC27773 cells.*

2.3. Harvesting of Cells and Protein Samples Preparation

At the end of incubation (8 h), cells were harvested by centrifugation at 10,000 x *g* for 10 min at 4°C and then washed twice with 40 mM Tris-buffer (pH 7.0). The cell pellets were re-suspended in appropriate volumes of 40 mM Tris buffer to achieve a final OD_{600nm} of 20. The bacterial cells were lysed mechanically by beating with 0.1 mm sterile zirconium beads using a mini-bead beater. The first beating cycle of 90 s was followed by three beating cycles of 60 s each. Cell suspensions were cooled down for 5 min on ice between each beating cycle. Cell debris was removed by centrifugation at 13,000 x *g* for 30 min at 4°C and the supernatant containing the cytosolic proteins was collected. The supernatant (protein sample) was stored at -80°C until use for proteomic analysis.

2.4. Comparative Proteomics Using iTRAQ and LC-MS/MS

2.4.1. Protein Quantification

Total protein concentrations in the extracts were quantified using 2D Quant kit. After protein quantification, eighty microgram of cytosolic proteins from each sample were

taken and precipitated by MeOH/CHCl3. Samples were centrifuged to obtain the protein pellets, re-suspended in 60 µl 0.5 M TEAB (Triethylammonium bicarbonate buffer), then reduced with 20 µl 100 mM TCEP (Tris 2-carboxyethyl phosphine hydrochloride) in 0.5 M TEAB. After reduction, those samples were alkylated with 20 µl (150 mM IAM iodoacetamide) in 0.5 M TEAB.

2.4.2. Trypsin Digestion and Isobaric Labelling of the Samples

Ten microgram of trypsin was added into each alkylated sample for protein digestion, and then incubated at overnight 37°C. After the incubation, the digests were dried down and resuspended each in 20 µl 0.5M TEAB and labelled according to the iTRAQ labelling protocol provided with the iTRAQ reagents-8plex kit (AB Sciex Pte. Ltd).

2.4.3. LC-SCX (Strong Cationic Exchange) Fraction

iTRAQ labelling was followed by the transfer of small amount of iTRAQ labelled and dried digest into a new Eppendorf tube and re-suspended in 0.1% TFA (Trifluoroacetic acid). The Eppendorf tube was then put into a conditioned C18 empore disc for shaking 3 h. The, the empore disc which contained Eppendorf tube was washed with 0.1% TFA containing 5% ACN (Acetonitrile). After washing, 50% ACN in 0.1% TFA was used to elute the peptides from empore disc. Then, dried down the empore disc and prepared 40 µl 0.1% formic acid for SCX fractionation. The SCX fractionation was performed using High-pressure liquid chromatography. Each empore-purified fraction from the SCX fractionation step (flow through, 1%, 5%, 10%, 20%, 30%, 40%, 60%, 80% and 100%) was the same as mentioned above.

2.4.4. LC-MS/MS (Liquid Chromatography-Mass Spectrometry)

LC-MS/MS was performed on a nanoAdvance UPLC coupled to a maXis impact mass spectrometer equipped with a CaptiveSpray source (Bruker Daltonik, Bremen, Germany). Two microliter sample was loaded on a C18AQ nano trap (Bruker, 75 µm × 2 cm, C18AQ, 3 µm particles, 200 Å pore size). The trap column was then switched in line with the analytical column (Bruker Magic C18AQ, 100 µm × 15 cm C18AQ, 3 µm particles, 200 Å pore size). The column oven temperature was 50 °C. Elution was with a gradient from 0% to 40% B in 90 min at a flow rate of 800 nl/min. Solvent A was LC-MS-grade water with 0.1% FA and 1% ACN; solvent B was LC-MS-grade ACN with 0.1% FA and 1% water.

Samples were measured in auto MS/MS mode, with a mass range of m/z 50-2200. One MS was followed by 10 MS/MS of the most intense ions. Acquisition speed was 2 Hz in MS and 10 or 5 Hz in MS/MS mode depending on precursor intensity. Precursors were selected in the m/z 400-1400 range, with charged states 2-5 (singly charge ions were excluded). Active exclusion was activated after 1 spectrum for 0.3 min.

2.4.5. Data Analysis

Peak list files (mgf format) were generated using DataAnalysis (Bruker), concatenated and submitted to an in-house Mascot server (v2.4) (Matrix Science, UK). The following search parameters were used: Taxonomy *Lactobacillus rhamnosus*; Enzyme semi trypsin; Cysteine modification carbamidomethyl; MS tolerance 0.02 Da; MS/MS tolerance 0.1 Da; 1 missed cleavage; instrument specificity ESI-QUAD-TOF. Mascot iTRAQ parameters included variable iTRAQ8plex (N-term, K, Y), with reporter ions defined as appropriate for the experiment. Peptides with a score below 20, and proteins with fewer than 2 peptides were discarded. Only unique peptides were used for quantitation. Normalisation was based on division by channel sum.

3. Results and Discussion

3.1. Growth under Stress Conditions

The experimental conditions used in this study impacted on the growth of *L. rhamnosus* in the expected manner. The control condition (1% lactose and pH 5.5) provided the ideal environment for growth and cells were in the exponential phase after 8 h (time of harvesting). This growth state represented a non-stressed cell condition; an increase in the culture OD_{600nm} ~1.0 was recorded (Fig. 2) and the pH was well stable at around 5.3. In contrast, the stress conditions restricted microbial growth; cultures grown at low pH (4.5) showed an increase of ~0.2 in the OD_{600nm} and the starved cells had a slight increase, i.e. OD_{600nm} ~0.05 after 8 h (Fig. 2). The results clearly showed that the two conditions used in this study impeded microbial growth. The observations of restricted growth in the absence of a known carbon source (glucose or lactose) and in low pH are consistent with other authors [4, 9]. Thus, cells harvested from the control condition were regarded as non-stressed whereas cells from the other two conditions were stressed, which allowed us to study their differences using proteomics tools.

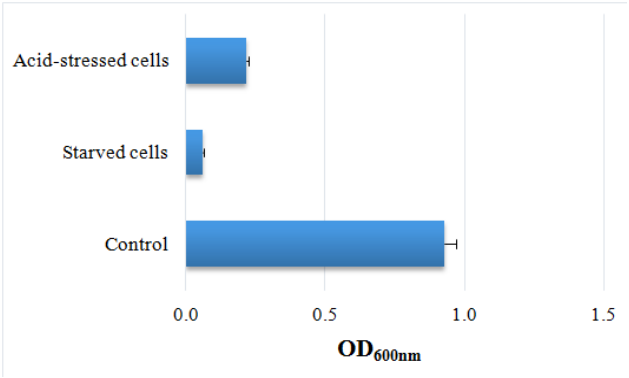

Figure 2. *Changes in OD_{600nm} values of L. rhamnosus ATCC27773 cultures grown under control (1% lactose, pH 5.5), starvation (0% lactose, pH 5.5) and acid stress (1% lactose, pH 4.5) conditions using modified MRS broth. Cells were incubated at 37°C for 8 h before harvesting for proteomics analysis. Error bars represent standard deviations.*

3.2. Changes in the Cytosolic Proteome of *L. rhamnosus* in Response to Starvation and Acid Stress

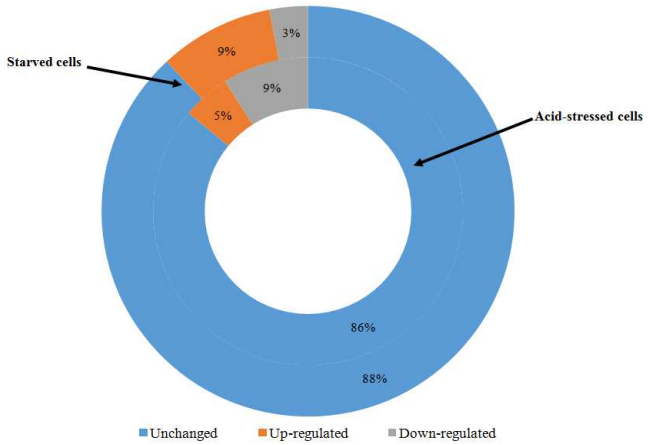

Figure 3. *Doughnut chart showing the up- or down-regulated proteins (using 1.5-fold expression factor) in the L. rhamnosus ATCC27773 cultures grown under lactose starvation (0% lactose, pH 5.5; outer circle) and acid stressed conditions (1% lactose, pH 4.5; inner circle) in comparison with control culture grown in MRS broth (1% lactose, pH 5.5) at 37°C and harvested after 8 h of incubation.*

Cytosolic proteins recovered from *L. rhamnosus* cells, grown under control (non-stress), starvation and acid stress conditions, were analysed using iTRAQ and mass spectrometry analyses. In total, 204 proteins were identified as associated with the cytosolic proteome of *L. rhamnosus*. The majority of the identified proteins were homologous with three strains of *L. rhamnosus* (GG, HN001, and LMS2-1). The changes in the proteomes generated from the non-stressed and stressed cells (starvation or acid) were compared. The comparison showed that 12% of proteins in the starved cells and 14% in the acid stressed cells were differentially expressed (Fig. 3). Interestingly, 9% of proteins increased and 3% of proteins decreased in response to starvation whereas 5% of proteins up-regulated and 9% of proteins down-regulated under the acid stress condition.

3.3. Effect of Starvation on Specific Protein Levels

Comparison of the proteome of starved cells with non-stressed cells proteome revealed that 25 proteins with up- or down-regulated values of these proteins. Among the differentially synthesized proteins, the levels of 19 proteins were increased and six proteins decreased in the starved cells (Table 1). Six proteins with increased intensity (2-fold or more) were associated with fatty acid biosynthesis, amino acid metabolism, gluconeogenesis, ion transport, transcription and cell division. Wang *et al.* [10] reported 2D profiles of the cytosolic proteins of *L. acidophilus* RD758 cells recovered from starved and non-starved conditions and identified 25 differentially expressed proteins.

Table 1. *List of differentially synthesized proteins in Lactobacillus rhamnosus ATCC 27773 under starvation and acid stress conditions*

Protein name [Homology match]	Function	Accession no.	Mass (Da)	Fold change	
				Starvation	Acid
Up-regulated					
Acyl carrier protein [*L. rhamnosus* HN001]	Fatty acid and polyketide biosynthesis	gi|199597462	8957	+2.55	+3.33
Glycine cleavage system protein H [*L. rhamnosus* GG]	Amino acid metabolism	gi|258508188	10787	+2.02	+2.41
Hypothetical protein LRH_11132 [*L. rhamnosus* HN001]	Unknown	gi|199597803	12741	+1.71	+1.72
NADH oxidase [*L. rhamnosus* R0011]	Energy metabolism	gi|418071707	49257	+1.50	
6-phospho-beta-glucosidase [*L. rhamnosus* LRHMDP2]	Glycolysis / gluconeogenesis	gi|421769753	54807	+3.11	
Phosphotransacetylase [*L. rhamnosus* HN001]	Acetate excretion/assimilation pathways	gi|199597298	35204	+1.58	
Possible acetyltransferase [*L. rhamnosus* LMS2-1]	Ion Transport	gi|229552863	12067	+1.71	
Glycerol-3-phosphate oxidase [*L. rhamnosus* LMS2-1]	Lipid biosynthesis	gi|229553666	67070	+2.02	
tRNA binding domain-containing protein [*L. rhamnosus* Lc 705]		gi|258539943	35206	+1.50	
HPr kinase/phosphorylase [*L. rhamnosus* HN001]	Sugar transport and phosphorylation system	gi|199597253	35325	+1.56	
Phosphocarrier protein HPr [Lactobacillus casei ATCC 334]	Sugar Transport	gi|116495240	9248	+1.63	
MarR family transcriptional regulator [*L. rhamnosus* GG]	Transcription	gi|258509122	18863	+3.02	
Lipoate-protein ligase A [*L. rhamnosus* HN001]	Energy metabolism	gi|199598387	38434	+1.65	
Oligopeptide ABC transporter periplasmic component, partial [*L. rhamnosus* MTCC 5462]	Energy metabolism	gi|417057226	10931	+1.63	
GMP synthase [*L. rhamnosus* LMS2-1]	Amino acid metabolism	gi|229553236	57934	+1.67	
Lysine transporter protein [*L. rhamnosus* GG]	Amino acid metabolism	gi|385827176	49813	+1.92	
Hypothetical protein HMPREF0539_1302 [*L. rhamnosus* LMS2-1]	Unknown	gi|229552046	92449	+1.50	
Hypothetical protein [Lactobacillus rhamnosus GG]	Unknown	gi|385827336	6678	+1.70	
Cell division protein GpsB [*L. rhamnosus* LRHMDP2]	Cell division	gi|421770856	15147	+2.48	-1.77
Catabolite control protein A [*L. rhamnosus* HN001]	Carbon metabolism	gi|199598113	36292		+1.53

Protein name [Homology match]	Function	Accession no.	Mass (Da)	Fold change	
				Starvation	Acid
Ribosomal subunit interface protein [*L. rhamnosus* ATCC 21052]	Ribosome assembly	gi|423079071	25768		+1.60
Short-chain alcohol dehydrogenase of unknown specificity [*L. rhamnosus* HN001]		gi|199598900	26216		+1.58
Glutaminyl-tRNA synthase b subunit [*L. rhamnosus* LMS2-1]	Amino acid metabolism	gi|229552360	16094		+1.72
2-dehydro-3-deoxyphosphogluconate aldolase/4-hydroxy-2-oxoglutarate aldolase [*L. rhamnosus* LMS2-1]	Pentose phosphate pathway, pentose and glucuronate interconversions, and arginine and proline metabolism.	gi|229551367	23099		+1.70
Conserved hypothetical protein [*L. rhamnosus* LMS2-1]	Unknown	gi|229552320	10359		+1.61
Down-regulated					
50S ribosomal protein L19 [*L. rhamnosus* LMS2-1]	50S ribosome assembly	gi|229552441	14555	-3.03	-2.98
Ribosomal protein S15P/S13E [*L. rhamnosus* HN001]	Ribosome assembly	gi|199598193	10307	-1.73	-2.36
L-lactate dehydrogenase (FMN-dependent) related alpha-hydroxy acid dehydrogenase [*L. rhamnosus* HN001]	Pyruvate metabolism	gi|199598503	39395	-1.52	-1.68
Tuf [*L. rhamnosus*]	Protein synthesis	gi|38488993	25919	-2.40	-1.53
Uracil phosphoribosyltransferase [*L. rhamnosus* HN001]	Pyrimidine metabolism	gi|199597583	22780	-2.10	
DNA-directed RNA polymerase, beta subunit [*L. rhamnosus* ATCC 21052]	DNA repair	gi|423078558	140187	-1.50	
Mannitol-1-phosphate 5-dehydrogenase [*L. rhamnosus* LMS2-1]	Fructose and mannose metabolism	gi|229551152	42605		-1.50
Hypothetical protein LRH_01568 [*L. rhamnosus* HN001]	Unknown	gi|199598045	13295		-1.52
Cell division protein FtsH [*L. rhamnosus* LRHMDP2]	Cell division	gi|421770643	78161		-1.63
Ribosomal protein S3 [*L. rhamnosus* HN001]	Ribosome assembly	gi|199598838	24933		-1.50
ABC-type dipeptide/oligopeptide/nickel transport system, ATPase component [*L. rhamnosus* HN001]	Ion Transport	gi|199597137	38150		-1.64
Glutamate--tRNA ligase [*L. rhamnosus* LMS2-1]	Gutamate metabolism, porphyrin and chlorophyll metabolism, and aminoacyl-trna biosynthesis.	gi|229552876	57443		-1.50
Glucosamine--fructose-6-phosphate aminotransferase [*L. rhamnosus* GG]	Cell envelope biosynthesis	gi|258507978	65788		-1.50
Diguanylate cyclase [*L. rhamnosus* R0011]		gi|418072587	44802		-1.69
Galactose-1-phosphate uridylyltransferase [*L. rhamnosus* HN001]	Galactose metabolism	gi|199596969	54599		-1.81
Translation initiation factor IF-2 [*L. rhamnosus* R0011]	Protein synthesis	gi|418070788	102395		-1.50
NADPH:quinone reductase related Zn-dependent oxidoreductase [*L. rhamnosus* LRHMDP2]	Energy production and conversion	gi|421768874	34777		-1.50
Hypothetical protein R0011_11275 [*L. rhamnosus* R0011]	Unknown	gi|418072350	299816		-2.31
DNA mismatch repair protein [*L. rhamnosus* HN001]	DNA repair	gi|199597366	71751		-1.50
Excinuclease ABC subunit A [*L. rhamnosus* R0011]		gi|418070522	84127		-20.78

A comparison of the differentially expressed proteins in various *Lactobacillus* species (e.g. *casei*, *plantarum*, *acidophilus*) in response to starvation showed very few similarity [6, 7, 8]. However, a pattern in the protein expression profiles of lactobacilli starved cells is noted. Up-regulation of proteins associated with energy and amino acid metabolism was clearly seen in this study and that was consistent with other authors [6]. Proteins involved in lipid biosynthesis (glycerol-3-phosphate oxidase and acyl carrier protein) increased in starved cells. This could be the result of a membrane adaptation response in the staved cells [10]. Up-regulation of several glycolytic enzymes, such as glyceraldehyde-3-P dehydrogenase, enolase, phosphoglycerate kinase and triose phosphate isomerase during starvation in lactobacilli reported in previous studies [8, 10], was not observed in *L. rhamnosus*.

The MarR family transcriptional regulator and 6-phosphate-beta-glucosidase were expressed in starved cells, at 3.02- and 3.11-fold, respectively, compared to cells grown in the presence of lactose. Higher expression of proteins involved in transcription and gluconeogenesis in response to starvation has been shown previously [7]. NADH oxidase, phosphotransacetylase, HPr kinase/phosphorylase, phosphocarrier protein HPr, lipoate-protein ligase A, GMP synthase, cell division protein GpsB and lysine transporter protein were also over-expressed in *L. rhamnosus* in response to lactose starvation.

Our study found a down-regulation of proteins in lactose-starved *L. rhamnosus* cells (see Table 1). The numbers of ribosomal proteins were fewer in the cells grown under

starvation conditions. This could be linked to the slow or restricted growth rates of *L. rhamnosus* in the absence of lactose. Proteomic analysis also revealed a down-regulation of certain proteins (L-lactate dehydrogenase (FMN-dependent) related alpha-hydroxy acid dehydrogenase, Tuf, uracil phosphoribosyltransferase and DNA-directed RNA polymerase, beta subunit) in starved *L. rhamnosus* cells. Overall, these responses in starved *L. rhamnosus* demonstrated its ability to survive and adapt under nutrient deficient conditions.

3.4. Effect of Low pH (Acid Stress) on Specific Protein Levels

Comparative analysis between the cytosolic proteomes of acid-stressed cells and the control showed 27 proteins were differentially expressed, including nine up-regulated and 18 down-regulated proteins (Table 1). The acyl carrier protein, glycine cleavage system protein H, catabolite control protein A (CcpA), short-chain alcohol dehydrogenase, ribosomal subunit interface protein, glutaminyl-tRNA synthase and 2-dehydro-3-deoxyphosphogluconate aldolase were overexpressed in *L. rhamnosus* in response to low pH. Among the down-regulated proteins were the 50S ribosomal protein L19, ribosomal protein S15P/S13E, ribosomal protein S3, Tuf, mannitol-1-phosphate 5-dehydrogenase, L-lactate dehydrogenase, galactose-1-phosphate uridylyltransferase, glucosamine--fructose-6-phosphate aminotransferase, cell division protein FtsH, cell division protein GpsB, glutamate--tRNA ligase, diguanylate cyclise, translation initiation factor IF-2, DNA mismatch repair protein and excinuclease ABC subunit A. It was well-documented that low pH conditions altered the expression of a number of proteins in *Lactobacillus* species: 33 in *L. casei* Zhang [11]; 40 in *L. reuteri* [12]; 30 in *L. delbrueckii* [13] and 15 in *L. sanfranciscensis* [14]. Results from previous studies show variations in the stress response proteins between species and strains.The protein CcpA is a master regulator of carbon and nitrogen metabolism in gram-positive bacteria [15]. Our results showed higher expression CcpA in acid-stressed cells. Zotta *et al.* [16] previously confirmed the involvement of CcpA in stress resistant and aerobic metabolism in *L. plantarum*. Glucosamine-fructose-6-phosphate aminotransferase (GlmS) was down-regulated in *L. rhamnosus* cells grown at low pH. The proteins associated with the cell envelope provide a first line of defence against bile and acid stress conditions. Lee *et al.* [17] showed down-regulation of proteins related to formation of the cell envelope, including GlmS. Our study also found decrease in cell division proteins (like cell division protein FtsH) under acid stress conditions, as reported by other authors [7, 10]. We assume the set of 27 differentially expressed proteins reported in this study could be unique to the acid stress response of *L. rhamnosus*.

Several ribosomal proteins were differentially expressed in *L. rhamnosus* in response to acid stress. Expression of the ribosomal subunit interface protein increased, whereas 50S ribosomal protein L19, ribosomal protein S15P/S13E and ribosomal protein S3 decreased in acid-stressed cells. It is difficult to assign specific ribosomal functions to individual ribosomal proteins due to the complex coordination of the ribosome. In general, the contribution of ribosomal proteins is essential for the assembly and optimal functioning of the ribosome. Wu *et al.* [11] observed a down-regulation of ribosomal protein L10 in *L. casei* Zhang under acidic conditions. Other reports also suggested the involvement of ribosomal proteins in stress responses [18]. The findings of this study supported and provided more evidence about the relationships between ribosomal proteins and acid stress.

3.5. Overlap of Differentially Expressed Proteins between Starvation and Acid Stress

This study also investigated the relationship between differentially expressed proteins under both conditions (lactose starvation and acid stress). A limited number of reports have shown overlapping between starvation and low pH stress responses in lactobacilli using proteomic analysis [2]. A total of 19 and nine proteins were over-expressed in response to starvation and acid stress, respectively, and only three up-regulated proteins were similar. The results suggest that up-regulation of 16 proteins was uniquely associated with lactose starvation in *L. rhamnosus*. Six proteins showed a relationship with acid stress only. Interestingly, only four proteins were commonly down-regulated under both conditions out of six and 18 negatively expressed proteins in starved and acid-stressed cells, respectively. Thus, again, demonstrating the individuality of stress conditions on proteome changes, i.e. two down-regulated proteins were found only in starved cells and 14 down-regulated proteins were found only in acid stressed proteins.

The three proteins up-regulated under both conditions were the acyl carrier protein, glycine cleavage system protein H and a hypothetical protein, LRH_11132. The acyl carrier protein is associated with fatty acid biosynthesis and glycine cleavage system protein H has functionality in amino acid metabolism, both these proteins showed more than a 2-fold increase in starved and acid-stressed cells. Four commonly down-regulated proteins in starved and acid-stressed *L. rhamnosus* cells were the 50S ribosomal protein L19, Ribosomal protein S15P/S13E, L-lactate dehydrogenase and Tuf. Lorca and de Valdez [2] detected seven proteins (26.3, 41.4, 48.7, 49.3, 54.5, 56.1, and 70.9 kDa) expressed in *L. acidophilus* during the stationary growth phase (generally associated with starvation) and the induction of nine proteins (14.1, 18.6, 21.5, 26.9, 29.3, 41.9, 42.6, 49.6, and 56.2 kDa) were reported as a result of low pH.

Comparative analysis of starvation stress and other environmental factors was very complex and variability existed in the information available [19]. There was no clear evidence that starvation adaptation increased tolerance to other stress conditions for all strains under any specific experimental conditions. Responses to starvation in lactobacilli are highly variable [20], however, generally, starved cells show more tolerance to other stresses [21].

Therefore, it was logical to assume there was some degree of overlapping in the responses between different stress conditions. Data presented in this study strongly supported this argument.

3.6. Alternative Carbon Sources Scavenging under Starvation

In this study we found that 12 proteins out of 19 up-regulated proteins in starved cells were associated with amino acid metabolism, lipids biosynthesis or energy metabolisms other than glycolysis. This was equal to two-thirds of the total up-regulated proteins in response to starvation. These observations provide further evidence to the previous theory that starved *Lactobacillus* cells hunted for alternative energy sources, i.e. amino acids, lipids or pentose sugars [7, 8]. . The over-production of the glycine cleavage system protein H, which play a role in glycine metabolism, and other amino acid metabolisms linked enzymes such as lysine transporter protein and GMP synthase in lactose starved *L. rhamnosus*, was consistent with previous reports. Growth in starvation conditions is strongly linked to the over-expression of the enzymes involved in amino acid metabolism [6, 7, 8]. Butorac *et al.* [6] reported up-regulation of two amino acid metabolism-associated proteins in 75-day-old starved *L. brevis* cells, showing an over-expression of nucleoside phosphorylase (cysteine and methionine metabolism) and pyrroline-5-carboxylate reductase (arginine and proline metabolism).

Previous investigations used different *Lactobacillus* species, growth conditions and harvesting time, therefore, while up-regulation of different enzymes involved amino acid metabolism were reported that made it difficult to reach a single conclusion at this stage. Thus, further research is needed to understand the preferences for amino acids by different *Lactobacillus* species and the factors that may affect their choice of a specific metabolic pathway.

4. Conclusions

The present study provided insights into the proteomic information of *L. rhamnosus* stress responses under starvation and low pH conditions. The results showed that 25 and 27 proteins were differentially expressed in response to lactose starvation and acid stress, respectively. A comparison of the differentially expressed proteins between the two conditions revealed that lactose-starved cells had 19 up-regulated and six down-regulated proteins whereas acid stressed cells had nine up-regulated and 18 down-regulated uniquely associated with each condition. Only three up-regulated and four down-regulated proteins showed an overlapping similarity in differentially-expressed proteins under both conditions. The data suggested *L. rhamnosus* used different physiological mechanisms to cope with starvation and acid stress. More proteins involved in amino acid metabolism and general stress responses were induced in response to starvation. Proteins linked to metabolic processes (carbohydrate metabolism) and cell divisions were differentially expressed during acid stress.

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Author Contributions

Navdeep Natt did experimental work and collected samples for proteomics analysis. Malik Hussain is first author/principal investigator and is responsible for manuscript preparation, data interpretation and all other aspects of the work.

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