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Research Article

DIFFERENTIAL PROTEOME STUDY OF PUTATIVE PROBIOTIC *LACTOBACILLUS FERMENTUM* BIF-19 STRAIN IN RESPONSE TO BILE STRESS

Aiswarya S. Panicker[1], Pradip V. Behare[1]*, Kanchan Munjal[1], Sudarshan Kumar[2], Jasmine Naru[3], Surender Singh[2], Preeti Rawat[2], Shveta Bathla[2], Bharat Bhushan[1], Manu Jamwal[4] & Ashok Kumar Mohanty[2]

[1]*Dairy Microbiology Division, National Dairy Research Institute, Karnal-132001, Haryana, India*
[2]*Animal Biotechnology Centre, National Dairy Research Institute, Karnal-132001, Haryana, India*
[3]*Department of Immunopathology, Post Graduate Institute of Medical Education and Research, Chandigarh-160012, India*
[4]*Department of Hematology, Post Graduate Institute of Medical Education and Research, Chandigarh-160012, India*

Abstract: Bile tolerance is remarkable property of an organism often used to screen new probiotic organism. Bile tolerance vary greatly among the cultures, some are resistant while others are sensitive. Elucidating mechanisms behind the resistant strains to adapt bile stress will help to identify suitable biomarkers of probiotic attributes that may narrow down the expensive and time-consuming clinical trials. *L. fermentum* BIF-19 strain isolated from infant feacal samples exhibited good *in vitro* bile tolerance property. The cytosolic proteins of BIF-19 grown in 12 mg/ml bile salt extracted by lysozyme treatment were subjected to 2-Dimensional Gel Electrophoresis and differences in spot intensities was compared by Image Master Platinum 7.0 software. A total of 185 and 192 spots were obtained respectively in control and bile treated samples. Fifty differentially expressed spots including 4 new ($p \leq 0.05$, ± 1.5 fold) were subjected to MALDI-TOF/TOF. Out of these, 12 up-regulated and 13 down-regulated proteins having good match between therotical and practical observed molecular weight, pI and significant MOWSE scores were evaluated for their involvement in biological processes of BIF-19. Analysis by DAVID Bioinformatics Resources 6.7 database revealed the role of these proteins/enzymes in metabolic and cellular pathway. Proteins involved in carbohydrate and lipid metabolism and ATP transport were up-regulated while proteins involved in nucleotide metabolism and cell division were down-regulated. Especially, phosphopyruvate hydratase (+ 1.95) and lactoylglutathione lyase (+ 1.79) involved in carbohydrate metabolism and phosphoenolpyruvate—protein phosphatase (- 1.93) and trigger factor (- 3.4) involved in transport and folding, sorting and degradation of proteins may be considered as key proteins during bile adaptive stress in *L. fermentum* strains. These proteins indicated gut adaptability in BIF-19 and can be used to select new putative probiotic strains.

Key Words: *L. fermentum* BIF-19; bile stress; probiotic; 2-DE; MALDI TOF/TOF

Introduction

Lactobacillus fermentum is a natural inhabitant of the human gastrointestinal tract (GIT) often isolated from dairy, non-dairy fermented foods, meat products and human feacal samples. Certain strains like *L. fermentum* VIR-003 PCC® and *L. fermentum* ME-3 DSM-14241 are used as probiotics based on their putative or proven health effects (West *et al.*, 2011; Mikelsaar and Zilmer, 2009). FAO/WHO defines probiotics as the live microorganisms which when administered in adequate number, confers health benefit on the host (FAO/WHO, 2002). Since, probiotic effects are strain specific and there is growing interest

Corresponding Author: **Pradip V. Behare**
E-mail: pradip_behare@yahoo.com
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in use of region or country specific strains that are easily adapted to the prevalent conditions, the focus of researchers across the world has shifted towards the isolation of putative probiotic strains from different geographical niche.

In order for a probiotic strain to exert beneficial effect to the host, it should be able to survive harsh gastrointestinal tract (GIT) conditions that comprise proteolytic enzymes, acidic pH and bile salt. Among these, bile salt is more detrimental to the probiotic strain while passing through the GIT. Therefore, tolerance to bile is used as one of the most important selection criteria for putative probiotic strain. Human liver secretes a litre of bile every day into the small intestine which contains 0.2 to 2% bile salts (Hofmann, 1999). Bile not only act as fat emulsifying and solubilizing agent, but also is an antimicrobial detergent that disorganize the structure of the cell membrane and cause dissociation of integral membrane proteins, resulting into cell death (Begley *et al.*, 2006).

Bile tolerance is remarkable property of probiotics that varies greatly among the cultures. Certain strains show resistance while others are sensitive. In recent time emphasis is given to elucidate mechanisms behind the resistant strains to adapt bile stress leading to ability of an organism to tolerate intestinal bile and as a result capable strain provides beneficial health effects to the humans. This will also help to identify suitable biomarkers of probiotic attributes that may narrow down the expensive and time-consuming clinical trials (Grossklaus, 2009). Proteomic techniques are useful to understand association of various processes or proteins involved in different pathways in potentially important strains. As far as probiotics are concerned, comparative proteome under different physiological conditions have been examined for *L. rhamnosus* GG, *L. plantarum* and *L. casei* (Koskenniemi *et al.*, 2011; Hamon *et al.*, 2011; Wu *et al.*, 2010) that provided large amount of information on the functional responses of these organisms under stressed conditions. Further, comparison of differentially expressed proteins for bile within the same strain in different conditions indicated bacterial adaptation to GIT conditions (Hamon *et al.*, 2011). Often bile tolerance is ascribed to presence of *bsh* gene (bile salt hydrolase enzyme), that helps bacterium to overcome damaging effect of bile salts. However, certain strains are reported to tolerate significant amount of bile, even if the *bsh* gene was absent (Moser and Savage, 2001), suggesting involvement of other proteins in bile tolerance process. It becomes inevitable to identify these proteins to evaluate their role in probiotic potential of the strain. Several proteins are produced during different growth phases of an organism, while some are specific and expressed under certain circumstances. Early stationary phase is more appropriate to understand the enhanced survival of probiotic bacteria in stressful conditions as the highest rate of protein biosynthesis occurs during this stage (Cohen *et al.*, 2006).

To our knowledge, this is the first study from India on differential proteome analysis of putative probiotic *L. fermentum* BIF-19 strain in bile containing medium. The information generated may be useful to understand BIF-19 adaptations to GIT and identify key proteins of probiotic attributes which would be used to screen new potential probiotic strains.

Materials and Methods

Bacterial source and growth conditions

Lactobacillus fermentum BIF-19 strain (NCBI accession no. KM396396) was isolated from infant faecal samples collected from Government hospital, Karnal, Haryana, India. The optimum growth temperature for BIF-19 was 37°C. It was always maintained in MRS broth and sterile skim milk at 4°C. For growth phase study, 0.2% culture was inoculated in MRS broth and incubated for 0, 3, 6, 9 and 12 hr at 37°C. Growth phase was established by plotting graphs of the values obtained from viable cell count by the following formula;

$$K = \frac{\log_{10} Nt - Log_{10} N_0}{Log_{10} 2t}$$

N_t- Final count (log cfu/ml), N_0- Initial count (log cfu/ml), t- time (hr), K- Growth rate constant (hr^{-1})

BSH activity and bile tolerance

Presence of *bsh* gene (NCBI gene bank accession no. HM 036119) in *L. fermentum* BIF-19 was evaluated by reported primers used for *L. fermentum* strain F1 (Zen and Nakanuma, 2010). Bile salt hydrolase activity was assessed by conjugated bile hydrolase (CBH) assay (Taranto *et al.*, 2003) while tolerance to bile salt was tested according to the method given by Gilliland *et al.* (1984) with slight modifications. The modifications included changes in bile concentration and time of bile exposure given to cell. The actively grown cells in MRS broth were harvested by centrifugation. The bacterial pellet containing approximately 10^{10} cfu/ml was resuspended in each 5 ml MRS broth tubes supplemented with 0, 3, 6, 9, 12, 15 and 18 mg/ml (w/v) ox bile (Himedia Laboratories Pvt. Ltd, Mumbai, India). Bile exposure was given for 0, 3 and 6 hr reflecting the time spent by food in the small intestine. Survival of *L. fermentum* BIF-19 was evaluated by plate count method by incubating the plates at 37°C for 48 hr.

Preparation of cellular protein fraction

The bacterial cells were grown in 100 ml MRS broth and harvested in stationary phase (1×10^{10} cfu/ml) by centrifugation at 10000 × g for 10 min. The pellet was divided and equally distributed in 50 ml MRS broth with 12 mg/ml (w/v) bile salt and without bile and incubated at 37 °C for 2 hr. For protein extraction, the cell pellets obtained were washed in Sodium phosphate buffer (pH 7.4), resuspended in 10 ml lysis buffer containing 0.1 mg/ml lysozyme and incubated at 37 °C for 1 hr to facilitate cell lysis. The lysed solution was centrifuged at 12000 × g for 30 min and the supernatant was concentrated. Protein content in samples was estimated by Bradford assay kit (BIORAD, US) using BSA as the standard.

2-DE

The proteins were subjected to IEF. Immobiline DryStrip gel (7 cm with linear range of pI 4-7, GE healthcare) was rehydrated overnight with 125 µl rehydration solution containing 300 µg of protein sample and 0.5% ampholytes. IEF was performed on Ettan IPGphor 3 electrophoresis unit (GE Healthcare Pvt Ltd.) using a 4-step program (150 V for 80 min, 1000 V for 31 min, 5000 V for 90 min, 5000 V for 24 min) reaching to a total of 7000 V hr. Prior to running the second dimension, the strip was equilibrated for 15 min in equilibration buffer containing 1% dithiothreitol in first step followed by 2.5% iodoacetamide for another 15 min. In second dimension, SDS-PAGE was carried out in 12% separating gel over layered with agar. The gels were run at 70 V in mini VE Vertical Electrophoresis system (Amersham Biosciences) and stained using Coomassie Brilliant blue dye. It was then de-stained using 30% acetone, 10% glacial acetic acid and stored in milli-Q-water at 4°C. The gels were scanned by Labscan and analysis was performed by Image Master Platinum 7.0 software (GE Healthcare Pvt. Ltd.). The individual protein spot intensity was normalized to the sum of intensities of all valid spots in one gel and then each protein spot was compared between the two gels. The changes in protein expression as a result of bile stress was considered as up and down regulated when changes in normalized spot intensities were of at least 1.5-fold at a significance level of $P \leq 0.05$, Student's t tests for paired samples.

Protein identification

The selected differentially expressed spots were excised manually by separate sterile surgical blades, destained using 100 µl of 40 mM ABC (ammonium bi-carbonate) and 100 µl of 40% acetonitrile followed by *in-situ* trypsin digestion (Gorg *et al.*, 2004). The digested peptides were desalted by zip tip (Merck, Bangalore) and vacuum dried. The desalted samples were reconstituted in 0.1% formic acid, loaded onto MALDI plate and analysed by MALDI TOF/TOF (ABSCIEX TOF/TOF 5800). The matrix of sinapinic acid 20 mg/ml (sinapinic acid in CAN:water:TFA, 50:50:0.1) was used. The MS analysis was performed by protein pilot software Mascot 3.0 with precursor tolerance at 150 PPM and MS/MS fragment tolaerance at 0.3 Da. The maximum missed cleavage was set to 1. The charge state for each peptide was +1. The proteins obtained were categorized on the basis of their molecular functions and their involvement in various biological processes. Further the involvement of some potential protein candidates in various pathways was assessed using different

software's such as PANTHER 9.0 (*http://www.pantherdb.org/*), MAS 3.0 (*http://www.pantherdb.org/*) and DAVID Bioinformatics resources 6.7 software (*http://david.abcc.ncifcrf.gov*).

Results and Discussion

Growth phase

Keeping in view, almost all the proteins necessary to cell survival are produced during the early stationary phase that plays crucial role in bacterial adaptations the growth phase for BIF-19 was established. The results of growth phase is shown in Figure 1. The early stationary phase for *L. fermentum* BIF-19 was obtained in 9 hr after calculation of K-value (Mean Growth Rate Constant), which was nearly zero (K = 0.05).

In order to evaluate physiological responses of a particular organism to given conditions, growth phase determination is important that varies from strain to strain. During the growth in stressed situation cell requires higher level of proteins for macromolecule synthesis and cell division. The proteomic growth phase study of *Lactobacillus plantarum* showed a higher relative abundance of proteins during the early stationary phase than the late stationary phase (Cohen *et al.*, 2006).

BSH activity and bile tolerance

In *L. fermentum* BIF-19 amplification product of 927 bp was obtained indicating ability of this strain to produce BSH enzyme (Figure 2). BIF-19 was also able to deconjugate primary salt i.e. sodium taurocholate (TC) and secondary salt i.e. sodium glycocholate (GCA) and sodium taurodeoxycholate (TDC) predicted from the zone of salt precipitation around the colonies on plates (Figure 3). Organisms that deconjugate primary or secondary bile salts did not show zone surrounding the colonies as in case of *L. fermentum* BIF 19 and *L. rhamnosus* GG, while *L. fermentum* ATCC 9338 *bsh* negative shown prominent zone was unable to deconjugate sodium taurocholate.

In vitro bile tolerance test indicated that BIF-19 count decreased with the increase in bile concentration and exposure time (Figure 4). Interestingly BIF-19 count increased up to 3 hr in

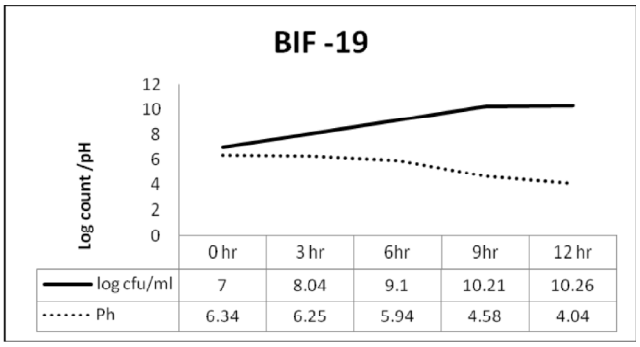

Figure 1: **Growth phase of *L. fermentum* BIF-19**

Solid line indicates that bacterial count remains static after 9 hrs i.e. BIF-19 entered stationary phase (K=0.05). Dashed line shows gradual decline in pH after 6 hrs.

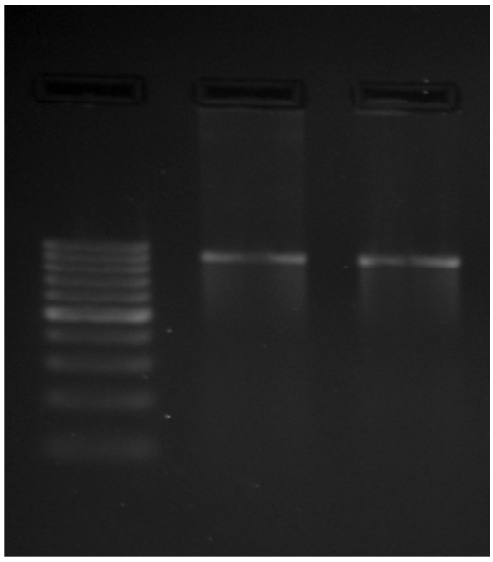

Figure 2: **PCR product of *bsh* gene in BIF-19**

Lane1 (L1) - molecular ladder (100 bp); Lane 2 (L2)- BIF-19 amplified product; Lane 3 (L3) - *Lactobacillus fermentum* ATCC 9338 amplified product (*bsh* negative). A PCR amplified product of 927 bp exhibited BIF-19 possesses *bsh* gene.

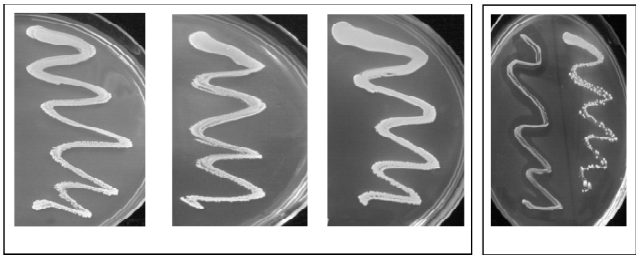

Figure 3: **Bile salt hydrolase activity of *Lactobacillus* species**

Figure in first box indicates *Lactobacillus fermentum* BIF-19 hydrolyzes three salts viz., Sodium taurocholate ii) Sodium taurodeoxycholate iii) Sodium glycocholate. In second box *Lactobacillus fermentum* ATCC 9338 did not hydrolyze Sodium taurocholate whereas *Lactobacillus rhamnosus* GG hydrolyzes the same salt.

bile concentration ranging from 3 to 15 mg/ml and remianed almost comparable up to 6 hr to that of conrol samples without bile salt. BIF-19 appreciably tolerated 12 mg/ml of bile salt although slight decline in count was observed after 6 hr. Bile concentration above 12 mg/ml i.e. 15 mg/ml and 18 mg/ml was stressful after 3 hr and 0 hr respectively as BIF-19 count started to decline. Based on *in vitro* bile tolerance test, 12 mg/ml concentration of bile was selected for differntial proteome analysis.

Differential proteome analysis

In prliminary studies we observed that, cytosolic proteins extracted by lysozyme treatment gave comparatively higher concentration of protein (1.2 µg/µl) than Q-proteome kit (0.5 µg/µl) and sonication method (0.14 µg/µl). The protein yield greatly varies between extraction procedure and several factors including bacterial strain, cultivation method, lab variation etc. affect protein extraction. As in our experiment, lysozyme treatment gave better yield, this method was used to extract proteins from BIF-19.

Initially, to check the distribution of proteins, the samples were run on broader range of IPG strips (pI 3-10). Most of the proteins were present within pI 4-6 (Figure 5). A total of 185 and 192 protein spots were obtained respectively in control and bile treated samples (Figure 6). Additional seven spots were evaluated by 3-D view indicated only four as new while three as redundant proteins that were identified as differentially expressed proteins (DEPs). For selection of DEPs mainly those with significant MOWSE scores, molecular weight and pI were considered. Fifty including 4 new differentially expressed spots ($p \leq 0.05$, ± 1.5 fold) (data not shown; available on request) were subjected to MALDI-TOF/TOF. Around 21 hypothetical proteins and proteins with MOWSE score less than 40 were eliminated from the study (data not shown). Among the four new spots, two (BT2 and BT3) were not matching in either pI or molecular mass. The remaining two spots BT-4 and BT-6 were the protein species of B37 and B46 spots respectively. Overall 12 up-regulated and 13 down-regulated proteins with a significant MOWSE score were used for further analysis (Table 1).

We used three bioinformatics tools to analyze the involvement of selected proteins in BIF-19. Proteins analyzed by Panther 9.0 were classified as hydrolases, oxidoreductase, enzyme modulators, lyase transferases, ligases, nucleic acid binding enzymes, kinases, isomerases and dehydrogenases (Figure 7A). These proteins were further categorized according to its biological significance in BIF-19 such as proteins involved in metabolic processes, biological regulation and others (Figure 7 B and Table 2). Majority of the proteins identified were found to be associated with metabolic process.

Analysis by MAS 3.0 produced comprehensive information on the biological functions of proteins (Table 3) in BIF-19 that matched with proteins of *E. coli* available in the database. DAVID 6.7 resulted in more relevant information (Table 4) with our culture as it uses data of similar *Lactobacillus* genus (*L. plantarum*). Table 5 represents combined results of up-regulated and down-regulated proteins analyzed by three gene ontology software and some of these having important role in BIF-19 are described below. Three different gene ontology softwares were used to generate missing information from any individual software.

Out of the fifteen DEPs, enzymes involved in glycolysis such as triosephosphate isomerase, phosphopyruvate hydratase and glyceraldehyde 3-phosphate dehydrogenase, were up-regulated. Triosephosphate isomerase converts glyceraldehyde 3-phosphate into dihydroxy acetone phosphate and further into glycerol. Triosephosphate isomerase is also involved in lipid metabolism. Various proteins involved in carbohydrate metabolism have been reported to be highly induced in response to bile stress in various micro organisms (Hamon *et al.*, 2011; Wu *et al.*, 2010; Lee *et al.*, 2008; Burns *et al.*, 2010; Alcantara and Zuniga, 2012). The enzyme glyceraldehydes 3-phosphate dehydrogenase links the glycolysis with pentose phosphate pathway which is reported to be the highest among the enzymes involved in glycolysis (Lee *et al.*, 2008). Carbohydrate and lipid metabolism are highly activated in BIF-19 because the cell needs more energy to survive in bile stressed condition due to the overproduction of proteins

Table 1
Selected bile responsive differentially expressed proteins in *L. fermentum* BIF-19

Spot ID	*Normalized volume with 12 mg/ml bile*	*Accession No.*	*Protein ID (Source)*	*Gene symbol*	*Uniprot No.*	*Theoretical Mol Wt. (Da)*	*Theoretical pI*	*Peptide numbers*	*% Sequence coverage*	*MOWSE E Score*	*Fold changes*
Up-regulated proteins											
B32	1.144 ± 0.310	gi|28377644	triosephosphate isomerase [*Lactobacillus plantarum* WCFS1]	***tpiA***	P27876	27126	4.62	11	69	211	+1.74
B37	0.145 ± 0.0374	gi|28377642	glyceraldehyde 3-phosphate dehydrogenase *[Lactobacillus plantarum* WCFS1]	*gapdh*	F9UM10	36644	5.30	9	43	174	+1.69
B36	0.286 ± 0.075	gi|28378847	ribokinase [*Lactobacillus plantarum* WCFS1]	***rbsK1***	F9ULK7	31749	5.18	6	32	130	+1.71
B48	0.152 ± 0.032	gi|28377085	oxidoreductase [*Lactobacillus plantarum* WCFS1]	*nox*	F9URQ8	31871	5.03	9	45	111	+1.53
B8	0.213 ± 0.079	gi|28379701	oxidoreductase (putative) [*Lactobacillus plantarum* WCFS1]	***nox***	**Q6LWG1**	23529	5.23	9	69	88	+2.2
B33	0.208 ± 0.056	gi|28378468	DegV family protein [*Lactobacillus plantarum* WCFS1]	*DegV*	F9UPE3	32183	5.40	9	51	76	+1.73
B46	0.347 ± 0.075	gi|28378683	ribosome recycling factor [*Lactobacillus plantarum* WCFS1]	*frr*	Q88VJ7	20581	5.85	5	41	73	+1.55
B16	0.157 ± 0.051	gi|28377645	phosphopyruvate hydratase [*Lactobacillus plantarum* WCFS1]	*eno*	Q88YH3	48057	4.61	6	27	57	+1.95
B50	0.126 ± 0.026	gi|149002170	iron-compound ABC transporter,ATP-binding protein [*Streptococcus pneumonia*]	*ABC-ATPase*	Q97NY0	8982	4.87	8	100	50	+1.52
B52	0.130 ± 0.026	gi|239758718	Tuf [*Lactobacillus plantarum*]	*tuf*	C5J217	30386	4.89	5	32	49	+1.51
B11	0.123 ± 0.043	gi|300767975	aspartate-semialdehyde dehydrogenase [*Lactobacillus plantarum subsp. Plantarum*]	***asd2***	E1TN69	38417	5.50	7	44	48	+2.09
B28	0.181 ± 0.051	gi|56807882	COG0346: Lactoylglutathione lyase and related lyases [*Streptococcus pyogenes*]	***gloA***	Q9A121	16155	5.66	8	55	44	+1.79

contd. table 1

Spot ID	Normalized volume with 12 mg/ml bile	Accession No.	Protein ID (Source)	Gene symbol	Uniprot No.	Theoretical Mol Wt. (Da)	Theoretical pI	Peptide numbers	% Sequence coverage	MOWSE E Score	Fold changes
Down-regulated proteins											
B31	1.368 ± 0.378	gi|254556014	elongation factor G [*Lactobacillus plantarum* JDM1]	***fusA2***	C6VN66	76951	4.80	16	46	230	-1.76
B18	1.084 ± 0.343	gi|28378036	phosphoenolpyruvate protein phosphatase [*Lactobacillus plantarum* WCFS1]	*PtsA*	F9UUK5	63185	4.69	16	52	208	-1.93
B38	0.327 ± 0.079	gi|300769680	universal stress protein [*Lactobacillus plantarum subsp. plantarum* ATCC 14917]	***uspA***	D7VCX8	17728	5.91	6	59	201	-1.64
B40	0.181 ± 0.043	gi|254556435	threonine-tRNA ligase 1 [*Lactobacillus plantarum* JDM1]	*thrS*	C6VPP0	73830	5.10	15	39	192	-1.61
B41	0.507 ± 0.116	gi|300767743	alkaline shock protein [*Lactobacillus plantarum subsp. plantarum* ATCC 14917]	*asp23*	D7VAG4	16056	4.77	6	61	144	-1.59
B26	0.118 ± 0.034	gi|28377497	ribosomal protein L10 [*Lactobacillus plantarum* WCFS1]	***rpmE2***	Q88Z52	17916	5.08	6	50	135	-1.81
B3	0.692 ± 0.376	gi|28378739	trigger factor [*Lactobacillus plantarum* WCFS1]	*Tig*	**Q88VE1**	49406	4.5	17	52	131	-3.4
B7	0.196 ± 0.077	gi|239758778	Tuf [*Lactobacillus plantarum*]	*Tuf*	Q88VE0	29722	4.82	6	42	117	-2.3
B21	0.064 ± 0.019	gi|254557334	dihydroorotase [*Lactobacillus plantarum* JDM1]	*pyrC*	C6VJ34	45677	5.76	11	58	86	-1.88
B30	0.352 ± 0.098	gi|28377744	bifunctional GMP synthase/ glutamine amidotransferase protein [*Lactobacillus plantarum*]	***gatB***	Q88XP6	57592	4.97	8	24	85	-1.77
B4	0.639 ± 0.282	gi|28379560	phosphoglyceromutase [*Lactobacillus plantarum* WCFS1]	***gpmA***	Q88YY8	26071	4.94	4	30	67	-2.6
B39	0.077 ± 0.018	gi|28377930	transcription regulator (putative) [*Lactobacillus plantarum* WCFS1]	***whiA***	Q88YI2	21804	5.72	5	38	57	-1.63
B5	0.170 ± 0.067	gi|28379266	cellobiose PTS, EIIB [*Lactobacillus plantarum* WCFS1]	***Pts***	F9UUK3	11391	4.62	4	39	56	-2.3

Hypothetical proteins and proteins that obtained less MOWSE scores are listed in supplementary Table 2

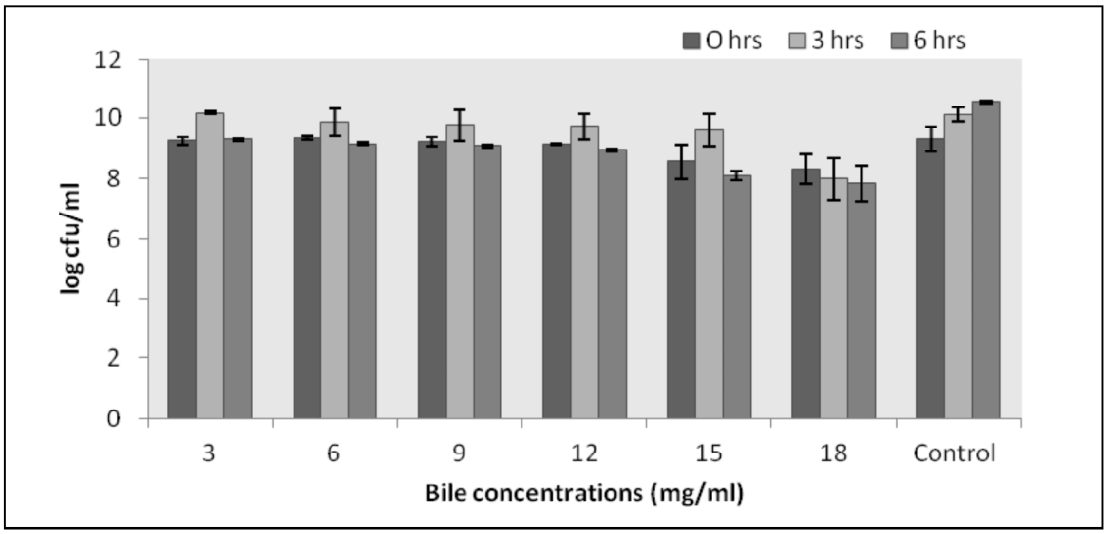

Figure 4: **Bile tolerance by *L. fermentum* BIF-19**

Increase in bile concentration decreased BIF-19 viable count. Bile concentration beyond 12 mg/ml was much stressful.

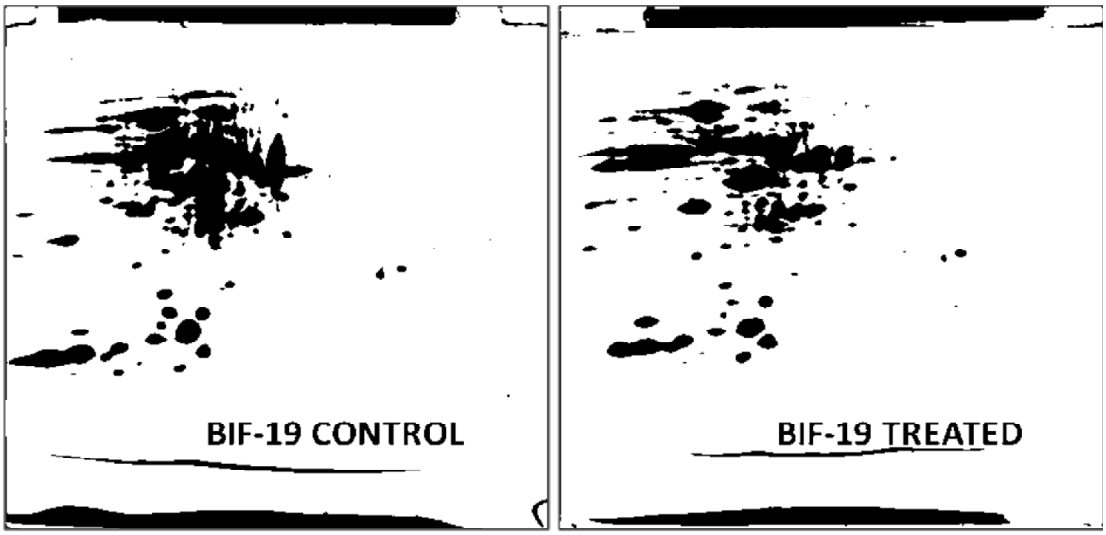

Figure 5: **2-DE of control and treated (12 mg/ml bile) sample of BIF-19 (pI 4-7, 7cm)**

Figures shows 2-DE protein spots of A. control without bile stress and B. bile treated samples

Table 2
Metabolic and biological role of differentially expressed proteins by PANTHER 9.0

Metabolic function	*Biological Regulation*
2,3-Bisphosphoglycerate-dependent phosphoglycerate mutase (*gpmA)*	
Phosphopyruvate hydratase (*eno)*	
Phosphoenol pyruvate- protein phosphatase *(ptsA)*	Elongation factor G (*fusA2)*
Triose phosphate isomerase (*tpiA)*	Ribosomal protein L10 (*rpmE2)*
Threonine-tRNA *ligase (thrS)*	Trigger factor *(tig)*
Glyceraldehyde 3-phosphate dehydrogenase *(gapdh)*	Elongation factor Tu (*tuf)*
Dihydroorotase *(pyrC)*	Ribosome recycling factor (*frr)*
Bifunctional GMP synthase/ glutamine amidotransferase protein (*gatB)*	Transcription regulator (*WhiA)*
Aspartate-semialdehyde dehydrogenase (*asd-2)*	
Lactoylglutathione lyase (*gloA)*	

Table 3
Biological functions of selected differentially expressed proteins by MAS 3.0

Protein (gene symbol)	*Biological function*	*GO term*
Triose phosphate isomerase (*tpiA*)	Pentose phosphate shunt	GO:0006098
	Lipid biosynthesis	GO:0008610
	Fatty acid biosynthesis	GO:0006633
	Glycolysis	GO:0006096
	Gluconeogenesis	GO:0006094
Phosphoenol pyruvate - protein phosphatase (*ptsA*) GO:0016310GO:0009401	Phosphorylation Phosphoenol pyruvate dependent sugar transport	
Bi-functional GMP synthase/ glutamine amidotransferase protein (*gatB*)	Phosphoenol pyruvate dependent sugar transport Galactose metabolism	GO:0009401
Trigger factor *(tig*)	Protein folding	GO:0019402
	Protein transport	GO:0006457
	Cell cycle	GO:0015031
	Cell division	GO:0007049 GO:0007049
Ribosome recycling *factor (frr*)	Protein biosynthesis	GO:0006412
	Translational termination	GO:0006415
Threonine-tRNA *ligase (thrS*)	Protein biosynthesis	GO:0006412
	Threonine tRNA amino acylation	GO:0006435
	tRNA acylation for protein synthesis	GO:0006418
	Regulation of protein biosynthesis	GO:0006417
Dihydroorotase (*pyrC*)	Pyramidine base biosynthesis	GO:0019856
	Pyramidine nucletide biosynthesis	GO:0006221
Phosphoglyceromutase (*gmpA*)	Glycolysis	GO:0006096
Enolase (*eno*)	Glycolysis	GO:0006096
Universal stress protein (*uspA*)	Response to stress	GO:0006950

GO- Gene Ontology

Table 4
Bioinformatics analysis results obtained using DAVID 6.7 software

Identified protein	*Gene symbol*	*UniProt ID*	*Function*
ABC transporter, ATP-binding protein	*ABC ATPase*	Q97NY0	ABC transporter
50S ribosomal protein	*rpmE2*	Q88Z52	Stress response protein
Bi-functional GMP synthase/glutamine amidotransferase protein	*gatB*	Q88XP6	Aminoacyl- tRNA biosynthesis
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	*gmpA*	Q88YY8	Glycolysis/ Gluconeogenesis
Elongation factor Tu	*Tuf*	Q88VE0	Protein biosynthesis Nucleotide binding
Triosephosphate isomerase	*tpiA*	P27876	Glycolysis/ Gluconeogenesis
Enolase	*Eno*	Q88YH3	Glycolysis/ Gluconeogenesis RNA degradation
Lactoylglutathione lyase	*gloA*	Q9A121	Pyruvate metabolism
Trigger factor	*Tig*	Q88VE1	Cell division, chaperone
Oxidoreductase	*Nox*	Q6LWG1	Cellular homeostasis
Ribosome recycling factor	*Frr*	Q88VJ7	Protein biosynthesis
Transcription regulator	*WhiA*	Q88Y12	Transcription regulation DNA binding

Table 5
Functional classes of differentially expressed proteins analyzed by three softwares

Functional category	*Identified protein*	*Uniprot No.*	*Fold change*	*Software used*
Up-regulated				
	triosephosphate isomerase (*tpiA*)	P27876	+ 1.74	MAS 3, DAVID
	phosphopyruvate hydratase (*eno*)	Q88YH3	+ 1.95	MAS 3, DAVID
	Lactoylglutathione lyase (*gloA*)	Q9A121	+ 1.79	MAS 3
	glyceraldehyde 3-phosphate dehydrogenase (*gapdh*)	F9UM10	+ 1.74	PANTHER
Transport	ABC transporter, ATP-binding protein (ABC-ATPase)	Q97NY0	+ 1.52	DAVID
Transcription and Translation	ribosome recycling factor (*frr*)	Q88VJ7	+ 1.55	MAS 3, DAVID
Cellular homeostasis	oxidoreductase (*nox*)	Q6LWG1	+ 1.53	DAVID
Lipid metabolism	triosephosphate isomerase (*tpiA*)	P27876	+ 1.74	MAS 3
Down-regulated				
Transport	phosphoenolpyruvate—protein phosphatase (*ptsA*)	F9UUK5	- 1.93	MAS 3
	bifunctional GMP synthase/ glutamine amidotransferase protein (*gatB*)	Q88XP6	- 1.77	MAS 3, DAVID
Nucleotide metabolism	Dihydroorotase (*pyrC*)	C6VJ34	- 1.88	MAS 3
Folding, sorting and	trigger factor (*tig*)	Q88VE1	- 3.4	DAVID
Degradation of	threonine-tRNA ligase (*thrS*)	C6VPP0	- 1.61	MAS 3
protein, Cell devision	elongation factor *G(tufB*)	C6VN66	- 1.76	MAS 3
Transcription and	ribosomal protein L10 (*rpmE2*)	Q88Z52	- 1.81	DAVID
Translation	transcription regulator (*whiA*)	Q88YI2	- 1.63	DAVID

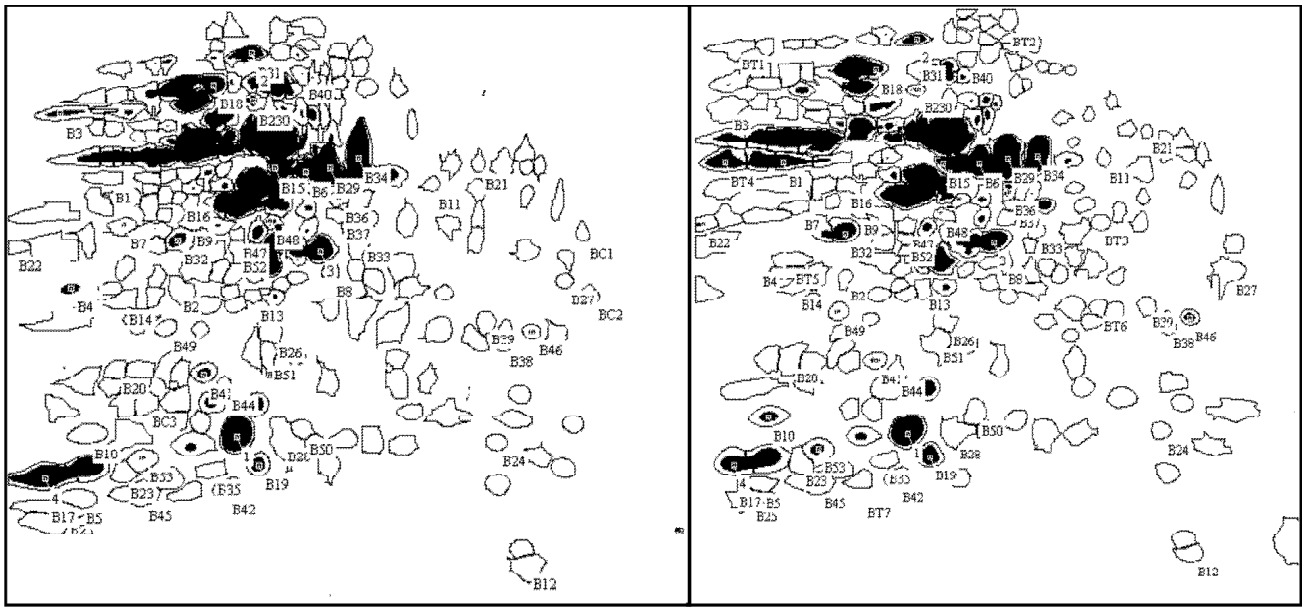

Figure 6: **2-DE gel images of differentially expressed proteins of BIF- 19**
A: Untreated cells (control) and B: Bile treated cells (12 mg/ml) of BIF-19 which was analysed using Image Master Platinum 7. The identified proteins are labelled by alphabates and names are listed in supplementary Table 1, differentially expressed protein spots with their names are presented in Table 1

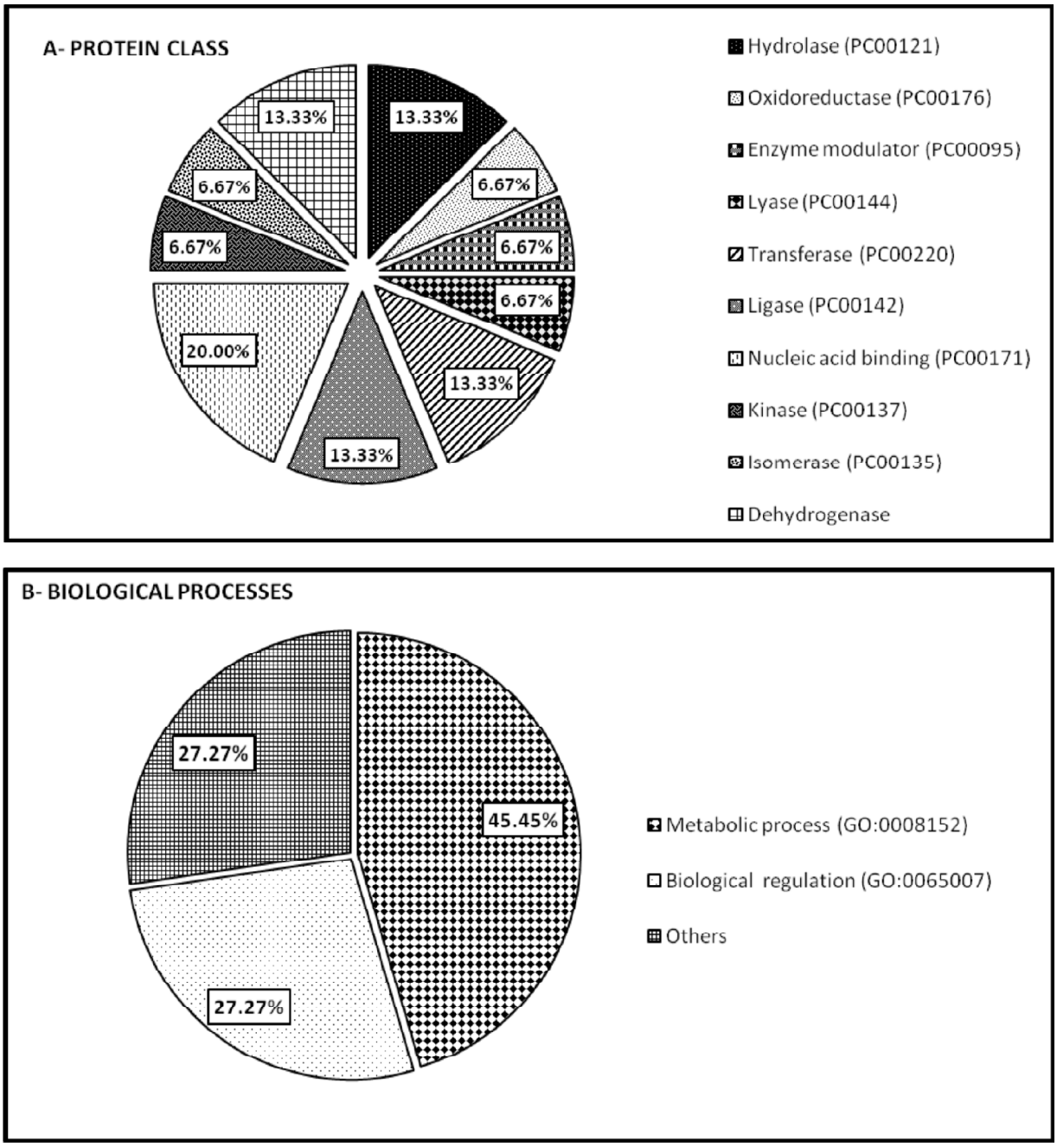

Figure 7: **Classification of identified proteins by PANTHER 9.0**

(A) Protein class and (B) biological processes (Proteins involved in metabolic and biological processes are mentioned in Table 2) Figure 4

associated with adaption and protection against stress (Huang *et al.*, 2011). Our finding is consistent with the effect of bile on *Lactobacillus reuteri*, that carbohydrate catabolism for energy production was enhanced (Lee *et al.*, 2008).

In ABC transporter, the ATP binding protein in BIF-19 was up-regulated as more energy is utilized for the transport of solutes across the membrane. The ABC transport system forms a super family of different membrane proteins including an extracellular substrate-binding protein, two ATP binding/ hydrolyzing subunits and two integral membrane subunits. The extracellular substrate binding protein helps in binding sugars, amino acids, minerals etc. and the extracellular substrate binding protein complex

connects with integral membrane subunits. The ATP binding/ hydrolyzing subunits supply ATP (energy) as a fuel to transport these solutes across cell membrane (Lee and Pi, 2010). ABC transporters are also reported to be involved in transport of harmful compounds through its efflux system and further leads to detoxification (Leverrier and Ridley, 2001). This result suggested that the transporter may possibly perform the exclusion of bile in *L. fermentum* BIF-19 in response to bile stress. Lactoylglutathione lyase also known as glyoxalase I involved in detoxification of methylglyoxal a reactive 2-oxoaldehyde was up-regulated. Methylglyoxal is a by-product of normal biochemistry (obtained as result of action of triosephosphate isomerise on dihydroxyacetone phosphate and methylglyoxal synthase on theronine) that is a carcinogen, mutagen and can chemically damage several components of the cell.

Proteome analysis also revealed the up-regulation of the enzyme-oxidoreductase involved in regulation of cell homeostasis. The same protein was reported to be expressed in a probiotic bacterium isolated from traditional home-made koumiss (Wu *et al.*, 2010). The protein is involved in maintaining the cellular homeostasis by changing the oxidation- reduction potential inside the cell during bile stress. Glucose is more efficient for energy production which is beneficial to cope up bile stress. ATP and reducing equivalents are essential to protect against bile stress, through pumping-out bile, repairing damaged protein and DNA and regulating the internal pH (Haoran *et al.*, 2014).

The protein dihydroorotase involved in nucleotide biosynthesis was down regulated which has been reported to be reduced in acid stress response of *Lactobacillus rhamnosus* GG during mid exponential growth phase (Koponen *et al.*, 2012) probably due to poor growth rate of the organism during stressed condition. The protein bifunctional GMP synthase/glutamine amidotransferase involved in amino acyl-tRNA biosynthesis was down regulated due to decreased biosynthesis of proteins for cell growth during bile stress. However, contrary to our results this enzyme was up-regulated in *Lactobacillus plantarum* during bile stress (Hamon *et al.*, 2011) indicating different lactic strains behaves differently during the stress. The protein phosphoenolpyruvate – protein phosphatase and cellobiose PTS, involved in phosphorylation and the further transport of sugar via PEP dependent sugar transport, was down regulated. This indicates the sugar transport across cell membrane by this system is affected due to the presence of bile salts whereas sugar transport by ABC transporter is activated as explained above. We further observed the down regulation of proteins involved in transcription, translation, folding, sorting and degradation of protein. The proteins such as elongation factor G, ribosomal protein L10, universal stress protein, transcription regulator and threonine-tRNA ligase are involved in transcription and translation. The ribosomal proteins, ribosome recycle factor, elongation factors, asparagine t-RNA ligase are reported to be affected in bile exposure (Lee *et al.*, 2008; Burns *et al.*, 2010; Sanchez *et al.*, 2005). Ribosome-associated proteins are key factors that promote the folding pathways of newly synthesized proteins. Expression of ribosomal protein L10 was found to be decreased by acid stress in *Lactobacillus casei* Zhang (Wu *et al.*, 2010). The increased rate of synthesis of elongation factor and universal stress protein were reported in *Lactobacillus casei* Zhang. In addition to its function in translation elongation, elongation factor Tu has been implicated in protein folding and/or protection from stress in *E. coli* (Caldas *et al.*, 1998). The protein trigger factor having chaperone function and cell division was down regulated. Chaperones guide conformational state of protein for the correct folding, translocation and assembly during modification during misfolding. The proteins/enzymes involved in the reproductive functions were reduced during bile stress, which indicates that the cell reduces its reproductive functioning to balance the system during survival in bile stress. These results can be validated by western blot, but we did not have access to antibodies for the DEPs of interest. Another interesting fact is that although we observed *bsh* gene in BIF-19 and the culture exhibited deconjugation of primary and secondary bile salt, Bile Salt Hydrolase (BSH) enzyme did not detect in differentially expressed proteins. This may be due to the fact that BSH is

produced inside the medium which might have been lost during the cytosolic protein extraction. Nevertheless, combined secretome and cytosolic study can give more comprehensive information.

In general bile stress had changed the protein profile of *L. fermentum* BIF-19 due to cell faces sudden stroke of bile. The metabolic pathway becomes highly activated as the cell requires more energy to survive in bile stress. Apart from this, the cellular homeostasis was up-regulated within the organism under bile stress. Nucleotide synthesis and cell division were suppressed under bile stress in order to balance the internal environment of the organism. Based on this information it is quite difficult to predict involvement of certain key enzymes, however, it has provided very useful information on bile adaptive proteins. The up-regulated proteins which are involved in active phase of metabolism probably help the cell to derive energy in an efficient way under the bile stress. Especially, phosphopyruvate hydratase (+ 1.95) and lactoylglutathione lyase (+1.79) involved in carbohydrate metabolism and phosphoenolpyruvate—protein phosphatase (- 1.93) and trigger factor (- 3.4) involved in transport and folding, sorting and degradation of proteins may be considered as key proteins during bile adaptive stress in *L. fermentum* strains. Further studies on health aspects by use of such strains required to be performed as this findings generated preliminary data on bacterial response to bile stress.

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Abbreviations

ABC, ATP-Binding Cassette; bsh, bile salt hydrolase; BSA, Bovine Serum Albumin; ; cfu, Colony Forming Unit; DEPs, Differentially Expressed Proteins; FAO, Food and Agricultural Organization; GIT, Gastrointestinal Tract; IEF, Isoelectric focusing; MALDI, Matrix-Assisted Laser Desorption/Ionization; MOWSE, Molecular Weight Search; MRS, de Man-Rogosa-Sharpe; MS, Mass Spectrometry; SDS-PAGE, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; TOF, Time of Flight; WHO, World Health Organization.

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