Iranian Journal of Microbiology
Volume 12 Number 5 (October 2020) 431-436

ORIGINAL ARTICLE

Proteomic analysis of *Lactobacillus casei* in response to different pHs using two-dimensional electrophoresis and MALDI TOF mass spectroscopy

Narges Dadfarma[1], Golgis Karimi[2], Jamileh Nowroozi[1], Naser Nejadi[3], Bahram Kazemi[2], Mojgan Bandehpour[4*]

[1]*Department of Microbiology, North Tehran Branch, Islamic Azad University, Tehran, Iran*
[2]*Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[3]*Proteomics Research Center, School of Paramedical Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[4]*Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

Received: May 2020**, Accepted:** August 2020

ABSTRACT

Background and Objectives: *Lactobacillus casei*, an acid-resistant bacterium, has a protective role against the pathogens. So we aimed to determine the proteome of *Lactobacillus casei* ATCC39392 strain in response to different pHs of 5 and 7 using proteomic analysis.
Materials and Methods: Supernatant and bacterial extraction of *Lactobacillus casei* ATCC39392 adapts at pHs 5 and 7 were isolated using sodium dodecyl sulfate–polyacrylamide gel and two-dimensional gel electrophoresis. The comparison of results showed that 7 protein spots were seen in pH 5 but not in pH 7. Afterward, they were excised and sent for Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) to be identified.
Results: Seven different proteins (four secretory and three structural) with different roles in human body health were identified. Prescribed proteins include putative cell wall associated Hydrolase, Glycoside Hydrolase, beta-N-Acetyl hexosaminidase, Histidine Kinase, Chaperonin, metal dependent Hydrolase and Lysozyme.
Conclusion: Seven isolated proteins with anti-cancer and digestive impresses are proper subjects in therapy or drug delivery approaches especially oral drug usage for protection against stomach acidic area.

Keywords: *Lactobacillus casei*; Acid tolerance; Anti-cancer; Digestive impresses; Two-dimensional gel; Mass spectrometry

INTRODUCTION

Lactobacillus casei is a Gram positive bacterium that is found at different sites of the body such as intestine and mouth (1); therefore, it is resistant to a wide range of pH and temperature. This particular species of *Lactobacillus* is dominantly used in the dairy products industry, including cheese and fermented milk as a nonstarter lactic acid bacteria (2, 3). The beneficial health effects of this bacterium as a probiotic is well documented in particular in animal studies (4). Briefly, *L. casei* by sticking to the intestinal wall, the site that intestinal lymph tissue is stimulated, regulates the immune system. Besides, there is a competition between *L. casei* and pathogens for food and space. Therefore, *L. casei* has a

*Corresponding author: Mojgan Bandehpour, Ph.D, 4 Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
Tel: +98-021-22439957
Fax: +98-021-22439956
Email: bandehpour@gmail.com

protective role against the pathogens. On top of this, the bacteria produce acids which in turn reduce the pH and lead to a reduction in the pathogen's bacteria growth. Bacteriocins are the other compounds of *L. casei*, which ruins the pathogens (5).

One of the potential criteria of *L. casei*, which makes it a good strain to be used in probiotic supplements is its acid tolerance (6). This feature is an essential factor as after consuming probiotics, they confronted with a strongly acidic environment in the stomach during the transition into the intestine (7). One of the useful tools which have been recently used widely to analyze information regarding the metabolites changes in the network of *Lactobacillus* is proteomics (6). Bacterial surface and extracellular proteins have a growing interest in the study of bacteria–host relationships, whether pathogenic or host beneficial. This interest caused an increase in the use of proteomics as a significant tool in the early characterization of bacteria. Therefore, in the present study we aimed to analyze the proteome of *L. casei ATCC39392* in pHs of 7 and 5 using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional electrophoresis and MALDI TOF (Matrix-Assisted Laser Desorption-Ionization-Time of Flight) mass spectroscopy.

MATERIALS AND METHODS

Strain and growth condition. *L. casei ATCC39392* was cultured in De Man Rogosa and Sharp (MRS) broth (27.57 grams per Liter) (ibresco Life Science, Iran). The amount of 60cc of MRS broth media transferred to a sterile Erlenmeyer and 300 λ of hydrochloric acid added to the broth to pH5. In the next step 100 λ of bacteria stock transferred to the acidic media and neutral one (pH 7) and incubated at 37°C for one week. The cultures were harvested by centrifugation (8000 rpm, 5 min, 4°C) at the late exponential growth phase (OD_{600}:0.6).

Identification of bacteria. To determine the morphologic characteristics of the colonies on MRS, gram staining was carried out. Also, we confirmed the bacterial species of *L. casei*, by specific amplification of a 666 base pairs fragment of the genome by CZF: 5'-CAGCGCTGGTGGAAGACTTG-3' and CPR:5'-TGCATTTCCCCGCTTTCATGACT-3' primers. The PCR reaction was prepared in a final volume of 25 µL that contained 1 µg extracted DNA, 13 µL Taq 2× Master Mix RED (Ampliqon), 1 pmol of each primer. PCR was performed as follows: 95°C for 5 min, 95°C for 30s, 60°C for 30 s, 72°C for 30 s and 30 cycles of extension. The PCR products were visualized in agarose gel electrophoresis with SyBR Green II staining.

Proteins preparation and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Bacterial cells grown in MRS at different initial pH levels for one week were harvested by centrifugation (8000 rpm, 5 min, 4°C) at the late exponential growth phase [approximately 10^9 colony forming units (CFU) mL^{-1}] (OD_{600}:0.6). The supernatant containing the secretion proteins was precipitated with 2 mL methanol 10% and stored at -20°C for further analysis. Then 100 µL of lysis protein buffer containing Tris 50 mµ, Glycerol 50%, Triton x-100 0.1%, and PMSF 1 mµ, and 2 µL of PMSF was added to the depositions from the centrifuge. After that, the samples were sonicated for 40 cycles, each lasted 30 seconds. Then, samples were mixed by 200 µL of acetone and stored at -20ºC for further analysis. After 24 hours, samples were centrifuged at 8000 rpm for 5 minutes. Then, supernatants were discarded, and 30 µL of deionized water and 1X of SDS-loading were added to each one and mixed gently. At last, all prepared samples were kept in thermoblock at 85°C for 10 minutes. Then electrophoresis was run on prepared SDS-PAGE 12% under the following condition; voltage=90, current=37, power=3.32. Afterwards, proteins were fixed by immersing the gels in an aqueous of 10% (v/v) trichloroacetic solution for 1 h and stained overnight in Coomassie blue stain [0.25% (w/v) Coomassie blue R-250, 50% (v/v) methanol and 10% (v/v) acetic acid]. After destaining the gel with a solution of 25% (v/v) methanol and 10% (v/v) acetic acid, the gel was visualized using a lightbox.

Two-dimensional polyacrylamide gel electrophoresis (2DE). Seventeen-centimeter immobilized pH gradient (IPG) strips with the nonlinear range of pH 3-10 (BioRad, USA) were used to perform isoelectric focusing (IEF). Rehydration step of IPG strips has been done overnight by loading of about 1 mg of protein extracts to a 300 µL total volume of rehydration buffer including 7 M urea, 2 M thiourea, 4% CHAPS, 0.2% Bio-Lyte pH 3-10, 50 mM DTT, and a trace amount of bromophenol blue. The pro-

gram of Protean IEF cell (BioRad) for focusing was the linear increase from 0 to 250 V for 20 min, followed by a linear increase to 10,000 V, and remained on 10,000 V to achieve a total 50,000 V h. Afterward, the IPG strips were equilibrated by a buffer containing 50 mM Tris-HCL–HCl pH 8.8, 6 M urea, 20% glycerol, 2% SDS, 0.01% bromophenol blue and 2% DTT for 15 min. Then, an equilibration buffer devoid of DTT which was supplemented with 2.5% iodoacetamide used to alkylated samples (15 min). For the second dimension of electrophoresis, equilibrated strips were placed on top of the handmade 12% SDS-PAGE gel slabs and sealing with 1% agarose. Electrophoresis was run using the standard Laemmli buffer system under the following condition; 16 mA/gel for 30 min and 24 mA/gel for approximately 5 h at 16°C until the bromophenol blue was 1 cm above the bottom of the gel. Lastly, a sensitive colloidal Coomassie Brilliant Blue G 250 (CCB) method was used to stain the gels.

Proteins identification by MALTI TOF mass spectrometry. We have compared the 2DE gels of proteome extraction of *L. casei* at pHs of 7 and 5. There were seven spots of differences between them. The seven interested gel slices were firstly washed and were reduced using DTT. Right alkylation by iodoacetamide they were incubated at 37°C with 125 ng sequencing grade trypsin (Promega) for an overnight. Each tryptic digest was analyzed by MALTI TOF mass spectrometry for the protein spots identification. Results analysis was performed using a mascotserver (http://www.mascot.org) against a protein database. Proteomic analysis was performed for whole-cell protein lysates from early-stationary phase of *L. casei* cultured in standard (MRSC, initial pH 7) and stressing (MRSC initially adjusted to pH 5) conditions.

RESULTS

Culture and Identification of bacteria. The obtained bacterium was approved by specific PCR. A PCR product of approximately 670 bp was considered to be the target gene. So the confirmed strain as *L. casei ATCC39392* has cultivated in the De Man Rogosa and Sharp (MRS) broth in two pHs 5 and 7 that showed the resistance of the bacteria to even more acidic conditions. Fig. 1 demonstrates the PCR product fragment amplification and confirms the genus and species of *L. casei*.

Bacterial proteome isolation at acidic pH. The results of the present study indicate remarkable changes in the cellular proteome of *L. casei ATCC39392* cultured under anaerobic and pH-control conditions at 37°C. Quantitative proteomic analysis of secretory proteins revealed that some of the proteins which mainly stressed response proteins were affected by pH. In other words, lower pH (pH5) upregulates the

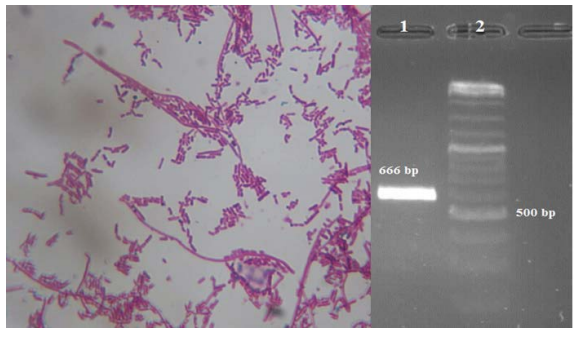

Fig. 1. Genus and species of *L. casei* confirmation, Left) Gram staining smear of *L. casei* culture, Right) Agarose gel electrophoresis of PCR product

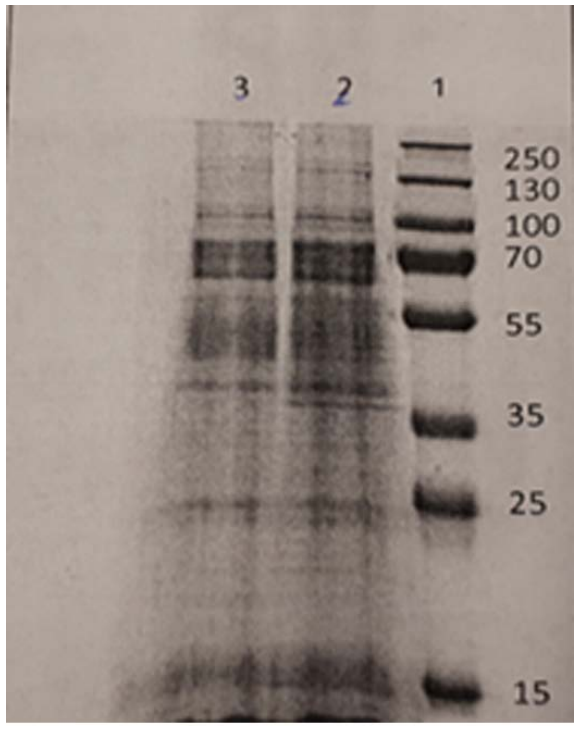

Fig. 2. Proteome analysis of *L. casei* using SDS-PAGE in response to pH 5 & 7 (lane1: Protein Marker, lane 2: pH 5, lane 3: pH 7)

secretion of enzymes in the proteolytic pathway. SDS-PAGE electrophoresis analysis of bacterial proteins cultured in two different pHs showed different protein patterns (Fig. 2).

The results of the Two-dimensional electrophoresis of bacterial total protein samples are indicated in Fig. 3. The gel images show the difference between structural and secretory proteins in two various culture conditions. The images analyzed with Image Master software recognized more than 100 differentially expressed proteins. The seven spots with the highest concentration were identified with mass spectroscopy.

Identification of protein spots. Analysis of seven isolated spots at pH 5 culture condition recognized by MALDI TOF mass spectroscopy. They obtained from mascot server analysis. Specifications of detected proteins are described in Table 1.

DISCUSSION

Following the role of *L. casei* as normal flora at acidic pH of the intestine and genital tracts, we decided to determine the difference between expressed bacterial proteins at two growth conditions. Therefore, proteomics of secretory and structural proteins isolated from this strain was used to characterize the strain response cultured at two different pHs 5 and 7. One of the acid-resistant proteins identified in this study belongs to the clan MA, Subclan MA (E), family M41. Includes the FtsH endopeptidase, which is known from bacteria, mitochondria, and chloroplasts. FtsHis a metalloprotease that is associate with ATP-dependent zinc for cytoplasmic and membrane proteins. A large number of earlier investigations studied on this protein in *Escherichia coli.* They reported that this protein plays a role in many biological processes including quality control of protein by degrading out-of-membrane proteins like the subunit alpha of the F1F0 ATP synthase complex and the type 2 secretion system translocon protein SecY and involved in choosing between lysis and lysogeny upon bacteriophage l infection, LPS biosynthesis, and cell division (8). FtsHis a homohexamer in the inner membrane. When a substrate degrades in the proteolytic chamber of the protease, FtsH uses its ATPase to unfold and translocate it. Therefore, misfolded and proper folded proteins are removed by Ftsh due to quality control or regulatory reasons, respectively. FtsH is considered as a key role protein in *E. coli* due to its role in quality control and other related processes (9).

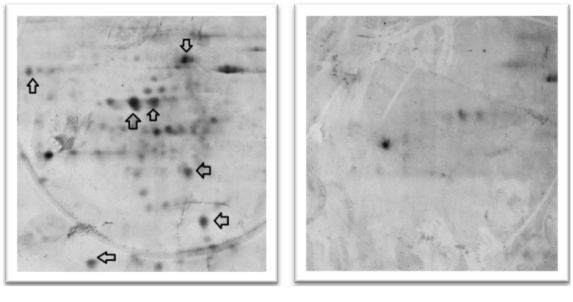

Fig. 3. Proteome analysis of *L. casei ATCC39392* in response to different pHs using two-dimensional electrophoresis. Analysis of images with Image Master software showed 7 green spots at pH5 (left) whereas they were not recognized in pH 7 (right) (arrows).

Recent studies identified genes involved in *Lactobacillus* stress responses, such as the methionine sulfoxide reductase (oxygen stress) (10), F1F0-ATPase (acid stress) genes (11), and molecular chaperone *groESL* and *dnaK* (heat stress). Hossein Nezhad et al. indicate a significant association between surface proteins of *L. casei* strain GCRL 12 with growth and survival at low pH (12). The results of the current study identified one secretory protein, which is re-

Table 1. Identified proteins from *L. casei ATCC39392* that were different in response to pH

Detected protein	pH 7	pH 5	Molecular Mass (kDa)	Location
putative cell wall associated Hydrolase	✓	--	85	Structure
Glycoside hydrolase	--	✓	70	Structure
beta-N-Acetyl hexosaminidase	--	✓	60	Structure
Histidine kinase	--	✓	40	Structure
Chaperonin	--	✓	80	Secretory
metal dependent Hydrolase	--	✓	60	Secretory
Lysosyme	--	✓	70	Secretory

sponsible for ATCC39392 to survive under low pH.

The protective effect of substances on the acid tolerance of *L. casei* was monitored by some earlier studies. Wu et al. (2010) elucidate exogenous aspartate increased the growth performance and survival rate of *L. casei* during acid stress. According to the results of the physiological data, cells supplemented by aspartate had higher intracellular NH4+ content, intracellular pH (pHi), H+-ATPase activity, and intracellular ATP pool (13). In some studies, variations in strains that respond to acid stress are seen in comparison to strains that are not exposed to acid stress. Scanning electron microscope showed that the surface of the stressed cell became unevenly rougher than unstressed ones. The authors speculated that it is caused by the induced expression of those surface proteins. Results of the comparison between the stress response of the parental strain *L. casei* Zhang and the acid-resistant mutant that obtained through adaptive evolution and represented increased acid tolerance was shown that the evolved mutant lb-2 exhibited an increase in concentrations of lactate and acetate, higher survival rate, higher intracellular pH (pHi) and NH4+ concentration, lower inner membrane permeability, higher amounts of intracellular arginine and aspartate than that of the parental strain. A new insight about proteins that are expressed by *L. casei* under stress conditions suggests a new mechanism perspective involved in bacteria adaptation and stress tolerance. These proteins have been identified to increase bacterial surviving under stress conditions (14). Although, surface and extracellular proteins of lactobacilli have important roles in the functioning of probiotics, little is known about the effect of stress on the secretome (15). In fact, recent studies mostly focused on S-layer producing species (16). One of the possible and important mechanisms in LAB for energy production in terms of ATP under stress condition is via modulating protein pathways of glycolytic (17). This modulation is species and stress type-dependent (18). The anti-cancer effect of *L. casei ATCC 393* has been shown in earlier studies. Tiptiri-Kourpeti et al. (2015) indicated apoptotic cell death with live *L. casei* and a decrease in viability of colon cancer cells which were affected by *L. casei* for 24 hours. They also indicated that 13 days of oral administration of *L. casei* was significantly suppressed the colon cancer cells'growth. All in all, these findings provide beneficial health evidence for LAB strains including tumor-inhibitory, anti-proliferative, and pro-apoptotic (19). Besides, glycoside hydrolase which identified in the present study has digestive function including catalyzing the hydrolysis of glycosidic bonds in complex sugars (20). This protein plays a wide range of enzymatic roles including anti-bacterial defense strategies (e.g., lysozyme), degradation of cellulose, and starch (amylase), normal cellular function, and pathogenesis mechanisms (e.g., viral neuraminidases). Besides, β-*N*-acetyl-D-hexosaminidases catalyze the removal of *N*-acetyl-D-galactosamine (GalNAc) or *N*-acetyl-D-glucosamine (GlcNAc) from the non-reducing ends of oligosaccharides, glycoproteins, and glycolipids (21).

The acidic pH resistant proteins of *L. casei* can be used for targeted drug delivery or drug design in future studies.

CONCLUSION

According to different expression proteins pattern in *Lactobacillus casei* in two pHs 5 and 7, we can suggest this bacteria or acid resistance proteins can be used in drug delivery programs.

ACKNOWLEDGEMENTS

This study was funded by the National Elites Foundation and Shahid Beheshti University of Medical Sciences.

REFERENCES

1. Morata de Ambrosini VI, Gonzalez SN, Oliver G. Study of adhesion of *Lactobacillus casei* CRL 431 toileal intestinal cells of mice. *J Food Prot* 1999;62: 1430-1434.
2. Adu KT, Wilson R, Nichols DS, Baker AL, Bowman JP, Britz ML. Proteomic analysis of *Lactobacillus casei* GCRL163 cell-free extracts reveals a SecB homolog and other biomarkers of prolonged heat stress. *PLoS One* 2018; 13(10): e0206317.
3. Banks JM, Williams A. The role of the nonstarter lactic acid bacteria in Cheddar cheese ripening. *Int J Dairy Technol* 2004; 57: 145-152.
4. Wang Y, Xie J, Li Y, Dong S, Liu H, Chen J, et al. Probiotic *Lactobacillus casei* Zhang reduces pro-inflam-

matory cytokine production and hepatic inflammation in a rat model of acute liver failure. *Eur J Nutr* 2016;55: 821-831.
5. Aktas B, De Wolfe TJ, Tandee K, Safdar N, Darien BJ, Steele JL. The effect of *Lactobacillus casei* 32G on the mouse cecum microbiota and innate immune response is dose and time dependent. *PLoS One* 2015;10(12): e0145784.
6. Wu R, Zhang W, Sun T, Wu J, Yue X, Meng H, et al. Proteomic analysis of responses of a new probiotic bacterium *Lactobacillus casei* Zhang to low acid stress. *Int J Food Microbiol* 2011;147: 181-187.
7. Cotter PD, Hill C. Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiol Mol Biol Rev* 2003;67: 429-453.
8. Kamal SM, Rybtke ML, Nimtz M, Giske C, Trček J, Dechamps J, et al. Two FtsH proteases contribute to fitness and adaptation of *Pseudomonas aeruginosa* clone C strains. *Front Microbiol* 2019;10: 1372.
9. Biswas S, Keightley A, Biswas I. Characterization of a stress tolerance-defective mutant of *Lactobacillus rhamnosus LRB*. *Mol Oral Microbiol* 2019;34:153-167.
10. Kilstrup M, Jacobsen S, Hammer K, Vogensen FK. Induction of heat shock proteins DnaK, GroEL, and GroES by salt stress in *Lactococcus lactis*. *Appl Environ Microbiol* 1997;63: 1826-1837.
11. Al-Naseri A, Bowman JP, Wilson R, Nilsson RE, Britz ML. Impact of lactose starvation on the physiology of *Lactobacillus casei GCRL163* in the presence or absence of tween 80. *J Proteome Res* 2013;12: 5313-5322.
12. Hosseini Nezhad M, Hussain MA, Britz ML. Stress responses in probiotic *Lactobacillus casei*. *Crit Rev Food Sci Nutr* 2015;55: 740-749.
13. Wu R, Sun Z, Wu J, Meng H, Zhang H. Effect of bile salts stress on protein synthesis of *Lactobacillus casei* Zhang revealed by 2-dimensional gel electrophoresis. *J Dairy Sci* 2010;93: 3858-3868.
14. Zhang J, Wu C, Du G, Chen J. Enhanced acid tolerance in *Lactobacillus casei* by adaptive evolution and compared stress response during acid stress. *Biotechnol. Bioprocess Eng* 2012;17: 283-289.
15. Kleerebezem M, Hols P, Bernard E, Rolain T, Zhou M, Siezen RJ, et al. The extracellular biology of the lactobacilli. *FEMS Microbiol Rev* 2010;34: 199-230.
16. Klotz C, O'Flaherty S, Goh YJ, Barrangou R. Investigating the effect of growth phase on the surface-layer associated proteome of *Lactobacillus acidophilus* using quantitative proteomics. *Front Microbiol* 2017; 8: 2174.
17. Hahne H, Mäder U, Otto A, Bonn F, Steil L, Bremer E, et al. A comprehensive proteomics and transcriptomics analysis of *Bacillus subtilis* salt stress adaptation. *J Bacteriol* 2009;192: 870-882.
18. Papadimitriou K, Alegría Á, Bron PA, De Angelis M, Gobbetti M, Kleerebezem M, et al. Stress physiology of lactic acid bacteria. *Microbiol Mol Biol Rev* 2016;80: 837-890.
19. Tiptiri-Kourpeti A, Spyridopoulou K, Santarmaki V, Aindelis G, Tompoulidou E, Lamprianidou EE, et al. *Lactobacillus casei* exerts anti-proliferative effects accompanied by apoptotic cell death and up-regulation of TRAIL in colon carcinoma cells. *PLoS One* 2016;11(2):e0147960.
20. Bourne Y, Henrissat B. Glycoside hydrolases and glycosyltransferases: families and functional modules. *Curr Opin Struct Biol* 2001;11: 593-600.
21. Liu F, Liu T, Qu M, Yang Q. Molecular and biochemical characterization of a novel β-N-acetyl-D-hexosaminidase with broad substrate-spectrum from the Aisan corn borer, Ostrinia furnacalis. *Int J Biol Sci* 2012;8: 1085-1096.