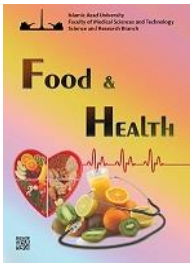

Food & Health Journal
Journal homepage: fh.srbiau.ac.ir

Auto and co-aggregation, hydrophobicity and adhesion properties of *Lactobacillus plantarum* strains isolated from Siahmazgi traditional cheese

Hassan Gandomi [1*], Azra Farhangfar [1], Afshin Akhondzadeh Basti [1], Ali Misaghi [1], Negin Noori [1]

[1] Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

ARTICLE INFO

Original Article

Article history:
Received 02 March 2019
Revised 01 May 2019
Accepted 06 May 2019
Available online 15 May 2019

Keywords:
Adhesion
Auto and co-aggregation
Hydrophobicity
Lactobacillus plantarum
Siahmazgi cheese

ABSTRACT

Lactobacillus plantarum was the most common species in the microflora of artisanal Siahmazgi white brined cheese with 41.6% occurrence among the total isolated LAB. In this study, the attachment properties of 5 different *L. plantarum* strains isolated from Siahmazgi traditional cheese were evaluated by in vitro tests including auto and co-aggregation, hydrophobicity, and cell adhesion. A relatively high amount of auto-aggregation ranged from 58.21 to 73.99% was seen in selected isolates. Co-aggregation was highly variable from 1.46 to 49%, depending on the pathogenic bacteria and *L. plantarum* isolates. Hydrophobicity was also highly different in tested strains ranging from 6.58 to 73.3%. Two isolates showed great affinity about 73% to chloroform. All the isolates presented high adhesion to Caco-2 cell line up to about 90%. In conclusion, five *L. plantarum* isolates showed appropriate attachment properties and could be good candidates for further studying, including safety evaluation, that support their use as probiotics.

1. Introduction

Probiotic food products are considered as functional foods which can improve consumers' health. According to the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), probiotics are live microorganisms which when administered in adequate amounts, confer health benefits to their host (1). Several lactic acid bacteria (LAB) strains are known to be beneficial for their host and have been chosen for use as probiotics. They are human gastrointestinal (GI) microflora and are classified as GRAS (2). Commonly used probiotic bacteria include several species of *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Lactococcus* and some species of *Enterococcus* (1). Among them, *Lactobacilli* are one of the main genera of LAB known for probiotic characteristics. They are found in a wide range of food products. *Lactobacillus plantarum* is a common inhabitant of the human GI tract and some strains are used as ingredients of probiotic foods (3). In order to meet the growing demand of the market, especially in developed countries, to produce the functional food products with active probiotic cultures, assessment of new probiotic strains is of great importance. It is necessary to screen and characterize numerous strains to obtain an ideal probiotic culture since not all lactic acid bacteria possess the health benefits for the host (4). In this way, there is a great interest in indigenous and traditional fermented foods from non-industrialized countries, especially dairy products, as a reservoir to search for new *Lactobacillus* strains with novel functional properties (5). For the beneficial impact on the health of the host, probiotic cultures must be ingested in sufficient amount (10^6 to 10^7 CFU/g). Probiotic microorganisms should overcome the physical and chemical barriers, especially the acid and bile stress as well as pancreatic enzymes during the gastrointestinal transition. It is known that adhesion of probiotic microorganism to the intestinal epithelium is needed for their colonization on the gastrointestinal tract epithelium and play an important role in preventing bacterial elimination by peristalsis and providing a competitive advantage in the ecosystem (6). Furthermore, auto-aggregation is suggested to be necessary for adhesion of probiotic microorganisms to the intestinal epithelium, and co-aggregation potential may

*Corresponding authors: Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.
Email address: gandomih@ut.ac.ir (Hassan Gandomi)

prevent colonization by pathogens (2). Moreover, a correlation hydrophobicity and adhesion ability have been previously reported (7). Siahmazgi cheese is a traditional cheese produced from raw sheep and goat milk in Northern provinces of Iran. This cheese ripens in bags made of sheep skin called Khik. No starter culture is added to raw milk during the production of this type of cheese and the process of ripening is conducted only by the indigenous flora. It is a popular traditional product among northern people due to its delicious taste and belief that it improves health. In the previous study, we identified the LAB flora isolated from this cheese using biochemical methods and 16s rDNA analysis (8). *Lactobacillus plantarum* was the most common species in the microflora of artisanal Siahmazgi white brined cheese with 41.6% occurrence among the total isolated LAB. The resistance properties of the isolates against acid, bile and gastrointestinal conditions were investigated in the previous study (Veterinary Research Forum accepted article). In this study, we aimed to evaluate the attachment properties of the *L. plantarum* strains isolated from Siahmazgi cheese, through the study of their auto-aggregation and co-aggregation, their hydrophobicity and their adherence to Caco-2 cell line.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Five *L. plantarum* strains used in this study, which were previously isolated from Siahmazgi traditional cheese and molecularly identified by the Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, University of Tehran (8). The isolates were grown in de man rogosa sharpe (MRS) broth (Merck, Darmstadt, Germany) at 37˚C for 24 hours. Two pathogenic strains (*Listeria monocytogenes* ATCC19118 and *Salmonella* Typhimurium ATCC14028) were also supplied by the Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, University of Tehran. They were grown in brain-heart infusion (BHI) broth (Merck, Darmstadt, Germany) in aerobic condition at 37˚C for 18 h.

2.2. Auto-aggregation and co-aggregation assay

Four mL of overnight culture containing 10^8 CFU/mL of selected *L. plantarum* strains was vortexed and incubated for 5 hours at room temperature. At intervals of 1 hour, 0.1 mL of the supernatant was removed and transferred to a new tube containing 3.9 mL PBS and then the optical density was measured at 600 nm. The auto-aggregation percentage was calculated as:

$$\textit{Auto-aggregation (\%)} = 1-(A_0 / A_t) \times 100$$

A_0: The optical density at 0 h
A_t: The optical density at 5 h

In order to assess the co-aggregation, equal volumes (500 µL) of various strains and each pathogen (*Salmonella* Typhimurium and *Listeria monocytogenes*) were mixed and incubated at room temperature. Absorbance values at 600 nm were measured during 5 hours of incubation and the percentage of co-aggregation was determined as:

$$\textit{Co-aggregation (\%)} = [(A_{pa} + A_{pr}) / 2 \times (A_{mix})] / (A_{pa} + A_{pr}) / 2 \times 100$$

A_{pa} and A_{pr} represent A600 of pathogens and *L. plantarum* strains, respectively and A_{mix} denotes A600 of the pathogens and *Lactobacillus* mixture.

2.3. Cell surface hydrophobicity

Percentage of cell surface hydrophobicity of bacterial isolates to hydrocarbons including xylene, chloroform, and toluene was assessed according to the method of (9). The cell pellets were harvested by centrifugation and washed 2 times with PBS and then a suspension of 10^8 CFU m/L was prepared in 3 mL 0.1 Mol/L KNO3. The optical density of the mixture was measured at 600 nm (A_0). One milliliter of the hydrocarbons was added to the mixture, vortexed for 5 minutes and incubated for 20 minutes at room temperature. After removing the aqueous phase, the optical density was measured at 600 nm (A_1). The percentage of the cell surface hydrophobicity (H %) was calculated as follows:

$$H\ \% = (1 - A_1/A_0) \times 100$$

2.4. Caco-2 adhesion

Adhesion of the *L. plantarum* strains to the human colon adenocarcinoma cell line was evaluated according to Chen et al. (9) with some modifications. The human intestinal Caco-2 cell line was procured from Pasteur Institute of Iran. The test was done on a 24-well tissue culture plate. The Caco-2 cells were seeded in 24-well plates with Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, USA) containing supplement incubated in 7% CO_2 at 37 °C for 13 days till the cell concentration reaches almost 80% confluence. The medium was replaced with fresh medium every 2 days. Twenty-four h before an adhesion assay, an antibiotic-free medium was used. Caco-2 cells were washed 2 times with PBS and 2 ml of DMEM without supplement was added to each well. Bacterial suspension of *L. plantarum* strains was harvested by centrifugation at 6000g for 15 min. The cell pellets were washed three times with PBS and then suspended in DMEM. The final concentration of bacteria was adjusted to 10^8 CFU m/L. Then, 1 mL suspension was added to Caco-2 monolayers. After 2 hours incubation in aerobic conditions at 25°C, monolayer was washed 3 times with PBS to detach the non-adhered bacteria. The cell line was lysed by triton X100. The suspension was plated on MRS agar plates to measure adhesion percentage.

2.5. Statistical analysis

Each experiment was replicated three times and data were

expressed as MEAN±SE. One-way ANOVA was used to compare different mean groups using SPSS version 229 (IBM Company, New York, United States). Tukey test used to assess significant differences between the groups $p<0.05$ was considered as significant.

3. Results and discussion

The results of the auto-aggregation test are shown in Fig. 1. All tested *Lactobacillus* strains revealed a high amount of auto-aggregation after 5 hours. F19 and F7 strains showed the highest (73.99%) and the lowest (58.21%) level of auto-aggregation, respectively. The auto-aggregation ability is one of the key factors that determine the ability of the probiotic strain to adhere to the oral cavity, gastrointestinal tract, and urogenital tract (10). All the *L. plantarum* strains studied in the present work revealed great auto- aggregation up to 74%. The auto-aggregation levels seen in this study are greater than those reported by Kaushik et al. (11) which was found to be 31% for indigenous *L. plantarum* Lp9, and, 40, and 46 % for standard probiotic *L. johnsonii* LA1 and *L. acidophilus* LA7, respectively. It has been reported that the auto-aggregation ability is associated with cell adhesion properties (12, 13).

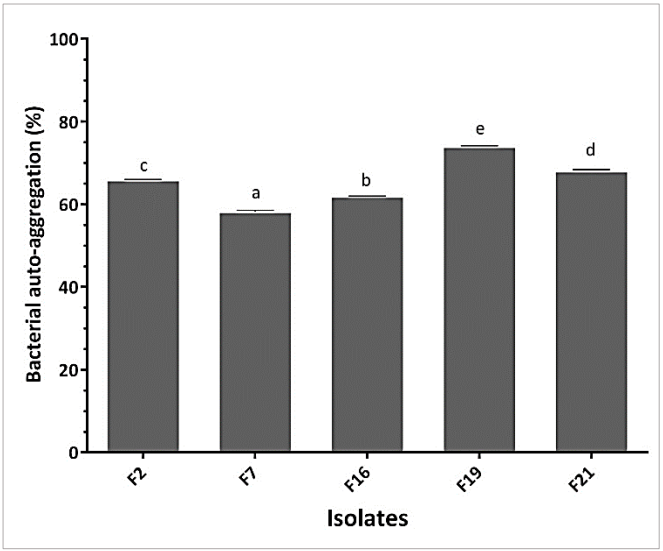

Fig. 1. Auto-aggregation ability of the selected *L. plantarum* strains isolated from Siahmazgi cheese. Values shown with different letters are statistically different ($p<0.05$).

The results of the co-aggregation test are shown in Fig.2. F16 and F7 Strains displayed a significantly higher percentage of co-aggregation against *S.* Typhimurium with 49.04 and 29.55% co-aggregation, respectively. The isolate *L. plantarum* F2 showed maximum adherence to *E. coli* and *L. monocytogenes* with 29.6 and 29.3% co-aggregation, respectively, while its co-aggregation with *S.* Typhimurium was only 1.46%. Furthermore, F21 isolate showed the lowest co-aggregation (2.66%) against *S. aureus*. Probiotic microorganisms that have the ability to co-aggregate with pathogens may be better able to kill undesirable bacteria because they could produce antimicrobial substances in very close proximity to them (14). The results of this study revealed that co-aggregation of the potential probiotic strains with bacterial pathogens was variable and depended on the species or strains which are in consistence with previous works (15, 16, 17). Furthermore, each isolate showed different amounts of co-aggregation with different bacterial pathogens. The ability of co-aggregation can be of great importance for *Lactobacillus* strains to inhibit the pathogen adhesion to the surface of epithelial cells and interferes with pathogen colonization (18).

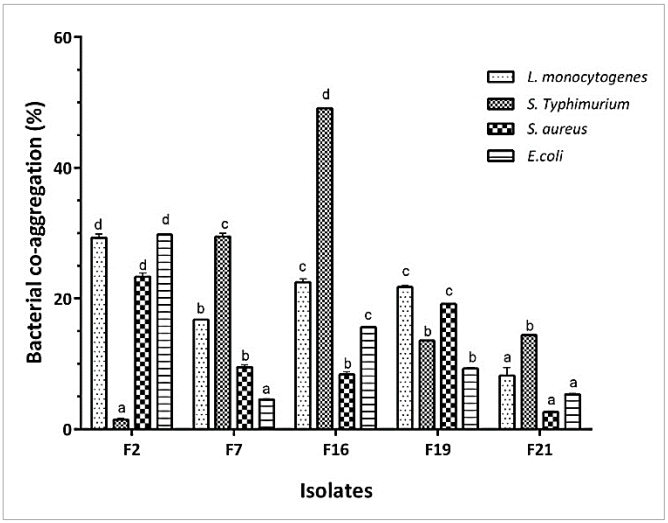

Fig. 2. Co-aggregation ability of the selected *L. plantarum* strains isolated from Siahmazgi cheese. Different letters indicate a significant difference between co-aggregation of each pathogen with different isolates ($p<0.05$).

The results of cell surface hydrophobicity of the isolates are shown in Fig. 3. Hydrophobicity was highly variable in tested strains ranging from 6.58 to 73.3%. The affinity of the selected isolates to xylene varied between 31.7 and 56.4%. *Lactobacillus plantarum* F2 and F7 strains had a high percentage of affinity to chloroform which were 73.3 and 72.3%, respectively, while strain F2 was most adherent to xylene with 56.5% hydrophobicity. Ability to adhere to the mucosa or intestinal epithelial cells is another important feature of the probiotic strains enables them to remain and colonize in the intestine (19). This ability prevents the removal of strains by the intestinal peristalsis contractions. Cell surface hydrophobicity can influence the adhesion of bacterial cells to the host cell layer. Adhesion to hydrocarbons like xylene, toluene, and n-hexadecane is considered as a biochemical marker for adhering to the gut epithelial cells. Xylene and toluene are polar solvents which indicate the hydrophobicity of the probiotic cells. *L. plantarum* isolates examined in the present work exhibited 31 to 56% hydrophobicity to xylene which is comparable to the hydrophobicity of standard probiotics *L. johnsonii* LA1 (46 %) and *L. acidophilus* LA7 (58 %) reported by Kaushik et al. (11). Bacterial cells with high hydrophobic properties usually form strong interactions with mucosal cells. Hydrophobic and hydrophilic appendices and other macromolecules can influence the cell surface

hydrophobicity to the hydrocarbon (19). It is stated that the presence of (glyco-) proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are associated with the presence of polysaccharides (20). In this study, there was no correlation between the amount of auto-aggregation and hydrophobicity which was previously mentioned in other studies (21, 22). Furthermore, the great affinity of the selected isolates to chloroform which is a polar acidic solvent describes the electron donor properties of the bacterial cell surface.

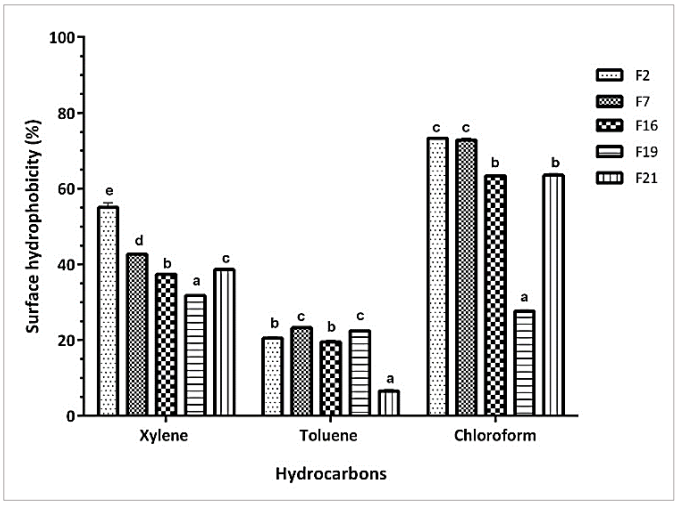

Fig. 3. Hydrophobicity (%) of the selected *L. plantarum* strains isolated from Siahmazgi cheese to different hydrocarbons. Values shown with different letters are statistically different ($p<0.05$).

The results of the isolates cell adhesion to Caco-2 cells are shown in Fig. 4. All the strains showed great adhesion ability. The most adhesive strain was F21 (89.29%), while the F19 strain showed the lowest adhesion percentage (73.75%). Although it is increasingly clear that probiotics can provide beneficial effects even without true colonization of the gastrointestinal tract, adhering to human intestinal epithelial cells confer beneficial effects to the host including inhibition of pathogen colonization in the gut and modulation of the immune system (23). Thus, the ability of the probiotics to adhere the GI epithelium is one of the important criteria for choosing potential probiotic bacteria. In this study, we evaluated the adhesion properties of the selected *L. plantarum* to Caco-2 cell line as this cell line exhibits various enterocytic characteristics in vitro (24). Although its tumoral nature can somehow limit the extrapolation of the results, this cell line offers a relevant tool for in vitro screening (3). The results of the adhesion assay showed excellent adhesion (higher than 70%) of the selected *L. plantarum* isolates to Caco-2 cells. The adhesion ability of the *Lactobacillus* strains reported by previous studies varied from 10-20% for *Lactobacillus* strains isolated from Uttapam batter (25) to 30-74% for *L. plantarum* strains isolated from a traditional fermented Korean beverage (1) and about 90% for *L. plantarum* isolated from quinoa seed (26). The adhesion ability of the present isolates is higher than reported values of 6.7% for *L. plantarum* ATCC 8014 (27) and 8.5% for *L. plantarum* 122E (28).

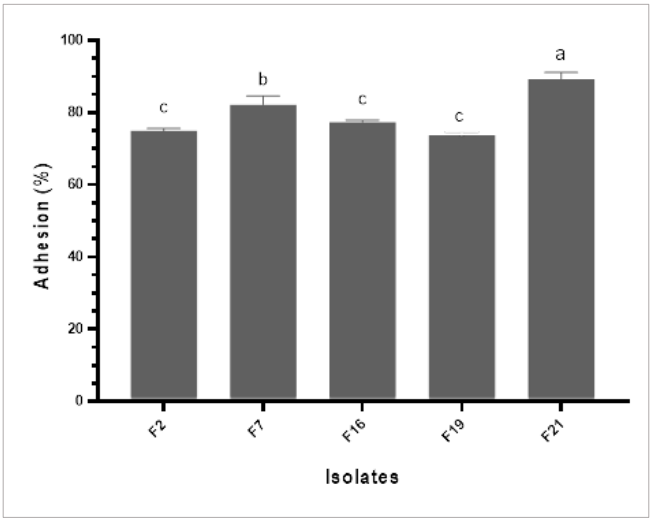

Fig. 4. The adhesion ability of the *L. plantarum* strains isolated from Siahmazgi cheese to Caco-2 cell line. Values shown with different letters are statistically different ($p<0.05$).

Furthermore, these selected *L. plantarum* isolates showed more adhesion to Caco-2 cells compared to standard probiotic *L. casei* Shirota and *L. rhamnosus* GG which presented adhesion values lower than 40% (1). Moreover, it was revealed that the adhesion ability of the selected *L. plantarum* isolates was strain dependent. This strain-dependent manner with the adhesion ability of bacterial strains belonging to the same genus/species is generally assumed for probiotic bacteria (29). Cell adhesion is a multistep process involving contact of the bacterial cell membrane and interacting surfaces (23). The adhesion mechanisms are not fully understood, however bacterial cell-surface associated proteins with mucus and intestinal cell binding properties have been identified and characterized in probiotic strains (30, 31).

4. Conclusion

In conclusion, five selected *L. plantarum* strains represented desirable auto and co-aggregation, hydrophobicity, and cell adhesion abilities. These isolates can be suitable probiotic candidates for using in functional foods including dairy products. Although *L. plantarum* is considered among the non-starter LAB flora in industrial cheese production, selected strains from Siahmazgi cheeses may be used as adjunct cultures in mixed starter preparations to deliver potential beneficial probiotic activities. However, further studies are still needed to evaluate them from both points of view of their safety and their ability to improve human health in well-designed controlled clinical trials.

5. Acknowledgements

This work was supported by the Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.

References

1. Argyri AA, Zoumpopoulou G, Karatzas KAG, Tsakalidou E, Nychas GJE, Panagou EZ, Tassou CC. Selection of potential probiotic lactic acid bacteria from fermented olives by in vitro tests. *Food Microbiology*. 2013;33:282-91.
2. Fernández MF, Boris S, Barbés C. Safety evaluation of *Lactobacillus delbrueckii* subsp. *lactis* UO 004, a probiotic bacterium. *Research in Microbiology*. 2005;156:154-60.
3. Bove P, Gallone A, Russo P, Capozzi V, Albenzio M, Spano G, Fiocco D Probiotic features of *Lactobacillus plantarum* mutant strains. *Applied Microbiology and Biotechnology*. 2012;96:431-41.
4. Turchi B, Mancini S, Fratini F, Pedonese F, Nuvoloni R, Bertelloni F, Virginia EV, Cerri D. Preliminary evaluation of probiotic potential of *Lactobacillus plantarum* strains isolated from Italian food products. *World Journal of Microbiology and Biotechnology*. 2013;29:1913–22.
5. Ayeni FA, Sánchez B, Adeniyi BA, Reyes-Gavilán CG, Margolles A, Ruas-Madiedo P. Evaluation of the functional potential of Weissella and *Lactobacillus* isolates obtained from Nigerian traditional fermented foods and cow's intestine. *International Journal of Food Microbiology*. 2011;147:97-104.
6. Del Re B, Sgorbati B, Miglioli M and Palenzona D. Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Letters in Applied Microbiology*. 2000;31:438-42.
7. Wadstrom T, Andersson K, Sydow M, Axelsson L, Lindgren S, Gullmar B. Surface properties of lactobacilli isolated from the small intestine of pigs. *Journal of Applied Microbiology*. 1987;62:513-20.
8. Partovi R, Gandomi H, Basti AA, Noori N, Borujeni GN, Kargozari M. Microbiological and chemical properties of Siahmazgi cheese, an iranian artisanal cheese: Isolation and identification of dominant lactic acid bacteria. *Journal of Food Processing and Preservation*. 2014;39:871-80.
9. Chen P, Zhang Q, Dang H, Liu X, Tian F, Zhao J, Chen Y, Zhang H, Chen W. Screening for potential new probiotic based on probiotic properties and α-glucosidase inhibitory activity. *Food Control*. 2012;3:65-72.
10. Bautista-Gallego J, Arroyo-Lopez FN, Rantsiou K, Jimenez-Diaz R, Garrido-Fernandez A, Cocolin L. Screening of lactic acid bacteria isolated from fermented table olives with probiotic potential. *Food Research International*. 2013;50:135-42.
11. Kaushik JK, Kumar A, Duary RK, Mohanty AK, Grover S, Batish VK Functional and probiotic attributes of an indigenous isolate of *Lactobacillus plantarum*. *PLOS One*. 2009;4: e8099.
12. Angmo K, Kumari A, Savitri, Bhalla TC. Probiotic characterization of lactic acid bacteria isolated from fermented foods and beverage of Ladakh. *LWT- Food Science and Technology*. 2016;66:428-35.
13. Martin R, Olivares M, Marin ML, Fernandez L. Probiotic potential of 3 lactobacilli strains isolated from breast milk. *Journal of Human Lactation*. 2005;21:8-17.
14. Kassaa IA, Hamze M, Hober D, Chihib NE, Drider D. Identification of vaginal lactobacilli with potential probiotic properties isolated from women in North Lebanon. *Microbial Ecology*. 2014;67:722-34.
15. Gupta A, Sharma N, Bajwa J. Evaluation of *Enterococcus faecium* Ch-1 isolated from Chuli- a traditional fermented apricot product of Trans Himalayan region. *SDRP Journal of Food Science & Technology*. 2017;2: 1-12.
16. Lee KW, Park JY, Sa HD, Jeong JH, Jin DE, Heo HJ, Kim JH. Probiotic properties of *Pedicoccus* strains isolated from jeotgals, salted and fermented Korean sea-food. *Anaerobe*. 2014;28:199-206.
17. Sahoo TK, Jena PK, Negar N, Patel AK, Seshadri S. In vitro evaluation of probiotic properties of lactic acid bacteria from the gut of *Labeo rohita* and *Catla catla*. *Probiotics and Antimicrobial Proteins*. 2015;7:126-36.
18. Jeronymo-Ceneviva AB, Paula AT, Silva LF, Todrov SD, Franco BDGM, Penna ALB. Probiotic properties of lactic acid bacteria isolated from water-buffalo mozzarella cheese. *Probiotics and Antimicrobial Proteins*. 2014;6:141-56.
19. Borah D, Gogoi O, Adhikari C, Kakoti BB. Isolation and characterization of the new indigenous *Staphylococcus* sp. DBOCP06 as a probiotic bacterium from traditionally fermented fish and meat products of Assam state. *Egyptian Journal of Basic and Applied Sciences*. 2016;3(3):232-40.
20. Kos B, Suskovic J, Vukovic S, 2, Simpraga M, Frece J, Matosic S. Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *Journal of Applied Microbiology*. 2003;94:981-7.
21. Devi SM, Archer AC, Halami PM. Screening, characterization and in vitro evaluation of probiotic properties among lactic acid bacteria through comparative analysis. *Probiotics and Antimicrobial Proteins*. 2015;7:181-92.
22. Kaewnopparat S, Dangmanee N, Kaewnopparat N, Srichana T, Chulasiri M, Settharaksa S. In vitro probiotic properties of *Lactobacillus fermentum* SK5 isolated from vagina of a healthy woman. *Anaerobe*. 2013;22:6-13.
23. Garcia-Ruiz A, Llano G, Esteban-Fernandez A, Requena T, Bartolome B, Morno-Arribas MV. Assessment of probiotic properties in lactic acid bacteria isolated from wine. *Food Microbiology*. 2014;44:220-5.
24. Fogh J, Fogh JM, Orfeo T. One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *Journal of the National Cancer Institute*. 1977;59:221-6.
25. Saraniya A, Jeevaratnam K. In vitro probiotic evaluation of phytase producing *Lactobacillus* species isolated from Uttapam batter and their application in soy milk fermentation. *Journal of Food Science and Technology*. 2015;52(9):5631-40.
26. Vera-Pingitore E, Jimenez ME, Dallan A, Belfiore C, Fontana C, Fontana P, Wright A. Screening and characterization of potential probiotic and starter bacteria for plant fermentation. *LWT–Food Science and Technology*. 2016;71:288-94.
27. Tuomola EM, Salminen SJ. Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *International Journal of Food Microbiology*. 1998;41:45–51.
28. Bianchi MA, Del Rio D, Pellegrini N, Sansebastiano G, Neviani E, Brighenti F. A fluorescence-based method for the detection of adhesive properties of lactic acid bacteria to Caco-2 cells. *Letters in Applied Microbiology*. 2004;39:301-5.
29. Lim SM, Im DS. Screening and characterization of probiotic lactic acid bacteria isolated from Korean fermented foods. *Journal of Microbiology and Biotechnology*. 2008;19:178-86
30. Sánchez B, Bressollier P, Urdaci MC. Exported proteins in probiotic bacteria: adhesion to intestinal surfaces, host immunomodulation and molecular cross-talking with the host. *FEMS Immunology and Medical Microbiology*. 2008;54:1-17.
31. Veléz MP, De Keersmaecker SCJ, Vanderleyden J. Adherence factors of *Lactobacillus* in the human gastrointestinal tract. *FEMS Microbiology Letters*. 2007;276:140-8.