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Phylogenomic and biochemical assessment of four presumptive probiotic lactic acid bacteria

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Abstract

The commercial usage of probiotic *Lactobacillus* strains found in traditional fermented food products have been expanded due to its therapeutics potential. The purpose of this study was designed to isolate, identify, characterize, and evaluate the probiotic abilities of four selected Lactobacilli strains from Inner Mongolian cheese. Four *Lactobacillus* strains were aseptically isolated on previously specified *de Man Rogosa* media from Inner Mongolian cheese. Isolates were initially identified by Gram-staining, motility, and catalase tests. Moreover, the presumed *Lactobacilli* strains were further evaluated for probiotic properties including acid and bile salt tolerance, auto-aggregation, and co-aggregation assays to analyze the adhesive abilities. Further, several phylogenetic analyses were performed to discover the S-layer conserved protein motifs and theoretical protein interaction network for functional annotations. The acid and bile tolerance test were investigated under pH (2.0 & 3.0) and 0.3% bile concentration at 0, 1, 2 and 3 hours of time intervals respectively. Our findings suggested that all four selected LAB strains showed substantial increased in tolerance against acid and bile. The ability of auto-aggregation among *Lactobacillus* strains range from 15.94% to 70.02%. However, *Lactobacillus* strain 3(8) showed the highest co-aggregation phenotype with *Listeria monocytogenes* (54.7%), and (40.8%) with *Staph. aureus* while strain K showed the strongest ability with *Salmonella typhi* (39.34%). Phylogenetic investigations revealed the discovery of four S-layer conserved protein motifs and essential protein interaction network among selected Lactobacilli strains. These breakthroughs promote novel perspectives concerning the use of inner Mongolian cheese as a rich source of probiotic bacteria in future researches.

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Introduction

Probiotics are widely known as a group of microorganisms that carry various positive effects with regards to human health. FAO/WHO has recognized probiotics which can be defined as "the class of living microorganisms that confers several advantages to its host when encountered in sufficient quantity (FAO, 2002).

A number of recent studies have demonstrated that the ability of probiotic could inhibit various chronic diseases such as hypertension, inflammatory bowel disease, acute diarrhea, irritable bowel syndrome, diabetes, and constipation (Weichselbaum, 2009). Multiple food products consist of probiotics are also known as functional foods, that can deliver several therapeutic effects, for example immune modulatory effects, hypoglycemic properties, antioxidant, anticancer, and antihypertension (Clare & Swaisgood, 2000; Umer Khan, 2014). Because of the medical and industrial importance of probiotics, a great interest emerges to isolate novel strains of probiotic that are actively associated with human health-promoting beneficial impact (Umer Khan, 2014).

The final products of probiotics contain numerous groups of essential enzymes and other nutrients such as vitamins and capsules, and microorganisms which is extremely favorable and beneficial to the host biosystem. By in large, the products of probiotics which is directly or otherwise related to human consumption are mostly synthesized in the fermented milk or directly available as oral medicine in the form of powders and tablets (Çakır, 2003). However, these oral capsules and tablets have not been approved for medicinal purposes rather they can be used only as beneficial health foodstuffs. Several mechanisms upon the protective effect of oral usage of probiotics microorganisms on the gut flora are being observed. Even so, many studies demonstrated the beneficial effects of probiotics to overcome gut microbial disorders; however, it remains a great challenge among food and drug specialist to confirm the clinical and medicinal effects of such products (Çakır, 2003; Ouwehand & Salminen, 1998).

Lactobacilli are widespread probiotics microbe in nature many with multiple species that contribute enormous applications in the field of food industry (Weichselbaum, 2009). The abundance of *Lactobacilli* are present in high carbohydrates containing substrate within diverse habitats including oral cavity, intestine, and vagina of human and animals and also on fermenting food most specifically cheese (Pot *et al.,* 1994; Saad, *et al.,* 2013). Most of the *lactobacillus* stains of are categorized as natural probiotics because they are purely fermentable, as well as aero-resistant to anaerobic, aciduric or acidophilic environment with intricate nutritional necessities (Desai, 2008). In the current study we demonstrated the isolation and biochemical characterization of probiotic *Lactobacillus* strain 3(8) from Inner Mongolian cheese. The ability of 3(8) strain as probiotics was further explained with different assays such as tolerance of 3(8) strain against acidic pH, bile tolerance, lactose utilization, auto-aggregation assay and co-aggregation assay. Moreover, we also carried out several in silico analysis to identify potential targets of probiotic *Lactobacillus* 3(8) strain at molecular level.

Materials and methods

Sample collection

Different Cheese samples were collected for isolation of *Lactobacillus species* from Inner Mongolia province of China. Multiple samples were collected inside clean and sterile bags of plastic and transported to the laboratory of Food Science and Engineering Jilin Agricultural University China for further processing. The samples were kept at 4°C in sterile sample bags till further use.

Isolation and identification of Lactobacillus species

For isolation of *Lactobacillus species*, 5g of each sample was weighed and mixed with 45ml of autoclaved solution of saline at a concentration of 0.85% w/v. Dissolving of samples was followed by homogenously shaking. The mixture obtained was subjected to serial dilution and then incubated on the agar plates of *de Man Rogosa* Sharpe (MRS;

Difco, USA) at 37°C for 48 h. After the aforesaid incubation period, all the isolates were further subjected to culturing in liquid MRS broth (Difco USA) with controlled conditions (37°C for 16 h). Once the required density was achieved, the cultures were then stored in 20% glycerol+ MRS broth at -80 °C until next use. The isolates were transferred from the refrigerator to the working lab following microscopic examination, catalase reactions and then gram staining respectively. The rod shape, non-motile, catalase-negative and gram positive were selected. All the strains were sub cultured twice prior to the following experiments.

Sensitivity against acidic pH

The *Lactobacillus* isolates were cultured in MRS broth for overnight at 37 °C. Out of the culture, 0.1 mL sample from the selected isolates were subjected to pH adjustment at pH 3.0 and 2.0 using 5 N HCl and then incubated on adjusted shaker at 37 °C for a period of 3 hours. Cultures were collected after 3 hours incubation and the density of bacterial growth was observed by estimating the absorbance at 600 nm with the help of a spectrophotometer (Nova Spec II, Pharmacia) (Singhal *et al*., 2010). The experiments were repeated in three independent biological replicates.

Resistance to Bile concentrations

To examine the activity of the selected *Lactobacillus* strains against bile concentrations, we cultured the aforesaid five strains in MRS broth (liquid) for overnight at 37°C. A saturated solution of the bile was previously in a separate chamber simply by melting the powdered bile extract purchased from (Oxoid).

The prepared solution of the Bile was subjected to filtration by using a sterilized 4-micron filter and then the solution was mixed with the cultures to get the required 0.3 % final concentration of bile. The Lactobacilli cultures were kept on shaker at 37 °C for 3 hours for incubation. After 3hours of incubation, thegrowth of the bacterial cultureswas measured by absorbance values at 600 nm with the help of a spectrophotometer (Nova Spec II, Pharmacia) (Singhal *et al.,* 2010). The experiments were repeated in three independent biological replicates.

Lactose utilization

Sterilized 10 ml of fermentation media was taken in sterilized test tube. Bacterial culture was inoculated to falcon tubes and then subjected to incubation at 37°C for a period of 22-46 hours. Post incubation of the cultures, the concentration of lactose usage was observed by estimating color change assay which is turned yellow from red (Ahmed & Kanwal, 2004).

Autoaggregation Assays

Autoaggregation test for the selected five *Lactobacillus* strains were conducted according to the instruction given by (Del Re *et al.,* 2000), as modified by (Kos *et al.,* 2003). The bacterial cultures were supplemented in MRS broth (liquid) for a period of 18 hours at 37°C.

The resultant solution was subjected to centrifugation at 5000 × g for a period of 15 minutes, and the cells precipitate were washed twice and then clean cells were resuspended in a solution containing phosphate buffer saline having pre-adjusted (pH at 7.0) in order to achieve theviable counts of the cells at approximately 10^8 CFU/ml.

The cellsuspension approximately (4ml) was dissolved by subjecting it to vortexing for a short period of 10 seconds and then finally auto-aggregation assay was carried out at room temperature. On multiple hourly intervals, the suspension of 100 µl was transferred to separate falcon tube with the addition of PBS (3.9 ml) and then the absorbance values at 600nm was calculated.

The capacity of Autoaggregation was measured according to following equation: $1 – (At / A0) \times 100$

Where At demonstrates the absorbance value at time t = 1,2,3,4 or 5 hours and A0 is the absorbance at time t= 0.

Coaggregation Assays

Co-aggregation test for the selected five *Lactobacillus* strains were conducted according to the instruction given by (Del Re *et al.,* 2000). The suspensions of the bacterial cells wereset up as explained in the previous section of autoaggregation test. The same volumes of *Lactobacillus* cultures present in broth media approximately (2 ml) and each pathogen strain were selected and subjected to mixing by vortexing for a period of 10 sec. Each bacterial suspension (4 ml) was used as the control independently. After mixing, the absorbance values of suspension at 600nm were observed after 5 hours of incubation at 37°C. The coaggregation percentage was measured according to the following equation following the instructions of (Handley *et al.,* 1987):

Coaggregation %= {(AX +AY)/2 – A (x+y)/(AX +AY)/2}] ×100

Where x and y denote each of the two strains in the control tubes and (x + y) the mixture.

Discovery of S-layer conserved protein motifs

The occurrence and identification of four conserved protein S-layer motifs within the selected *Lactobacillus* strain 3(8) using the online tool of Multiple EM for Motif Elicitation (MEME) which is freely available online at (http://meme-suite.org/). The sequence of *Lactobacillus* strain 3(8) 16sRNA was uploaded to online server of MEME and the parameters were selected to one, incidence of one motif per sequence; 2-50 amino acids, motif breadth range; and three, most range of motifs were identified. All different parameters follow with the default values.

Protein interaction map and functional Annotations

The putative *Lactobacillus* strain 3(8) S-layer protein sequences were added to the online server of STRING data server (version 10; https://string-db.org/).

The setup of the organism was selected as *Lactobacilli*. The set of genes exhibiting maximum Bitscores were utilized to create the interaction map by plotting the tightly (blue), partially (red) and slightly (green) interacting proteins involved in specific cellular functions. The information of functional annotation regarding different domains was pasted manually from results of the blasts.

Statistical analysis

For all the experiment, tests were performed in triplicate for statistical analysis. All data were described as mean ± standard deviations. Statistical comparisons were made using the statistical software package Statistix 8.1. The significant differences between treatments were tested by analysis of variance (ANOVA) followed by a comparison between treatments performed using least significance difference (LSD) method, with levels of significance $p< 0.05$.

Results and discussion

Tolerance to Low pH and Bile concentration

The survival of *Lactobacilli* was less in pH 2.0 in comparison to pH 3.0 as demonstrated in (Fig. 1 and 2).

Table 1. Autoaggregation percentage of *Lactobacillus* strains.

Strains	Percent auto aggregation on hourly bases			
	1 Hour	2 Hour	3 Hour	4 Hour
K	29.58±0.69^{c}	44.18±0.33^{c}	48.33±0.31^{c}	56.77±0.74^{b}
L	20.59±0.55^{d}	24.69±0.67^{e}	46.30±0.40^{d}	55.04±0.57^{c}
N	15.94±0.40^{e}	40.76±0.54^{d}	49.03±0.55^{c}	49.04±0.66^{d}
3(8)	48.38±0.35^{a}	59.92±0.57^{a}	62.16±0.75^{a}	70.02±0.33^{a}
LGG	32.17±0.39^{b}	49.95±0.48^{b}	51.24±0.54^{b}	54.64±0.52^{c}

Values in the same column with the same following letters do not significantly differ ($p < 0.05$); ±standard deviation.

Most of the strains are sensitive to pH 2.0 but the survival rate was observed less sensitive to pH 3.0. All five strains exhibited a significant growth and survival at pH 2.0 and 3.0. However, in case of pH 2.0, the presence of non-viable bacterial cells wasalso obtained after 1 hour which depicts that most of the bacterial strains were attenuatedin severe acidic pH environment.

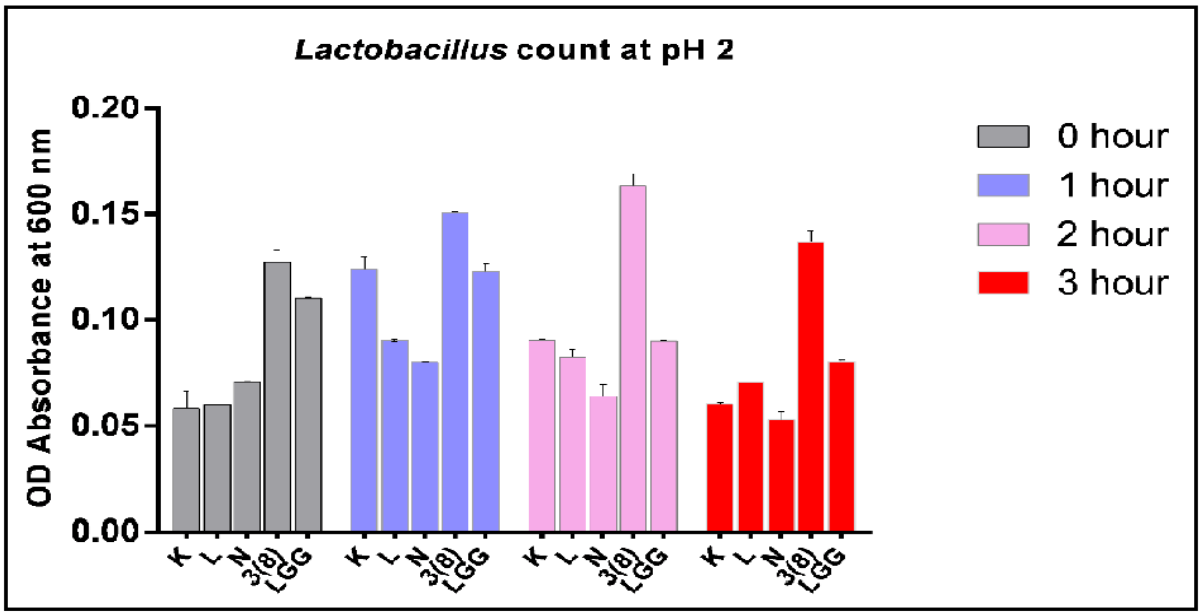

Fig. 1. Tolerance of isolated *Lactobacillus*to pH=2.0.

A recent report demonstrated thatthe disruption of various biomolecules including, DNA, protein and fatty acids can efficiently occur due to the presence of hydrochloric acid (HCL) in human stomach (Sahadeva *et al.,* 2011). Furthermore, the environments created by low pH can directly suppress the cell viability, metabolism and growth reduction of *Lactobacilli*. Several other researches also suggest that bacterial growth intensity could also lead to growth reduction due to the open exposure of pH 2.0 and gastric acid at 3 hours incubation (Mandal *et al.*, 2006; Sahadeva *et al.,* 2011).

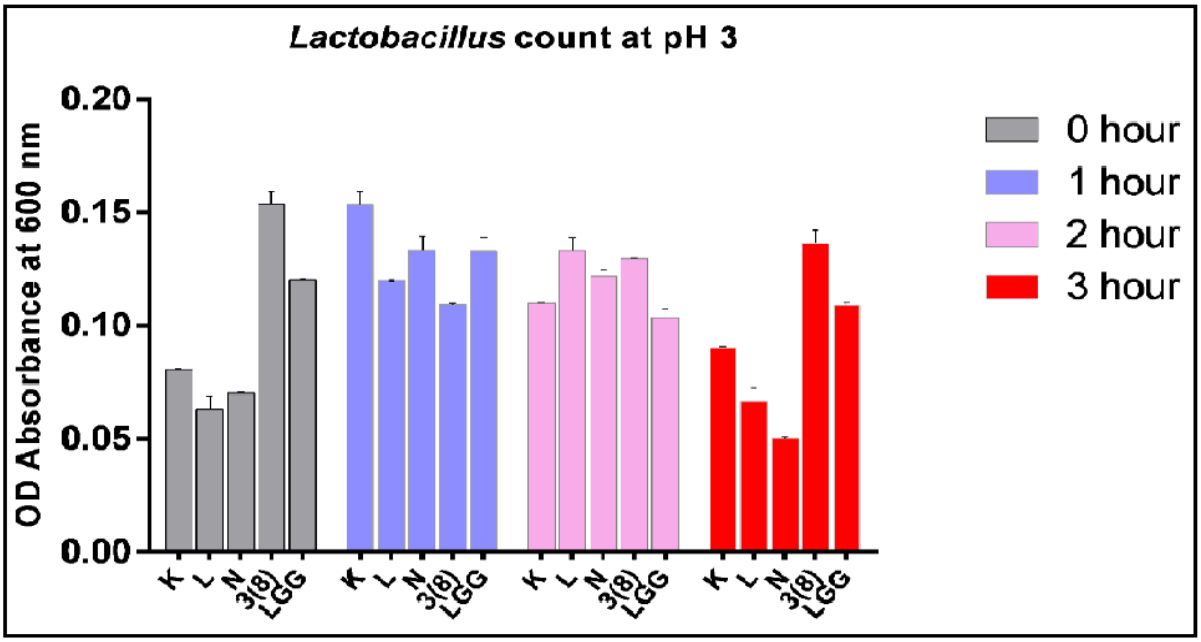

Fig. 2. Tolerance of isolated *Lactobacillus* to pH=3.0.

In addition, the recent reports of (Prasad *et al.,* 1998) and (Chan *et al.,* 2011) the optimum stage of acid resistance was kept at pH 2.0 and 3.0 for 3 hours incubation, because it actually resembles the adaptation of bacterial accommodation within the human stomach (Prasad *et al.,* 1998; Sahadeva *et al.,* 2011). In our study, we have found that the growth of bacterial strains reduced by promoting the incubation time at pH 2.0 and it did not affect bacterial growth at a higher pH 3.0. No further significant decline in the absorbance (OD) of bacterial density was shown with the declining of pH level (Fig.1 and 2). Hence, we speculated that it is more likely due to the adaptation competence of *Lactobacilli strains* against acid during MRS broth incubation.

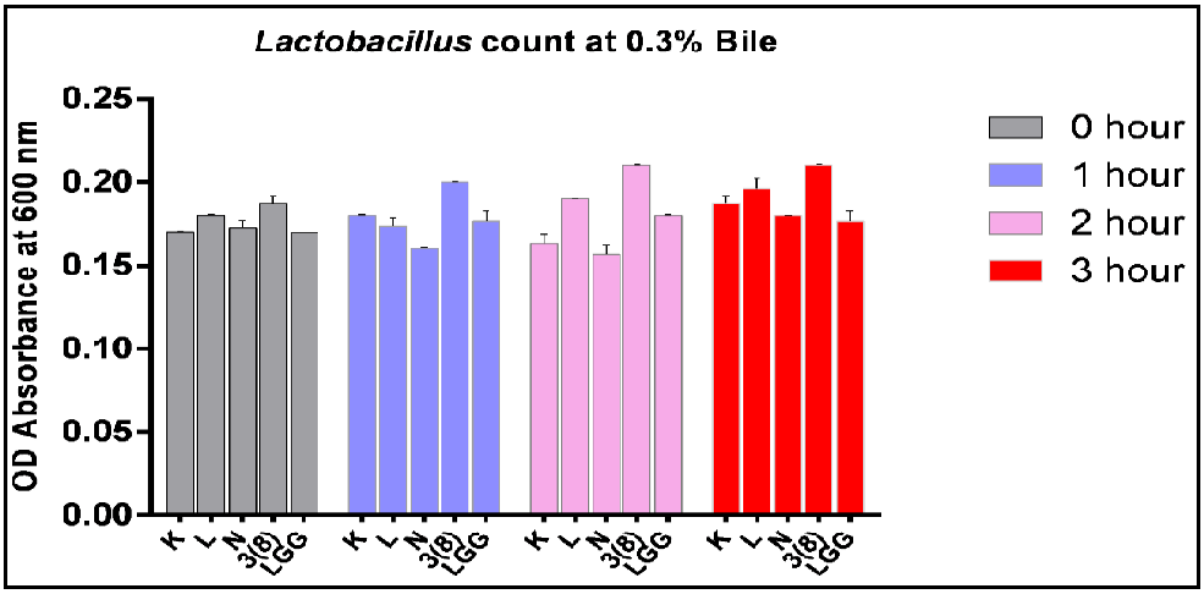

Fig. 3. Tolerance of isolated *Lactobacillus* to 0.3% Bile.

In the case of bile tolerance evaluation, the growth rate of the selected strains in culture media can be measured. Following the bacterial strain exposure to different bile salts, the breakdown of cellular homeostasis appeared which leads to the detachment of lipid bilayer and other several essential proteins present on the cell membrane, causingthe bacterial content reductionwhich ultimately reached to cell death as described by (Mandal *et al.,* 2006). Among all isolated strains in our study the growth and tolerance of strain 3(8) can survive efficiently against low pH and 0.3% bile concentration as compared to other strains (Fig. 3).

Auto aggregation assay

We observed that all selected strains demonstrated strong autoaggregation phenotype.

The isolated strains were examined for autoaggregation assay (Table 1). The process of Auto-aggregation is basically related to phenotype-based cell adherence characteristics (Pelletier *et al.,* 1997; Kos *et al.,* 2003). As per the instructions of (Del Re *et al.,* 2000), *Lactobacillus* strains indicating lower than 10% values are designated as non-autoaggregating. We observed that all selected strains demonstrated strong autoaggregation phenotype because at 1 h of time interval, all the isolated *Lactobacillus* strains presented a considerable autoaggregation phenotype higher than 10%. However, at the time interval of 2 hours all the strains surpassed this percentage. Our results showed that autoaggregation assay was increased linearly over time.

The strain 3(8) showed higher autoaggregation assay between 3 to 4 hours as compared to other strains as well as the reference strain LGG. Particularly, the probiotic strains showed increased autoaggregation capabilities in comparison to pathogenic strains (Collado *et al.*, 2007).

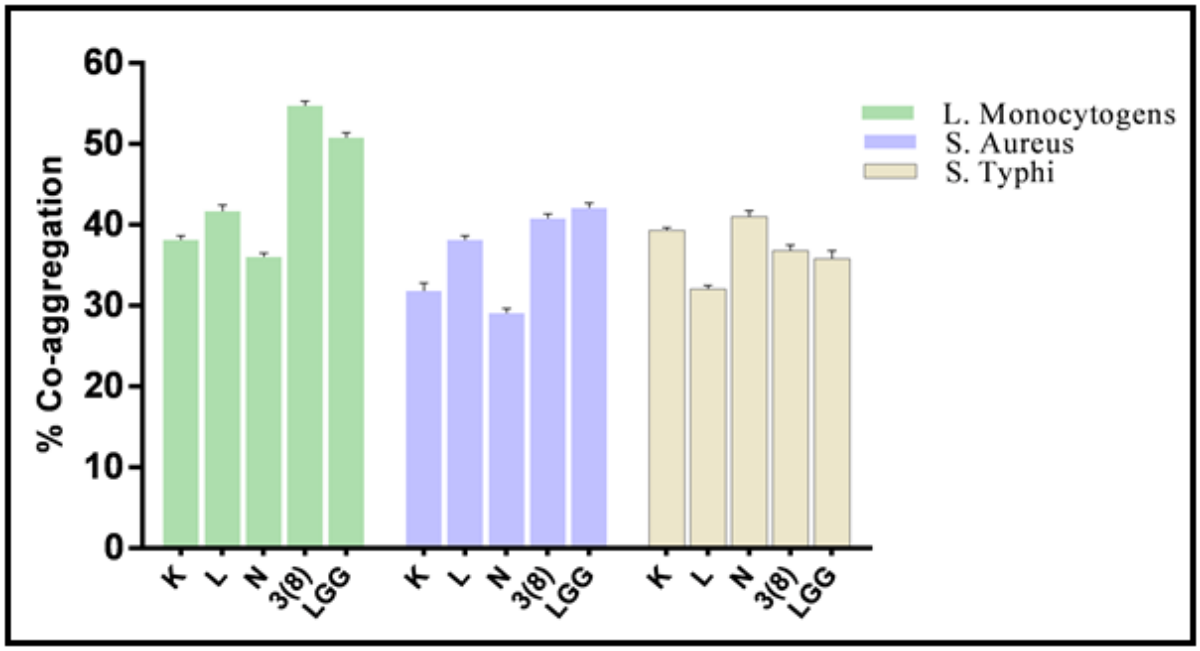

Fig. 4. Percent (%) of Coaggregation *Lactobacillus* strains to three pathogens.

Our finding result indicates that strain 3(8) showed excessive potential to attach to the epithelial cells of bacteria as well as to mucosal surface. A previous study demonstrated that the ability of phenotype-based cell adherence to epithelial cells and mucosal surface is a significant characteristic of many probiotic bacteria (Bao *et al.,* 2010; Kotzamanidis *et al.,* 2010). Moreover, a number of studies have investigated several intricate details of bacterial adhesion to intestinal epithelial cells such as structure, composition and forces of interaction (Pelletier *et al.,* 1997; Del Re *et al.,* 2000; Bao *et al.,* 2010; Kotzamanidis *et al.,* 2010). Altogether, our findings suggested that *Lactobacillus* strain 3(8) demonstrated sufficient autoaggregation capacity which can be helpful in identifying and isolating unidentified probiotics in future studies.

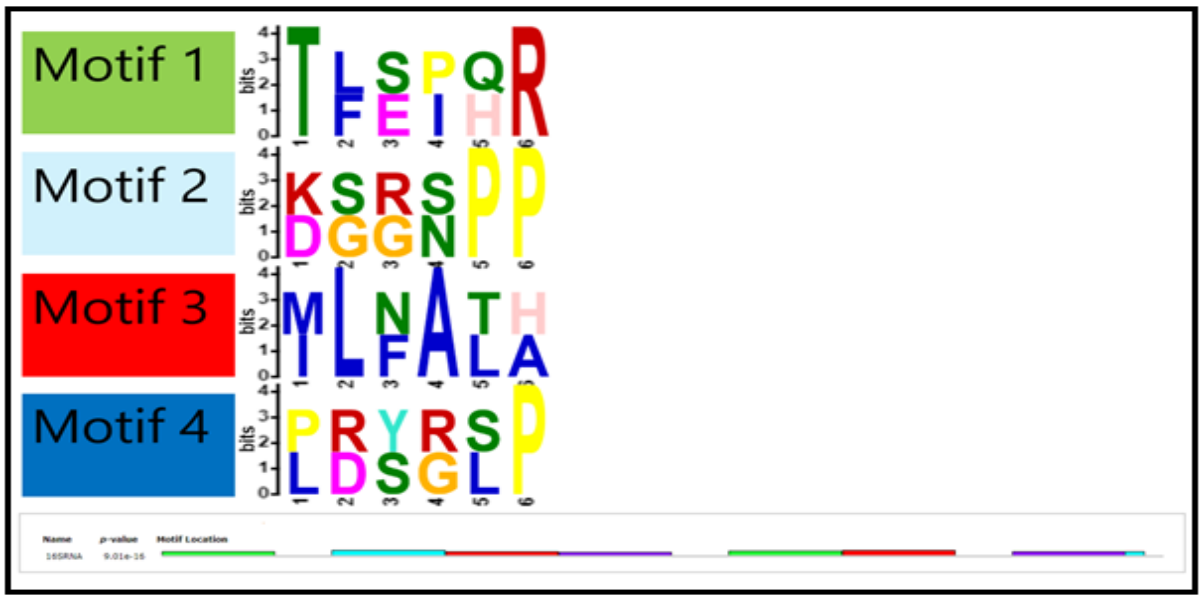

Fig. 5. The conserved S-layer protein motifs of Probiotic *Lactobacillus* 3(8) strain. Motif 1-4 were described in different color pattern including green, light blue, red and dark blue respectively. The composition and conservation of amino acids were demonstrated in capital alphabets.

Coaggregation assays

The coaggregation properties between the isolated strains and foodborne pathogen are shown in (Fig. 4). Among the tested strains, strain 3(8) showed highest coaggregation abilities with L. monocytogenes (54.7%). All the tested strains were highly coaggregated with L. monocytogenes (36.0 – 54.7%). Among all the strains, strain N showed the least coaggregation ability with S. Aureus (29.07%), L. monocytogenes (36.00%) and the strain L showed the least coaggregation ability with S. Typhi (32.07%). According to (Bao *et al.,* 2010), the coaggregation capacity is directly linked with strain-specificity.

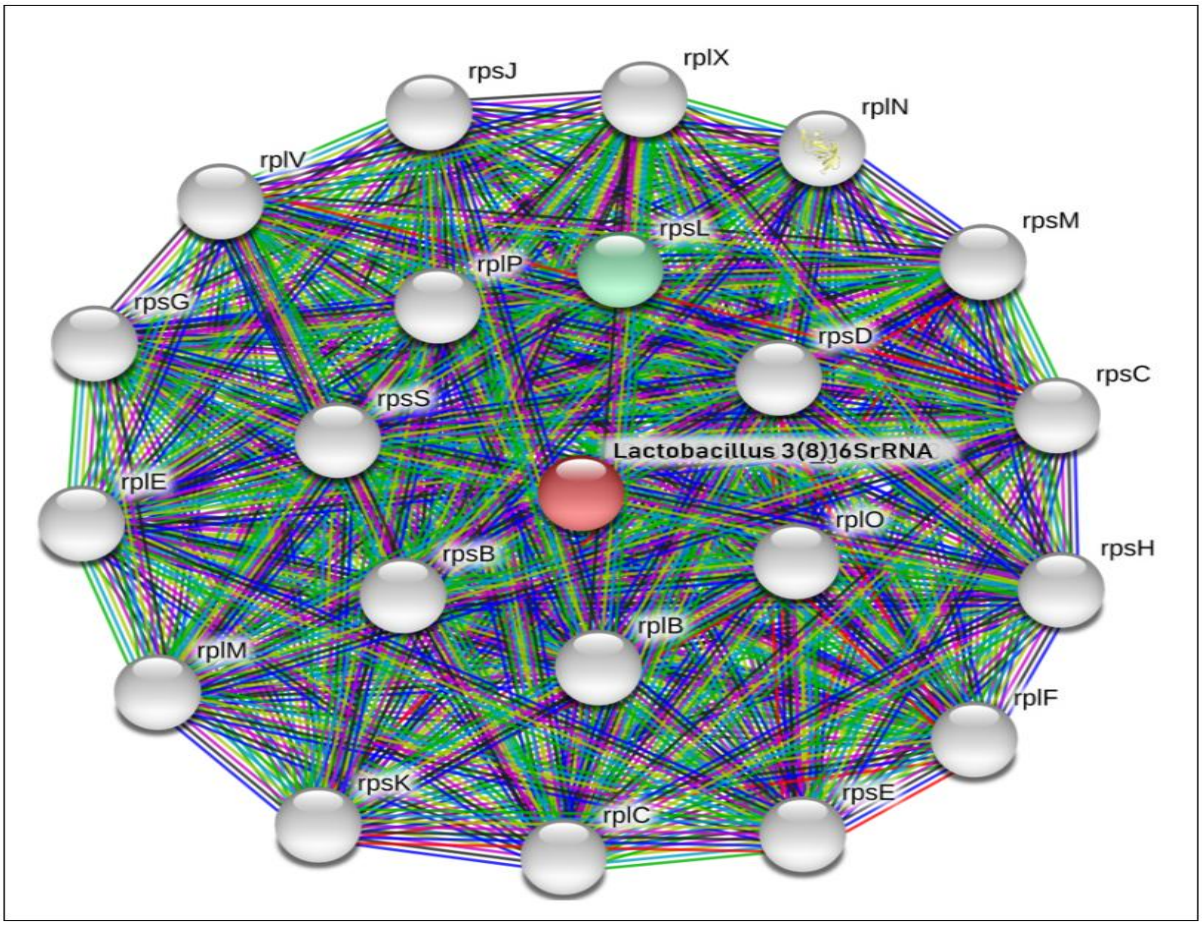

Fig. 6. The in silico protein-protein interaction map of *Lactobacillus* strain. The interaction map was created with available online tool STRING (https://string-db.org/) by plotting the tightly (blue), partially (red) and slightly (green) interacting proteins involved in specific cellular functions.

It is also important to understand that the coaggregation abilities found in most of the *Lactobacillus* species exhibiting potential pathogenic propertiescould also reduce the gut microbiota colonization and hence produce a substantial defense mechanism in urogenital and gastrointestinal tract of the host microbe against sever infective agents. On the basis of these findings, the ability of Coaggregation could therefore be identified as probiotic bacteria which are mainly attributed to lactic acid bacteria (Kos *et al.,* 2003; Collado *et al.,* 2007). In our study all the strains showed greater coaggregation with *L. monocytogenes*.

This property may be related to the identification of a combined species biofilm since combined species biofilms of *L. monocytogenes* and *L. plantarum* was previously reported by (Van der Veen and Abee, 2011). Therefore, we speculated that the selected *Lactobacillus* strains could be used as probiotic studies and for the production of mixed species biofilms in future researches.

Occurrence and Identification of S-layer protein motifs in Lactobacillus

The presence of prokaryotic surface layers proteins components also known as the proteinaceous cell envelope like structures ubiquitously originated in *Archaea* and Gram-positive/negative bacteria (Sára and Sleytr, 2000). These proteinaceous components synthesized the outermost layer in the bacterial cell, which is infrequently enclosed only by capsule (Fouet *et al.,* 1999). The (S) layers subunit composition are generally made up of hydrophobic and acidic amino acids, however, they are less in the number of amino acids containing sulphur thereby leading to a low isoelectric point (p*I*) value of the proteins (Sára & Sleytr, 2000; Ahmad *et al.,* 2019). *Lactobacillus* strains are the group of micro-organisms that are generally known as Gram-positive in nature with non-pathogenic properties and are collectively characterized to synthesize the end product "lactic acid" which is the main precursor of carbohydrate metabolism (Felis & Dellaglio, 2007). Apart from its use in diary, food additives and feed fermentations, the characterization of lactic acid bacteria have attracted the interest of several health-related (probiotic) abilities of numerous strains. These Lactobacillus strains have proved crucial for the efficient delivery of widely known pharmacological agents and prophylactic molecules, for example vaccine antigens signaling molecules etc in humans (Velasquez-Manoff, 2015). In our study we have investigated the occurrence and identification of four conserved protein S-layer motifs within the selected Lactobacillus strain using the online tool of Multiple EM for Motif Elicitation (MEME) which is freely available online at (http://meme-suite.org/). MEME allows the discovery of various set of conserved motifs which are present frequently in complex form of similar proteins and is regularly linked with various cellular functions. The principle of MEME introduces the identification of different crucial motifs as position-dependent amino acids that identify the probability of each possible amino acid at each position within the protein sequence at specific positions. The composition of the identified motifs within *Lactobacillus* S-layer protein was described in different colors patterns (Fig. 5). Previous studies described that several *Lactobacillus* genus *but not all contains S-layer protein domains*. Nonetheless, a number of biochemical studies have shown that a number of *Lactobacillus strains including amylolyticus*, *gigeriorum,* *kefiranofaciens*, *pasteurii* and *ultunensis,* consist of predicted S-layer producing protein genes in genomes which completely or partially sequenced. Therefore, it is essentially important to investigate further studies on genes related to S-layer protein producing genes in future researches.

Probiotic Lactobacillus 3(8) interaction network with other proteins

The interaction network between several surface components of the probiotic and its host cells could direct alteration of gut functions (Velasquez-Manoff, 2015). Symbiont bacteria that are mainly colonized in the gut have undergone co-evolution with their respective host, and constitute a ray of molecular interaction-based schemes which are participating directly in immune system development, adherence potential and epithelial barrier function (Vindigni*et al.,* 2016). The most productive ability of the probiotic bacteriaisthe activation of interaction-based pathways, which is most likely linked with adherence capabilities to the target cells. We therefore identified the potential Probiotic *Lactobacillus* 3(8) interaction network with other functionally active proteins by carrying out several in silico analysis with the help of freely available online tool STRING (https://string-db.org/) according to the instructions given by (Szklarczyk *et al.,* 2018). The interaction network plotted against the *Lactobacillus* strains suggested various candidate proteins directly or otherwise linked with multiple immune response, cell cycle regulation, cellular functions and signaling and cell death and proliferation. The projected interaction map explained the tightly, partially, and slightly association of potent proteins which are indicated in shades of blue, red and green color respectively. Major important checkpoints can be seen in (Fig. 6) which depicts the strong interaction between *Lactobacillus* 3(8) strain and rpsS, rpsB, rpiB, rpiO,

rpsL and rpIB proteins which are known as crucial candidates in various cellular pathways of probiotic bacteria. Furthermore, the loosely attachment between *Lactobacillus* 3(8) strain and rpIX, rpsC, rpIE, and rpsJ can be found which is indirectly related to post transcriptional regulation pathways in probiotic bacteria.

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