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Screening of Potentially Probiotic *Lactobacillus* Possessing Surface Layer Protein from Iranian Traditional Dairy Products

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Abstract

One of the most commonly observed surface structures on the prokaryotic cell envelopes is monomolecular crystalline array of proteinaceous subunits termed Surface Layers or S-layers. Due their self assembly ability and the highly ordered, regular structure down to the nanometer scale, S-layers were demonstrated to possess a great potential for nanobiotechnological applications. Some probiotic bacteria, such as *Lactobacillus* spp. have been found to possess S-layers. In this perspective, the objectives of this study were isolation and identification of *Lactobacillus* species from traditional dairy products carrying surface (S) layer protein and investigation of important prerequisite of probiotic interest, such as the capability to survive at low pH and in presence of bile salts. The protein profile of intact *Lactobacillus* isolates was analyzed in SDS-PAGE. The protein bands of 45–60 kDa were present in two of the 25 isolates, suggesting the presence of S-layer proteins in these strains. The above mentioned proteins were recovered in the guanidine hydrochloride extracts. After dialysis, the extracted proteins showed a significant band of apparent molecular mass of 50–60 kDa in SDS-PAGE. The strains possessing S-layers protein was identified both biochemically and by 16S rRNA sequencing. The 16S rRNA gene sequence analysis revealed that two isolates exhibited maximum similarity with the 16S rRNA sequence of *Lactobacillus brevis* spp. These two isolates were resistant to acid and bile in which their viability in acidic condition were between 51.6% and 77.8% and bile resistance of two isolates was approximately 99% after 24 h.

Keywords: Dairy products; *Lactobacillus* spp; S-layer; Probiotic.

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Introduction

The cell walls of many *Bacteria* and *Archaea* are covered by a crystalline structure known as surface layer (S-layer). Molecules of S-layer are composed of only one (glycol) protein subunit, varing in mass from 40 to 200 kDa, and are assembled into array with oblique, square or hexagonal symmetry. Under appropriate conditions monomers can reassemble in solution or on solid support into S-layer- like structures with the authentic symmetry pattern [1, 2]. Diverse functions have been proposed for S-layers, such as acting as molecular sieves, protective coats, molecular ion traps, cell shape determinants, and promoters for cell adhesion and surface recognition [3]. Due their self assembly ability and the highly ordered, regular structure down to the nanometer scale, S-layers were demonstrated to possess a great potential for nanobiotechnological applications [4].

Among lactic acid bacteria, the S-layer seems to be a typical surface structure in several *Lactobacillus* species, e.g., in *L.acidophilus*, *L. helveticus*, *L. casei, L. brevis, L. buchneri, L. fermentum, L. bulgaricus and L. plantarum* [5,6]. Interest in *lactobacilli* S-layer has been reinforced by claimed and demonstrated probiotic properties for human and animal consumers. Probiotics are alive microorganisms that when ingested in adequate amounts, exert health benefits to the consumer, other than those related to nutritional effects.

S-layer proteins of *lactobacilli* differ from common S-layer proteins in their smaller size (25–71 kDa) and high predicted pI value (9.4–10.4). The lattice symmetry of *Lactobacillus* S-layer proteins is oblique or hexagonal type [4]. Potentially probiotic *lactobacilli* are excellent candidates for health-related applications like alive oral vaccines and their S-layer proteins could be used as carriers of antigens or other medically important molecules, possibly in combination with immunostimulatory or adhesive molecules [6, 7, 8].

Lactobacilli have been isolated from various environments riched in carbohydrate or protein such as plants, food stuffs, silage and sewage, and they have been found in the gastrointestinal and genital tracts of humans and animals, where they form part of the normal flora [6].

In this work, the main aim was to find new S-layer positive *lactobacilli*. In future we decide investigating characteristics of these surface layer proteins for later use. From this point of view, the current study aimed to isolate and identify *Lactobacillus* spp. from traditional dairy products and then detect surface layer protein among them. In order to investigate probiotic characteristics of these isolated strains, bile and acid tolerance through growth were carried out. The acid and bile tolerances are two fundamental properties that indicate the ability of a probiotic microorganism to survive and passage through the gastrointestinal tract, resisting the acidic conditions in the stomach and the bile acids at the beginning of the small intestine [9, 10].

Materials and Methods

Isolation of lactobacilli from traditional dairy products

Ten samples (10 g each) of dairy products, including different kinds of yogurt and cheese were prepared from traditional workshops of bonab county, Tabriz. The samples were stored at 4˚C and brought to laboratory within 24 h, then they were inoculated into 100 ml of MRS broth (Merck, Germany) . After anaerobic incubation at 37°C for 24 h, 20 ml of the broth was centrifuged at 1000× g for 15 min and the pellet was resuspended into 10 ml of PBS adjusted to pH 2.5 with HCl. After 2 h of anaerobic incubation at 37°C, the medium was centrifuged at 4000 ×g for 20 min, the pellet resuspended in PBS (pH 7) and 0.1 ml of the culture were spread plated on MRS agar

medium (Merck, Germany) and the plates were incubated under anaerobic conditions at 37°C for 48-72 h. In order to avoid preliminary time consuming isolation steps and interference of yeast, *lactobacilli* colonies were selected on MRS agar containing 50 µg/ml Nystatin [11, 12].

Colonies characterized by different morphologies were isolated and purified on MRS agar medium. All the isolates were examined by microscopy, gram staining and catalase reaction. Gram positive and catalase negative rods were maintained in MRS broth with 30% sterile glycerol and stored at -70°C. The main purpose of this study was screened for presence of surface layer protein among isolates, so in the next step the presence of S-layer in *lactobacilli* isolates obtained from traditional dairy products was examined.

The well-known probiotic bacterium, *Lactobacillus acidophilus* ATCC 4356 was used as positive control. This bacterium that have nanostructure of S-layer, was obtained from culture collection of Iranian Reserch Organization for Science and Technology (IROST).

Detection of S-layer proteins

Pure culture strains were grown in MRS broth for 24 h followed by harvesting of cells by centrifugation at 16,000 × g for 2 min. Each cell pellet, equivalent to 1 ml of culture with equal OD, was dissolved directly in 50 ml of Laemmli sample buffer, boiled for 5 min and analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [13]. After electrophoresis, the gels were stained with Coomassie brilliant blue [14, 15].

The extraction of S-layer proteins

For extraction of S-layer protein of selected isolates, the bacteria were harvested at the end of log phase (with optical density of 0.7 at 695 nm) by centrifugation (15000 ×g, 15 min at 4°C) and washed twice with chilled distilled water. The end log harvested cell pellets were treated with 4M guanidine hydrochloride (Merck, Germany) in 50 mM Tris-HCl buffer (pH = 7.2), (1 g of harvested cell pellets was suspended in 10-15 ml of 4M guanidine hydrochloride (GHCl) for 1 h at 37°C. The extracted S-layer protein was collected by centrifugation (18000 ×g, 15 min, 4°C). The supernatant containing the S-layer protein was dialyzed over night at 4°C against two liters of 50mM Tris- HCl- buffer (pH = 7.2). Dialysed proteins were suspended in sample buffer for further analysis by SDS–PAGE. In all cases, SDS–PAGE was performed by the method of Laemmli [13, 16].

Bile salt and acid tolerance of selected lactobacillus isolates

Bile salt tolerance of selected *lactobacilli* with S-layer was ascertained in MRS broth (control) and in MRS broth containing 0.3% bile salt (sodium deoxycholate, Merck, Germany) incubated at 37°C for 3, 6, 24 h. After incubation, cells were serially diluted 10-fold and the residual viable population was determined by plate counting on MRS agar after 48–72 h. Overnight MRS broth cultures were utilized as an inoculum at 1.0% (v/v). The initial bacterial concentration was 10^6–10^8 $CFUml^{-1}$. The experiment was repeated twice for each strain and the data was presented as mean [17,18 , 19].

Also low pH tolerance of these *lactobacilli* was examined. The bacteria were grown overnight in MRS broth. A 0.1 ml aliquot of the bacterial culture was inoculated into 10 ml of MRS broth adjusted to pH values of 6.5 and 2.5 with HCl. The samples were incubated for 3 h at 37°C. MRS broth with pH 6.5 was used as a control. The initial bacterial concentration was 10^6–10^8 $CFUml^{-1}$.Ten fold serial dilutions were made from each 1ml sample using sterile saline solution and then, one hundred micro liter of each dilution was plated onto MRS agar and incubated at 37°C under a 5% CO2 concentration for 24 to 48 hours; then, the

survival rate of the *Lactobacillus* strains was measured by counting the cells. Approximately 30-250 colonies appeared after 48 h of incubation at 37°C. The experiment was repeated twice for each strain and the data was presented as mean [17, 18, 19].

Identification of S-layer carrying lactobacilli

The isolates were identified according to their morphological, cultural, and biochemical characteristics based on Bergey's Manual [20]. The used tests were: Gram reaction, catalase test, gas production from glucose, growth at 15 and 45°C in 1 week; acid production from carbohydrates (1% w/v) and ammonia production from arginine.

The selected isolates were identified via 16S rRNA sequencing. Genomic DNA of selected isolates was extracted from bacterial colonies by set buffer method. The 16S rRNA gene from the genomic DNA was amplified by PCR using the following forward and reverse primers of 16S rRNA, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1496R (5′-TACGGYTACCTTGTTACGAC-3′). Both strands of the PCR product were sequenced by dideoxy chain termination method. The closest known relatives of the isolates were determined by performing database searches. A phylogenetic tree and neighbor-joining phylogeny were constructed by use of MEGA soft ware package version 5.0 and bootstrapping was used to estimate the reliability of the phylogenetic reconstructions (1,000 replicates).

Statistical analyses

In section of bile salts and acid tolerance, mean values were calculated for each treatment and the results are presented with standard errors of means or standard deviations (SD). Independent sample T-test used to compare treatments and control conditions. Statistical significance was attributed to $P<0.05$.

Results and Discussion

Isolation of lactobacilli from traditional dairy products

In this study screening of acid tolerant strains in presence of acidic phosphate buffer (pH=2.5) led to the cultivation of 25 isolates of *Lactobacillus* spp. from different traditional dairy products. Most of them were isolated from cheese.

The traditional dairy products can be possibly a good source of potential probiotic organisms. In Iran, a number of researchers have reported the isolation of LAB from traditional dairy products like doogh, butter, kashk and cheese [11,12 , 21].

Screening for the presence of S-layer proteins and their extraction with 4M GHCl

The presence of a putative S-layer on the bacterial cell surface can be deduced from the occurrence of protein band in the protein profile of intact bacteria [14, 15]. The *Lactobacillus* isolates of this study were analyzed with SDS-PAGE. The presence of protein bands in the range of 45-60 Kda in protein profiles of bacterial lysate, indicating the presence of structure of S-layer. In this study, protein bands of 45–60 kDa were present in 2 of the 20 isolates (KM3, KM7) , suggesting the presence of S-layer proteins in these strains (Figure 1).

The surface noncovalently bound proteins were extracted with 4M guanidine hydrochloride and after dialysis against Tris-HCl buffer, a white aggregate could be seen in the dialysis bag. SDS-PAGE analysis of the extracted proteins showed a band with molecular weight of 50–60 kDa (Figure 1). The S-layer of *L.acidophillus* ATCC 4356 was used as positive control.

Among LAB, S-layer proteins have been found so far only in the genus *Lactobacillus*. In some of these bacteria, adhesive properties of S-layers to matrix components have also been linked to protective functions against invasiveness of pathogenic bacteria as well as

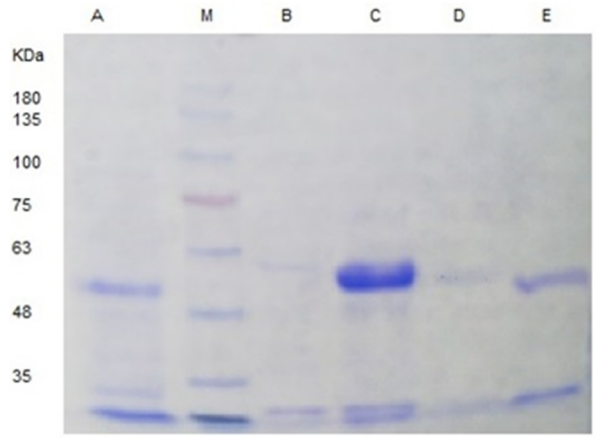

Figure 1. SDS-PAGE of S-layer proteins in two strains (KM3 and KM7), (A) S-layer protein of *L.acidophillus* ATCC 4356, (B, D) protein profile of intact bacteria, KM3 and KM7, respectively. (C, E) dialysed S-layer protein after treatment of bacterial cells (KM3 and KM7, respectively) with 4 M GHCl

to the probiotic properties of health benefiting bacteria [4, 22, 23]. Adhesive properties and possible therapeutic applications of S-layers have made it as appropriate candidate for targeted live antigen delivery vehicles to host tissues [24].

Bile and pH Tolerance of selected lactobacillus isolates

KM3 and KM7 strains were isolated from traditional dairy products and commercial probiotic strain, *L.acidophillus* ATCC 4356 as a positive control survived in MRS broth supplemented with 0.3% sodium deoxycholate as bile salt and this concentration had no inhibitory effect on their growth capability at different times (3, 6 and 24 h) of incubation at 37° C compared with control condition (MRS broth without bile salt) (Figure 2).

Moreover, viability of probiotic bacteria during passage through the stomach is an important parameter to reach the intestine and provide beneficial effect [10]. In this study, the effect of pH on *L.acidophillus* ATCC 4356, KM3 and KM7 strains was tested and the number of viable cells and survival percentage were determined (Table 1). The viability of all strains was reduced at pH (2.5) compared with that at control pH (6.5). *L.acidophillus*

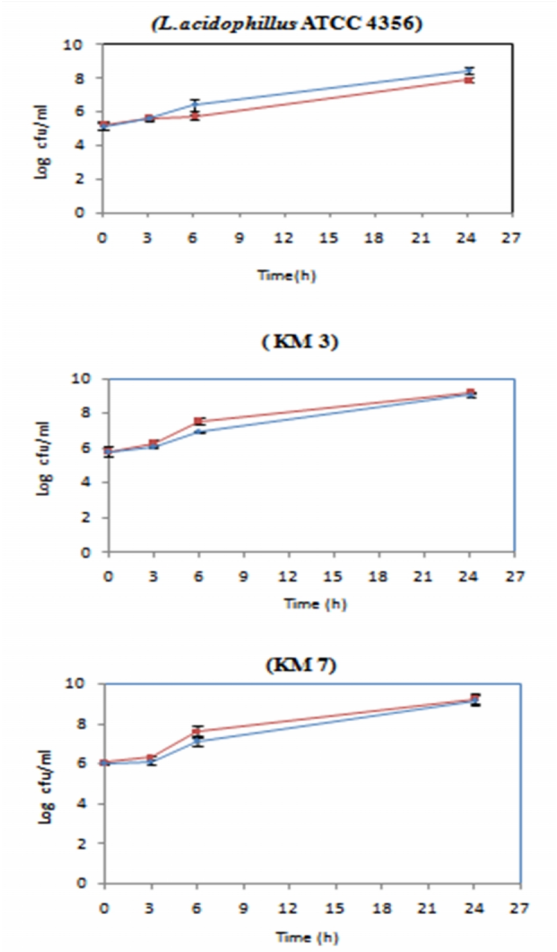

Figure 2. Bile Tolerance of commercial probiotic strain, *L.acidophillus* ATCC4356, KM3 and KM7 strains isolated from dairy products. (■) control condition, (◆) 0.3% bile salt

ATCC4356 has been shown to be more resistance to acid stress (92.4%) than the two native ones. Among two native isolates, KM3 showed better viability than the KM7 (77.8% and 51.6% survival percent, respectively).

In this study we could screen *Lactobacillus* spp. possessing surface (S) layer protein from Iranian traditional dairy products. Morever,the isolated strains can be identified as potential probiotic strains with respect to in vitro acid and bile experiments. Obviously, this important prerequisite of probiotic interest should be assessed in vivo too in order to ascertain the real capacity of the strains to survive through the gastro-intestinal tract.

Table 1. Acid tolerance of Commercial probiotic strain, *L.acidophillus* ATCC4356, KM3 and KM7 strains isolated from dairy products

	Log cfu/ml (viable percentage)		
	L.acidophillus ATCC 4356	KM3	KM7
Control (pH 6.5)	5.56± 0.59 (100%)	6.36± 0.33 (100%)	7.63± 0.32 (100%)
pH 2.5	5.14± 0.02 (92.4%)	4.94± 0.02 (77.8%)	3.94± 0.13 (51.6%)

Each value represents mean +/- SEM (n = 2)

Although the amount of S-layer carrying *lactobacilli* was relatively low, but introduction and development of native strains with surface layer protein and specific probiotic features can be very valuable. This work gives further insight on the probiotic properties of *lactobacilli* carrying S-layer against pathogens. Bile salt tolerance is considered as one of the essential properties required for lactic acid bacteria to survive in the small intestine. The concentration ranges of human bile are 0.3% to 0.5%, however 0.3% is considered as a critical concentration for screening of resistant strains [25, 26]. The acid resistance of the selected isolates might be beneficial for their use as a probiotic as they might withstand gastric stress and survive in high acid food for longer periods without large reduction in numbers.

Identification of the Isolates

The almost complete sequence of 16S rRNA gene was determined and the results demonstrated that two strains (KM3 and KM7) were members of the genus *Lactobacillus* and exhibited maximum similarity to 16S rRNA sequence of *Lactobacillus brevis spp.* "The sequence of KM3 isolate has been submitted to the DDBJ/EMBL/GenBank databases under accession No. KP219433". The phylogenetic position of two isolates (KM3 and KM7) is shown in Figure 3.

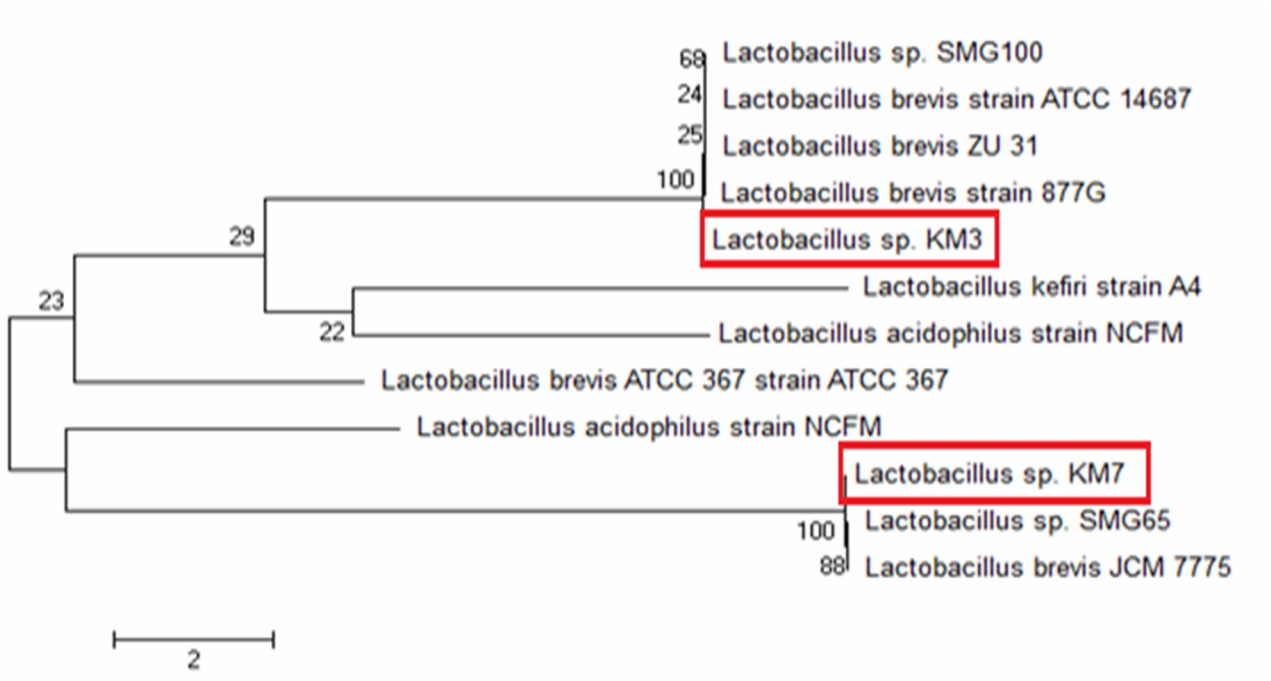

Figure 3. Phylogenetic tree based on the 16S rDNA sequencing of isolates. A phylogenetic tree based on the 16S rDNA sequencing of isolates and other reference strains. The tree was constructed by using the neighbor-joining method

S-layers have been found from several species of the genus *Lactobacillus* [27] such as *L.brevis* that is phylogenetically distant from *L.acidophilus* group [28]. *L. brevis* is often detected in the oral cavity and feces of humans [29]. *L. brevis* ATCC 8287 which has been shown to possess S-layer protein isolated from green fermented olives. This probiotic strain has a GRAS (Generally Recognized As Safe) status and would be used as a live oral vaccine [30, 31].

In this study, our aim was isolation and identification of S-layer carrying *Lactobacillus* for later use as vaccine vectors and/or probiotics..

For vaccine development, as well as for nanobiotechnological applications, in the future study, we must purify the S-layer protein of selected isolates, amplify their genes by polymerase chain reaction (PCR), and sequence the structural and regulatory regions of the S-layer gene.

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