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Full Length Research Paper

Motal cheese of Iranian nomadic tribes as an untouched source of potentially probiotic Lactobacilli

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Several studies have revealed the positive impacts of probiotic microorganisms on human health and physiology. Since the diversity of human microbiota is influenced by factors such as lifestyle and nutrition, looking for appropriate supplies to maintain the balance of gastrointestinal flora should be considered as high priority. In the present study, *Motal* cheese, a special kind of traditional cheese made inside goat skin casing, was intended as a source for isolation of potentially probiotic lactobacilli. The putative bacteria were tested for their acid and bile resistance, antimicrobial potential and antibiotic resistance. The isolates were eventually identified on the basis of 16S rDNA gene sequence and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Two distinct candidates were recognized which best matched with *Lactobacillus acidophilus* and *Lactobacillus plantarum.* According to the outcomes, *Motal* cheese was confirmed as a valuable source of probiotic microorganisms.

Key words: *Motal* cheese, genetic identification, *Lactobacillus*, probiotic.

INTRODUCTION

After the completion of the Human Genome Project, researchers were deeply surprised with the number of protein-coding genes which was nearly 20000, whereas to fulfill the complicated functioning of human body at least 100000 genes are required (Pennisi, 2012). Afterwards, it was deduced that genomes of symbiotic microorganisms provide traits which humans are not needed to evolve on their own (Gill et al., 2006; Lamont et al., 2011). The Human Microbiome Project (HMP) is running and its major goal is to understand the range of human genetic and physiological diversity, the microbiome and the factors that influence the distribution and evolution of the constituent microorganisms (Turnbaugh et al., 2007).

Today, by the increasing use of rather uniform foodstuff such as industrial dairy products and junk foods, the elimination of gut flora diversity becomes more concerning since the uniformity of individuals' gastrointestinal flora could increase the risk of new pandemics. On the other hand, the adaption of beneficial bacteria originated from local or traditional foods to their geographical hosts is a co- evolutionary process which takes place during a long period of time (Barzegari and Saei, 2012).

Then, taking the advantages of these bacteria as a part of natural assets is of central importance. Regardless of

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Figure 1. *Motal* and *Motal* cheese produced in nomadic tribes of Azerbaijan province- Iran.

the improvements in living standards, some tribes in Iran have preserved their early way of living and tend to have nomadic lifestyle. Some of these tribes in the north west of Iran and also Turkey and Azerbaijan Republic produce a special type of cheese so-called *Motal* cheese (Öksüztepe et al., 2005; Kamber et al., 2007). In Azeri language, *Motal* is referred to churns which are made of intact skins of flayed goats or sheeps (Figure 1). *Motal* cheese, because of its particular way of production and ripening seems to be an appropriate source of potentially probiotic bacteria. In this regard, the present study aimed at the isolation and *in vitro* evaluation of *Motal* cheese *Lactobacillus strains*. The potentially probiotic isolates were conducted to morphological and genotypic tests and assessed for their resistance to acid and bile. The acid and bile resistant bacteria were subjected to antibacterial and antibiotic resistance tests. 16S rDNA sequencing and PCR-RFLP were applied for genetic identification of the antibiotic sensitive *Lactobacillus* isolates.

MATERIALS AND METHODS

Cheese samples

Ten samples of *Motal* cheeses were aseptically collected from different nomadic tribes in Azerbaijan province- Iran and transferred to the laboratory. The samples were placed at 4°C in 50 mL falcon tubes.

Production of traditional cheese starter

Traditional cheese rennet is derived from ruminants' abomasums as follows: all of the inner contents of lamb abomasums are removed using cooled boiled water. Then, it is treated by rock salt and wrapped in cheese cloth and floated in appropriate quantity of cooled boiled water. Finally, to remove the unpleasant smell, extracts of some local aromatic plants and sugar is added.

Production of *Motal* cheese

There are two main methods for making *Motal* cheese in nomadic tribes in Azerbaijan province-Iran which are slightly different. Sometimes the hairy part of *Motal* is used for this purpose and when this happens, there is no need to inoculate rennet. However, since this kind of cheese generally contains hair, most people do not find it attractive. The using of the hairless side of *Motal* for cheese production is more common which in this case rennet is required.

For production of *Motal* cheese, one spoon full of the prepared rennet is added to 5 kg of unpasteurized goat or sheep's fatty milk with a temperature of 30°C, approximately. After an hour when milk coagulated, produced whey is drained and cheese curds are cut to small parts. To drain completely, the curds are collected within clean cheesecloth. Then, the packs of curd are hung for eight hours at room temperature to dry completely. The curds in the cheesecloth are then pressed with a weight placed on them for 24 h allowing more whey to be drained. Then, they are broken into pieces and 2% (w/w) of salt is added. The pieces are pressed under the same condition another 24 h. simultaneously, a goatskin churn is moistened to make it flexible. The next day, the curds are crushed and kneaded with raw milk and then tightly stuffed into the cleaned and salted goatskin through its neck (Figure 1). The curd filling is topped by salt, and the casing's opening is closed with a woolen string. Then the goatskin casing is buried in soil during the winter until the next year when nomads return to the same place. Some local ranchers also store *Motals* in cool places such as caves or cellars at approximately 10°C to ripen.

Isolation of bacteria

Cheese samples of 20 g were homogenized with sterile sodium citrate solution (2% w/v) at 45°C in a Stomacher for 2 min. Decimal dilutions were prepared in sterile 0.1% peptone water (Himedia labs, India), then 500 µL of each dilution was inoculated in 10 mL of MRS broth (Scharlau, Spain) and incubated at 37°C for 24-48 h in aerobic and anaerobic conditions. In order to screen lactobacilli for resistance to low pH, 1 mL of fresh enriched cultures was inoculated in 10 mL phosphate- buffered saline (pH = 2.5) and incubated at 37°C for 3 h (Erkkilä and Petäjä, 2000).

Survived bacteria were subsequently revived by inoculation into 10 mL MRS broth and incubation in 37°C for 24 h. Then for selecting bile resistant lactobacilli, 1 mL of culture was inoculated in MRS broth, enriched with 0.3% (w/v) Oxgall (Ox-Bile LP0055; Oxoid) and incubated at 37°C for 4 h (Maragkoudakis et al., 2006).. Serial dilutions of the enriched cultures of acid and bile resistant cultures were prepared. 100 µL of 1/100000 dilution were plated on MRS-agar and incubated at 37°C for 24-48 h under anaerobic condition. Then single colonies were exposed to catalase test and Gram staining. Gram positive, rod shaped and catalase negative isolates were subcultured in MRS broth and stored at -70°C in a mixture of MRS broth- skim milk (10% w/v) and glycerol (30% v/v).

Antibiotic susceptibility of the isolates

The resistance of the isolates to clinically important antibiotics including Methicillin (5 µg), Chloramphenicol (30 µg), Vancomycin (30 µg), Tetracycline (30 µg), Erythromycin (15 µg), Ampicillin (10 µg) was investigated by standard disc diffusion method (Bauer et al., 1966) according to the guidelines of the National Committee for Clinical Laboratory Standards using Mueller Hinton agar. Antibiotic discs were purchased from Padtan Teb Co. (Tehran, Iran) and antibiotic susceptibility tests were carried out according to the producer's guideline. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 35218 served as reference strains for disk diffusion test. Experiment was carried out in triplicate. Finally, the

isolates were classified into three groups: resistant, intermediate and sensitive.

Evaluation of antimicrobial activity of the isolates

The antibacterial activity of the isolates was evaluated by disc diffusion method (Bauer et al., 1966; Sadigh-Eteghad et al., 2011). T he overnight cultures of lactobacilli isolates in MRS broth were centrifuged at 1800 g for 5 min and filter sterilized (0.2 µm filter). 10 ml of this liquid was evaporated to 2 mL using rotary evaporator (Heidolph, Germany). The blank discs of 6 mm diameter were immersed in cell free supernatant and placed on inoculated Nutrient Agar (Sigma, USA) with approximately 100 thousand to 10 million cells of the following indicator bacteria: *Escherichia coli* PTCC 1276, *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 25923 and a wild isolate of *Escherichia coli.* Positive results were identified after overnight incubation at 37°C by clear zones around the discs. For discrimination of non-bacteriocin and bacteriocin related inhibitory effect on bacterial growth (Rodas et al., 2003), pronase treated discs were recruited (Sigma Deisenhofen, Germany). Experiment was performed in triplicate.

Molecular identification of isolates

Total genomic DNA was extracted from overnight broth cultures of the strains by QIAamp DNA mini kit (QIAGEN, 51304) according to the manufacturer's instructions. Previously reported primers (Lane et al., 1985) were used for amplification of 16S rDNA. The primers were: 16F: 5'-AGA GTT TGA TCM TGG CTC AG-3' and 16R: 5'-TAC CTT GTT AGG ACT TCA CC-3'. PCR amplification was carried out in a final volume of 50 µL using an Eppendorf thermal cycler. Each reaction contained 40 ng of DNA, 0.5 µM of each primer and 25 µL of 2X master mix Amplicon^{TM} (Amplicon, Herlev, Denmark). PCR program consisted of an initial denaturation step at 95°C for 4 min which was followed by 35 cycles of: denaturation at 94°C for 50 s, annealing at 59°C for 50 s and extension at 72°C for 80 s and a final extension step of 72°C for 5 min. PCR products were detected by 1% (w/v) agarose gel electrophoresis in TAE buffer stained with SYBR Green (DNA safe stain, Tehran, Iran). For discrimination of the isolates, PCR-RFLP on 16S rDNA amplicons was performed. For this purpose, PCR products were digested by *TaqI* enzyme (Fermentas, ER0671). Each reaction contained 7 µL of purified PCR product, 2 µL of 10X enzyme buffer, 5 units of restriction enzyme and the final volume was reached to 20 µL with nuclease free water. Digestions were carried out at 65°C for 3 h and digested products were electrophoresed on a 1.5% agarose gel and visualized with SYBR Green dye. Then, PCR products with different digestion patterns were sent to Macrogen Company for sequencing. Eventually, NCBI nucleotide BLAST tool was employed to compare the results with reported lactobacilli 16s rDNA sequences. The antibiotic resistant isolate was excluded from sequencing process.

RESULTS AND DISCUSSION

Isolation of bacteria

The screening of the acid and bile resistant isolates in a condition similar to harsh environment of human gastrointestinal tract (GIT) led to the attainment of 21 acid and bile resistant isolates which nine Gram positive, rod shaped and catalase negative isolates including SL, GO, A10L, H10L, M, B, A, YO and CS were realized as lactobacilli.

We believe that preliminary screenings such like, is essential for the isolation of probiotic bacteria. Since, the first priority in this regard, is the ability to survive simulated condition of the human digestive system. Although the encapsulation of lactobacilli isolated from far exogenous sources (example, environmental samples) can increase their survival rate by protecting them from human GIT conditions (Millette et al., 2007; Saarela et al., 2000), they fail to mimic exact symbiotic needs expected from probiotics and the long term persistence of them may be affected. Consequently, one important aim of this study was to isolate the promising strains which have established a symbiotic relationship with human GIT during long period of co- evolutionary process. Perhaps, these kinds of symbionts have been trafficked between food sources and native people repeatedly.

Antibiotic susceptibility of potentially probiotic isolates

The excessive use of antibiotics can change the bacterial resistance patterns in different regions. Since the resistance genes can naturally migrate among bacterial communities, the application of antibiotic resistant bacteria as probiotics may lead to the same outcome (Temmerman et al., 2003).. Accordingly, probiotic bacteria should exhibit an acceptable degree of sensitivity to conventional antibiotics. The unsupervised use of antibiotics in urban areas of Iran has resulted in an inevitable bacterial resistance and altered the diversity of the microbial flora but this is not the case in the rural areas. Thus, screening in the rural areas could provide a better opportunity for isolation of new promising isolates. In the inhibition zone evaluation, eight of the nine isolates from the previous step demonstrated a satisfactory level of sensitivity to six tested antibiotics and were categorized as sensitive or intermediate (Bauer et al., 1966). As expected, the reference strains including *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 35218 demonstrated standard diameters of inhibition zones for all tested antibiotics. However, isolate A had an inhibition halo <13mm for Erithromycin and <11mm for ampicillin and according to NCCLS guidelines was considered as resistant isolate to these two antibiotics (Figure 2).

Analysis of antimicrobial activity

Another feature of beneficial probiotic lactobacilli is their inhibitory effects on the growth of pathogenic bacteria. In the present study, all the nine candidates were further assessed for their antimicrobial activity. Figure 3 indicates their inhibitory effects on the indicator pathogenic bacteria. SL, M and B exhibited more suppressing effects on wild *E. coli,* isolate B inhibited *S. typhimurium* more intensely and GO had a higher restrictive impact on both *E. coli* PTCC 1276 and *S. aureus*. Based on the results *S.*

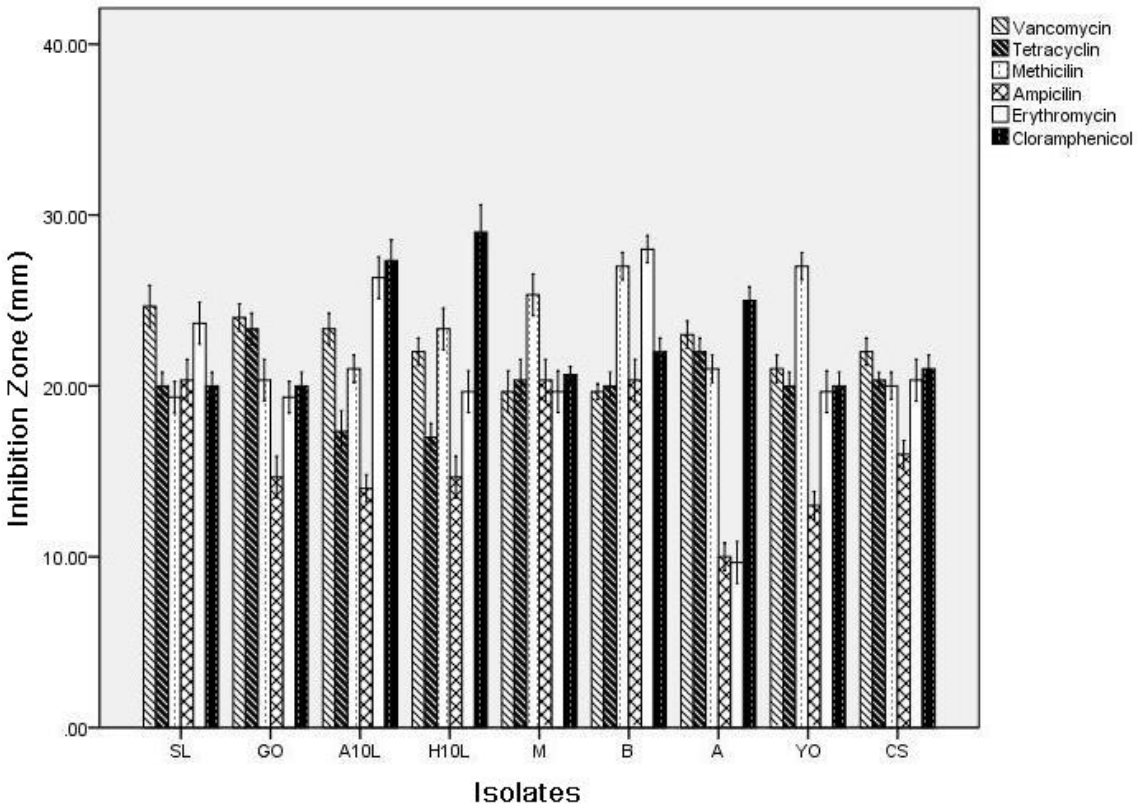

Figure 2. The inhibition zone of routinely used antibiotics (Vancomycin, Tertacycline, Methicillin, Ampicillin, Erythromycin, Chloramphenicol) against putative isolates SL, GO, A10L, H10L, M, B, A, YO and CS.

aureus were the most tolerant bacteria to *Lactobacillus* inhibition compared to the other pathogenic bacteria. The suppressing effects of lactobacilli on pathogenic microorganisms could be due to several factors such as production of H_2O_2, organic acids and particularly bacteriocins (Larsen et al., 1993; Nakai and Siebert, 2003; Millette et al., 2007). Recently, bacteriocin proteins have attracted a great deal of interest for their food preservative and antibacterial properties. To confirm the bacteriocin mediated inhibition of bacteria, pronase treatment was applied (Todorov et al., 2004). Only two isolates (YO and GO) were found to inhibit the indicator bacteria via bacteriocin effect, while other isolates caused inhibition by non-bacteriocin effect(s).

Molecular identification of lactobacilli

According to the FAO/WHO guideline for the evaluation of probiotics in food, *in vitro* speciation of the isolates was established using a combination of phenotypic and genetic tests (FAO/WHO, 2002). Identification of Lactobacillus genus by 16S rDNA gene amplification which is highly conserved in some part has been frequently described in the literature (Moreira et al., 2005). The mentioned gene also has very variable regions that can provide species specific signature (Ben Amor et al., 2007). In this study, PCR amplification of 16S rDNA gene was utilized for genotyping of lactobacilli at the genus level. Consequently, amplicons with approximately 1500 bp in size, corresponding to the full length of 16S rDNA gene in different isolates were obtained. Afterward, PCR- RFLP was used for discrimination of the isolates. Two distinct clusters were created when the 16S rDNA fragments were digested by *TaqI* enzyme (Figure 4). 16S rDNA amplicons of YO and GO as representatives of each cluster were subjected to sequencing. Isolate A, because of its resistance to Erythromycin and Ampicillin, also other six isolates due to lack of bacteriocin mediated inhibition on pathogenic bacteria were not considered for sequencing. Using BLAST software (http://www.ncbi.nlm.nih.gov/BLAST) the sequencing results were compared with those deposited in NCBI GeneBank as 16S rDNA gene of different *Lactobacillus* species. Accordingly, YO and GO were found to be 100% similar to *L. acidophilus* and *L. plantarum*, respectively.

Conclusion

Iranian *Motal* cheese, a unique type of traditional cheese, because of its particular way of production seems to be a valuable source of advantageous microorganisms. In this regard, some local samples were assessed for presence

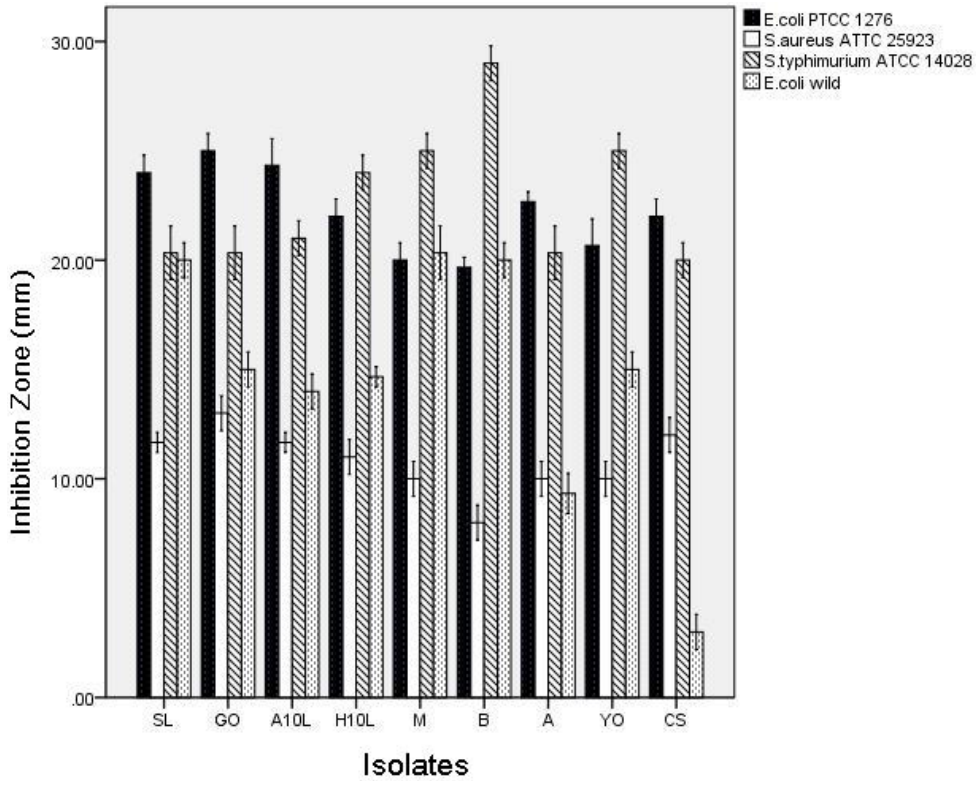

Figure 3. The inhibitory effect of putative isolates (SL, GO, A10L, H10L, M, B, A, YO and CS) on pathogenic bacteria including *E. coli* PTCC 1276, *S. aureus* ATCC 25923, *S. typhimurium* ATCC 14028 and a native *E. coli*.

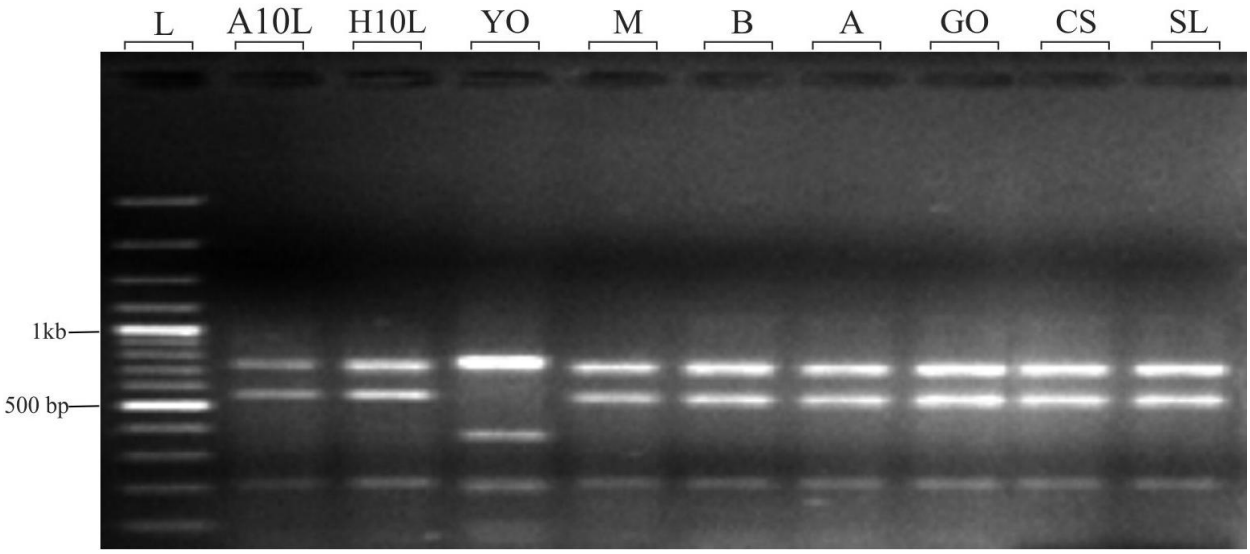

Figure 4. Gel electrophoresis of 16S rDNA amplicons digested by *Taq*I which revealed two distinct categories among 9 putative isolates.

of potentially probiotic lactobacilli. Morphological, biochemical and molecular investigations resulted in isolation of acid and bile resistant *Lactobacillus* strains which can survive in the harsh condition of gastrointestinal tract. Bacteriocin mediated antimicrobial properties and sensitivity to commonly used antibiotics were the other outstanding features of the chosen bacteria. It is deduced that, *Motal* cheese is an attractive source of probiotic microorganisms and could be used as dietary supplement for human health promotion.

REFERENCES

Barzegari A, Saei AA (2012). Designing probiotics with respect to the native microbiome. Future Microbiol. 7: 571-575.

Bauer AW, Kirby WM, Sherris JC, Turck M (1966). Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45: 493-496.

Ben Amor K, Vaughan EE, De Vos WM (2007). Advanced molecular tools for the identification of lactic acid bacteria. J. Nutr. 137: 741S-747S.

Erkkilä S, Petäjä E (2000). Screening of commercial meat starter cultures at low pH and in the presence of bile salts for potential probiotic use. Meat Sci. 55: 297-300.

FAO/WHO (2002). Guidelines for the evaluation of probiotics in food. Report of a Joint FAO/WHO Working Group. http://www.fao.org/es/ESN/food/foodandfood_probio_en.stm.

Gill SR, Pop M, DeBoy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE (2006). Metagenomic analysis of the human distal gut microbiome. Science. 312: 1355-1359.

Kamber U (2007). The traditional cheeses of Turkey: eastern Anatolia region. Food Rev. Int. 24: 148-174.

Lamont RF, Sobel JD, Akins RA, Hassan SS, Chaiworapongsa T, Kusanovic JP, Romero R (2011). The vaginal microbiome: new information about genital tract flora using molecular based techniques. BJOG. 118: 533-549.

Lane DJ, Pace B, Olsen GJ, Stahl DA, Sogin ML, Pace NR (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. PNAS. 82: 6955-6959.

Larsen AG, Vogensen FK, Josephsen J (1993). Antimicrobial activity of lactic acid bacteria isolated from sour doughs: purification and characterization of bavaricin A, a bacteriocin produced by Lactobacillus bavaricus MI401. J. Appl. Microbiol. 75: 113-122.

Maragkoudakis PA, Zoumpopoulou G, Miaris C, Kalantzopoulos G, Pot B, Tsakalidou E (2006). Probiotic potential of Lactobacillus strains isolated from dairy products. Int. Dairy J. 16: 189-199.

Millette M, Luquet F, Lacroix M (2007). In vitro growth control of selected pathogens by Lactobacillus acidophilus and Lactobacillus casei fermented milk. Lett. Appl. Microbiol. 44: 314-319.

Moreira JLS, Mota RM, Horta MF, Teixeira SMR, Neumann E, Nicoli JR, Nunes ÁC (2005). Identification to the species level of Lactobacillus isolated in probiotic prospecting studies of human, animal or food origin by 16S-23S rRNA restriction profiling. BMC Microbiol. 5: 15-25.

Nakai SA, Siebert KJ (2003). Validation of bacterial growth inhibition models based on molecular properties of organic acids. Int. J. Food Microbiol. 86: 249-255.

Öksüztepe G, Patır B, Çalıcıoğlu M (2005). Identification and distribution of lactic acid bacteria during the ripening of Şavak tulum cheese. Turk. J. Vet. Anim. Sci. 29: 873-879.

Pennisi E (2012). ENCODE project writes eulogy for junk DNA. Science. 337: 1159-1161.

Rodas AM, Ferrer S, Pardo I (2003). 16S-ARDRA, a Tool for identification of Lactic Acid Bacteria isolated from grape must and wine. Syst. Appl. Microbiol. 26: 412-422.

Saarela M, Mogensen G, Fonden R, Matto J, Mattila-Sandholm T (2000). Probiotic bacteria: safety, functional and technological properties. J. Biotechnol. 84: 197-215.

Sadigh-Eteghad S, Dehnad A, Shanehbandi D, Khalili I, Razmarayii N, Namvaran A (2011). Identification and characterization of a Streptomyces sp. isolate exhibiting activity against multidrug-resistant coagulase-negative Staphylococci. Vet. Res. Comm. 35: 477-86.

Temmerman R, Pot B, Huys G, Swings J (2003). Identification and antibiotic susceptibility of bacterial isolates from probiotic products. Int. J. Food Microbiol. 81: 1-10.

Todorov SD, Van Reenen CA, Dicks LM (2004). Optimization of bacteriocin production by Lactobacillus plantarum ST13BR, a strain isolated from barley beer. J. Gen. Appl. Microbiol. 50: 149-157.

Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI (2007). The Human Microbiome Project. Nature. 449: 804-810.