



Comparison of the Sensitivity of Phenotypic and PCR Method For The Detection Of Lactobacillus Acidophilus Isolated From Aushari Chesse

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ABSTRACT

Aushari (Pesta) cheese is one type of traditional cheese that is very common in Kurdistan region in Iraq, the identification of strains deals with ripening cheese is very important, in order to know the accurate strain that are responsible of the last flavor of the cheese. Classically, the microbial ecology of Cheeses has been studied by using traditional microbiological analysis based on the use of phenotypic test (1). However, this approach is not always appropriate to study complex microbial communities, because different species within a genus can demonstrate the same fermentation patterns and growth requirements (2). Also, different culture conditions and the diversity of strains can create difficulty in reproducing the results of phenotypic tests (3). Molecular techniques have allowed the precise and rapid identification and typing of bacterial strains, providing new ways to check for their presence and monitor their development (4). Among molecular techniques that have improved the discriminatory power of identification, Polymerase Chain Reaction (PCR) finds wide application in detection of lactobacilli (5). A variety of PCR methods are now available for the identification of bacterial strains (6). Variations in the lengths and sequences of rRNA intergenic spacer regions (ISRs) and Random Amplified Polymorphic DNA (RAPD) analysis have been successfully applied to the lactobacilli, including *L. acidophilus* (7). One of the most widely applied approaches deals with the use of 16S rRNA and its encoding genes as target molecules. PCR amplification of 16S rDNA -targeted primers have successfully been used for the detection and identification of Lactobacillus species (8) and (9), the aim of this study was: comparison the sensitivity of culturing and 16srRNA PCR to identify the species level of *Lactobacillus acidophilus* isolated from Aushari cheese.

Keywords: Aushari cheese, PCR, RAPD, Lactobacillus Acidophilus Isolated.

1. Materials and Methods

Sampling And Isolation Of *L. Acidophilus*: total of 21 fresh Aushari cheese samples were collected from different villages of Sulemania and Erbil, then 10g of each sample were Wight

aseptically and homogenized in 90 ml of distilled water ,the isolation of *Lactobacillus* was performed on solid selective media (10) .The streak plate method was used to isolate the lactic acid bacteria. For this purpose a loop full of each sample was streaked on MRS plates with 10 % lactose solution and the plates were incubated at 37 °C for 24 h. After incubation, the culture was observed for growth, single and isolated colonies were picked and sub cultured on MRS agar and incubated at 37 °C for 24 h to obtain pure culture of the isolate. Simultaneously the smears were prepared and stained with Gram's stain and examined under microscope for the staining characteristics and morphology of the colony.

Biochemical test: The strains isolated from different cheese sample were purified and characterized by biochemical tests according to the methods of (11), which include : Carbohydrate Fermentations, Indole production , Catalase Test, Nitrite reduction test. Gelatinase test, Arginine test, Gas production from glucose, Growth at Different Temperatures, and at different NaCl concentrations and Production acid from litmus milk.

Genomic DNA Isolation: Genomic DNA was prepared using the following procedure of (7).with some modification. Ten ml overnight cultures were prepared in MRS broths. Cells were harvested in a micro centrifuge for 5 min at 6000 rpm. After this, they were suspended in 200 µl 1xTE buffer (pH8) Containing 25% sucrose and 30 mg/ml lysozyme. The cell Suspensions were then incubated for 1 h at 37 °C. After the incubation, 370 µl, 1x TE (pH 8) containing Proteinase K (1mg/ml) and 30 µl, 10% SDS were added. The samples were then incubated for 1 h at 37 °C. Cells were lysed by the addition of 100 µl 5M NaCl and 80 µl CTAB/NaCl solution (10% Cetyl Tri methyl Ammonium Bromide, 0.7 M NaCl), respectively. Lased samples were incubated for 10 min at 65 °C with equal volume of chloroform (chloroform/isoamyl alcohol:24/1),Chloroform extractions were performed twice. The aqueous phase was transferred into a clean tube and the genomic DNA was precipitated by the addition of isopropanol (one equal volume) after addition 10% of the volume by ammonium acetate and then incubates in freezer for 30 minutes.the precipitated DNA was transferred into a fresh tube adding 500 µl of (70%) ethanol, and washed.Finally, it was dissolved in TE buffer.

2. PCR Analysis

The primers used were obtained from Nottingham university (England) and it s sequences was 16S1(sense)F AGSTGAACCAACAGATTCAC as forward primer and 16S2(antisense)R ACTACCAGGGTATCTAATCC as reverse primer which are *L. acidophilus* specific primer were applied for the identification of the *L. acidophilus* . PCR reactions were performed with 1×PCR buffer; 2.5 mM MgCl₂; 50ng DNA, 0.3 µM each primer; 0.25 mM (each) dNTP; 25 U/ml Taq DNA polymerase (sigma). Amplification consisted of 35 cycles: 1 min at 94°C, 1 min ,1 min at 55°C, and 1 min at 72°C. The amplified products were electrophoreses in 1% agarose gel and were subsequently visualized by UV illumination after ethidium bromide staining.

3. Results And Discussion

Phenotypic Identification Of *Lactobacillus Acidophilus*: the selective medium MRS at 37°C anaerobically had highest recovery in the isolation of *Lactobacillus acidophilus* strains, they are Gram-positive bacterium, rod shaped, ordered in chains with small circular white color colonies. The isolates were Catalase negative, unable to produce NH_3 from Arginine, possess no gelatinase enzyme and able to produce acid from Litmus milk also they unable to reduce nitrite and did not produce (CO_2) from glucose, these results are in agreement with (11) and (12). All of the isolates have the ability to grow at 26% NaCl concentration.

The isolates which gave the bright orange were accepted that they can produce ammonia from arginine. Phenotypic tests are often not definitive for species identification because different species within a genus can demonstrate the same fermentation patterns and growth requirements (Andrighetto et al., 1998). Also, different culture conditions and the diversity of strains can create difficulty in reproducing the results of phenotypic tests (Tannock, 1999). The precise identification of microorganisms is a prerequisite to select new strains among several bacteria isolates. Intra specific differentiation of bacteria is highly relevant for the selection of starter and probiotic cultures. Besides typing methods are very helpful in distinguishing patent protected strains, as for the safety aspects. It is also important to isolate Non Starter Culture isolates from cheese to identify the accurate strain associated with any kind of cheese.

Genomic DNA Isolation From Bacteria: Suitable yields of genomic DNA were obtained from repeated experiments with average yields between (50-95 $\mu\text{g}/\text{ml}$) from 10ml broth culture media (figure 1). The purity was also found good ranging between 1.7-1.8 determined by spectrophotometer ratio A_{260}/A_{280} , the molecular weight of DNA was estimated using 1% agarose gel electrophoresis. This method was found to be very efficient to obtain suitable amount of DNA for the PCR. However obtaining this result was not an easy task, because *L. acidophilus* is one of the Gram-positive species and unlike Gram-negative bacteria, Gram-positive species are much more resistant to cellular lyses resulting from the extensive concentration of peptidoglycan within the cell wall.



Fig 1 represent the DNA extracted from seven samples of *L.acidophilus*.Gel electrophoresis was performed on 1.2% Agarose gel and run at 90 Volt /cm for one hours Lane 1 was standard molecular weight marker 50Ug/ml and lanes 2-7 represent the isolates

PCR Reaction : The success of PCR assays for reliable identification of lactobacilli both at genus and species level is chiefly dependent upon the designing of the primers targeted against specific genes .With the increasing number of primers, the manufacturer of a bacterial dairy product is faced with the challenge of selecting the most suitable primer set. One of the target gene used in this study was the 16srRNA gene because it s very effective for species identification of lactobacilli. This pair of primers produce amplified band in an expected size (785 bp) from specific region of *L.acidophilus* (figure 2). This PCR assay provides a more rapid, specific and more sensitive alternative to conventional culture bacteria ,besides the same results obtained when apply the PCR Reaction using the DNA extracted from the cheese directly this mean that it can be used for detection of inaccuracies in labeling of species contained within commercial probiotic products.

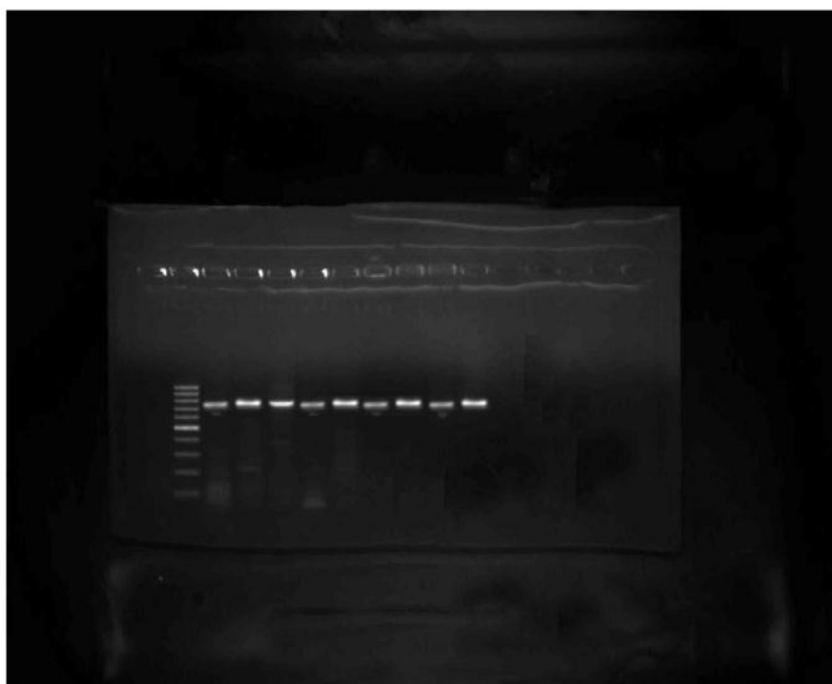


figure 2 represent the amplified products of *L.acidophilus* isolates using the primer targeting the 16SrRNA gene which could be distinguished by the presence of a band with molecular size of 785 bp ..Lane 1. 1kb DNA ladder Lanes3,4,5, and 6 represent the isolate of *L.acidophilus* strains Electrophoresis was performed on 1.2% Agarose gel and run with 90 volt for one hour.

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