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Annals of Biological Research, 2012, 3 (5):2213-2216
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0976-1233
CODEN (USA): ABRNBW

Isolation and Molecular Study of Potentially Probiotic Lactobacilli in Traditional White Cheese of Tabriz in Iran

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ABSTRACT

Probiotics are live microorganisms which transit the gastrointestinal tract and in doing so benefit the health of consumer. The isolation of probiotic bacteria from different native sources especially from traditional cheese was mostly suggested. The objective of this study was the isolation of potentially probiotic Lactobacilli from traditional cheese presented in Iran, Tabriz market. In total, 50 samples of traditional cheese were collected randomly from Tabriz market. After enrichment in MRS broth, the bacterial flora was screened for its acid and bile resistance. Then, the single colony was picked up from MRS agar for the evaluation and molecular identification. The modified ARDRA technique was exploited for the identification of isolates of lactobacillus at the level of species. After ARDRA, two isolates were sequenced. The isolated lactobacilli belonged to L. Fermentum and L. plantarum. The results showed that the traditional Tabriz cheese contained the superior strain of probiotic lactobacilli. So, ARDRA method is a rapid and accurate method for molecular evaluation of lactobacilli.

Keywords: Lactobacillus, probiotic, ARDRA, traditional cheese.

INTRODUCTION

In spite of advances in the development of industrial techniques and equipments and despite the increases in production and in variety of industrial cheese in Iran, several traditional cheese types are being produced and consumed in different regions of the country. The traditional white cheese in Tabriz markets are commonly called Lighvan cheese. It was traditionally and commonly made from raw ewe's milk and occasionally made from raw goat's milk, raw cow's milk and/or a mixture of them. Because of its pleasant organoleptic properties, this type of cheese is popular and widely consumed all over Iran and is enjoying high economical and nutritional value [1].

Numerous microorganisms including bacteria, yeasts and molds are present in cheeses which form a complex microbial ecosystem. Lactic acid bacteria (LAB) are involved in the cheese manufacture and ripening. Among LAB, numerous genera such as Lactobacillus, *Streptococcus*, *Enterococcus* and *Leuconostoc* are involved [2]. The genus Lactobacillus has a long history of safe use, especially in the dairy industry, and plays a major role in the production of fermented milk products. Lactobacilli are important organisms recognized for their fermentative ability as well as their health and nutritional benefits. They produce various compounds such as organic acids, diacetyl, hydrogen peroxide, and bacteriocin or bactericidal proteins during lactic fermentations [3].Over the past few decades, an increased drive has existed for the isolation of novel *Lactobacillus* strains which exert a beneficial health effect when ingested by humans. Such strains are termed [4]. Probiotic terms derived from Greek words Pro (favor) and bios (life) [5]. Probiotics are live microorganisms which transit the gastrointestinal tract and in doing so, they benefit the health of consumer [6]. Beneficial effects conferred by lactobacilli include the inhibition of pathogenic

organisms such as *Salmonella*, *Shigella* and *Helicobacter*. Furthermore, lactobacilli have been associated with numerous other health benefits such as the reduction of lactose intolerance and increased immune response. A beneficial role for lactobacilli has also been implied in cancer and especially in the case of colon cancer [4, 7].

The Probiotic bacteria selected for practical applications must retain the characteristics for which they were originally selected (Salminen et al., 1996)[8]. These include the growth and survival during transit through the stomach and small intestine [9-11]. Consequently, it is necessary to test the stability of these characteristics during ingestion to ensure that they are retained in the host. Therefore, the initial screening and selection of probiotic strains must include the testing of acid and bile tolerance [9].

To analyse and rapidly identify bacteria from microbial communities, classical physiological and biochemical tests are not adequately efficient, since bacterial population involved often has similar nutritional requirements and grows under similar environmental conditions. Therefore, a clear identification within the species by simple phenotypic tests may sometimes be difficult. The development of molecular techniques has opened up new perspectives for characterizing strains from fermented dairy foods [12-13]. Among PCR-based methods, 16S rDNA PCR-RFLP analysis is easy, rapid and inexpensive way to identify microbial species such as yeast, acetic acid bacteria, and also few Gram positive bacteria [14].

According to the WHO guideline, currently, the isolation and characterization of new strain of probiotic bacteria and lactobacillus in particular is facilitated through the use of molecular markers [15]. Therefore, in the present work, modified ARDRA technique was exploited for the identification of isolates of lactobacillus at the level of species. For the precise identification and also the confirmation of preliminary identification, molecular method ARDRA and sequencing of l6S rDNA genes were used. Indeed, we developed a reliable and fast method that was carried out using the bioinformatics-assisted ARDRA technique.

MATERIALS AND METHODS

Screening of lactobacillus isolates

In total 50 cheese samples were used for the isolation of lactobacilli. Samples were collected randomly from retailers in different regions of Tabriz. Each sample containing 20 g of cheese was homogenized within sterile sodium citrate solution (2% w/v) at 45°C in a Stomacher 400 Circulator (Seward, London, UK) for 2 min. Then a volume of 1 ml of each dilution was added in 20 ml of MRS broth and incubated at 37ºC for 24 h. In order to screen the Lactobacilli for its resistance to low pH, 1ml of fresh enriched culture was inoculated in phosphate- buffered saline (PBS, pH 3) and incubated at 37°C for 3 hours. In order to select bile resistant Lactobacilli, 1ml of PBS was inoculated in MRS broth, enriched with 0.3% (w/v) Oxgall (Ox-Bile LP0055; Oxoid), incubated at 37 ºC for 3 h. Serial dilutions were prepared from the enriched cultures of acid and bile resistant cultures. A volume of 100µl of 10^{-5} dilution were placed on MRS-agar and incubated at 37°C for 24-48 h under anaerobic condition (BBL Gas Pak Anaerobic System Envelopes, USA). Then single colonies were subjected to catalase test and gram staining procedure. Gram positive, rode shaped and catalase negative isolates were subcultured in MRS broth and stored at MRS broth with skim milk (10%) and glycerol (30%) in -70 °C.

DNA Extrraction and PCR Amplification of 16S rDNA

Extraction of total genomic DNA was carried out according to the method described by Atashpaz et al. [16]. Ten milliliter of an overnight MRS broth culture was used for this purpose. The extracted DNA was then suspended in 50 nuclease free water and evaluated on a 1% agarose gel electrophoresis. A pair of primers 16F 5-AGAGTTTGATCMTGGCTCAG-3 16R 5-TACCTTGTTAGGACTTCACC-3was used for direct amplification of an approximately 1500 bp fragment containing 16S ribosomal RNA gene. that reported previously [17]. PCR reaction was carried out in a final volume of 50 µl on an Eppendorf thermal cycler. Each reaction contained 40 ng of DNA, 0.5 µM of each primer and 25 µl of 2X PCR master mix (cinagene Tehran Iran). PCR program consisted of an initial denaturation step at 94°C for 5 min which was followed by 35 cycles of: denaturation at 94°C for 1 min, annealing at 55°C for 50 s, and extension at 72°C for 90 s, with a final extension step of 72°C for 10 min. PCR products were detected by 1% (w/v) agarose gel electrophoresis in TAE buffer stained with ethidium bromide.

ARDRA and bioinformatic analysis

The 16S rDNA sequences of all lactobacillus species of NCBI database were aligned and their digestion patterns were predicted for *Taq*I enzyme. Five µl of each purified PCR products were digested using *Taq*I (Fermentas, ER0671) enzyme. Digestions were carried out at 65°C for 3 h. The components used for the digestion reaction were: 2 µL of 10X enzyme buffer, 5 units of each enzyme, 5 µL PCR product and up to 20 µL with deionized water. Virtually digested sequences were compared with the ARDRA results to reveal the polymorphism of unknown

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isolates and identification of species. Accordingly, several reported 16s rDNA sequences in the NCBI Gene bank were subjected to the virtual digestion with the *TaqI* using GeneDoc software. The virtually digested pattern was compared with the experimental ARDRA result to show any differences in the digestion pattern of isolates.

Sequencing of PCR products

The PCR products were sent to Macro gene Company (Korea) for sequencing. Initial studies were performed by chromas software (version 2.13). Furthermore, the NCBI Online BLAST Tool was employed for the comparison of sequencing results, considering 16s rDNA sequences of different *Lactobacillus* species.

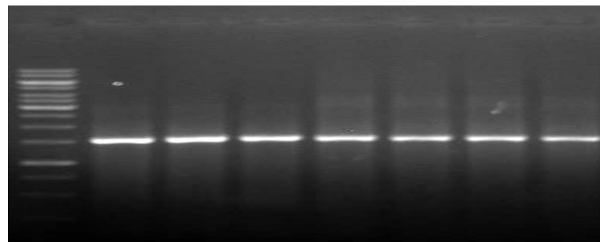
RESULTS AND DISCUSSION

Screening of Acid and Bile Resistant Isolates

Human gastrointestinal tract has a very unpleasant condition for successful delivery of useful bacteria into intestinal environment. Most of microorganisms are sensitive to the low pH of gastric juice or alkaline condition of intestine. So resistance to the acid and bile is a basic consideration in the screening of probiotic organisms. In order to simulate the gastrointestinal condition, the isolates from 50 cheese samples were subjected to low pH (3) for 3 h and bile salts (0.3%) for 3 hours. Screening resulted in the selection of 13 acid and bile resistant isolates.

PCR amplification of 16s-rDNA gene

Amplified Ribosomal DNA Restriction Analysis (ARDRA) is an accessible aid for the identification of *Lactoacillus* bacteria [12].Therefore, the PCR amplification of 16S rDNA gene was performed in order to confirm the lactobacillus in the genus level. Neither the inhibition of the amplification nor the restriction was observed. Fragments of approximately 1500 bp corresponding to the almost full length of 16S rDNA were obtained in different lactobacilli species, as expected (fig 1).



FigI., The PCR amplification of 16 s rDNA and electrophoresis in 1% agaros.

ARDRA Analysis and Identification of Lactoacilli

In this study, the identification of new isolates is achieved using the evaluation of bioinformatically digested pattern of the GenBank sequence as a reference. So, the result of bioinformatic-assisted ARDRA method exhibited high discrimination efficiency with *TaqI* enzyme, and accordingly the same profile was created when the 16S rDNA amplicon of the 13 isolates were cleaved using the *TaqI* enzyme (Fig 1). Comparison between the ARDRA profiles of virtual reference strains and unknown isolates revealed two distinct lactobacilli with higher homology to lactobacillus fermentum and lactobacillus planetarium.

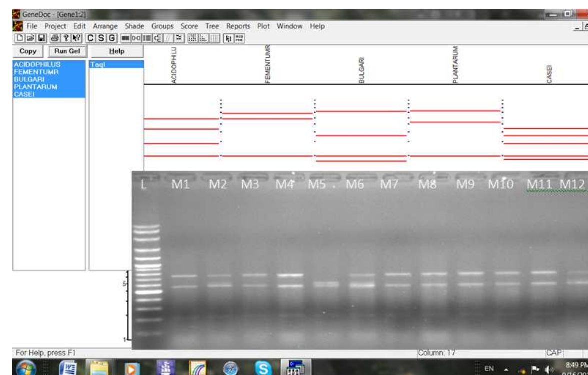


Fig2. The ARDRA pattern of 16s rDNA and analysis with bioinformatics software Gene Doc

Sequencing of 16s-rDNA
The PCR product the 16S rDNA gene was sequenced BLAST done with software (this sentence needs rewriting) (http://www.ncbi.nlm.nih.gov/BLAST), the determined sequences were compared with the sequences deposited in NCBI GenBank as 16S rDNA of lactobacilli species. Comparison of results revealed that the distinguished lactobacilli including M1 and M5 are 100% similar to *Lactobacillus planetarium* and *Lactobacillus fermentum* respectively.

CONCLUSION

The isolation of probiotic bacteria from different native sources especially from traditional cheese was mostly suggested. Based upon our findings, the preliminary screening of Tabriz cheese that gets ready by traditional process seems to be so valuable source in introduction of new promising probiotic bacteria.

Acknowledgement
This study was supported by the Islamic Azad University, Tabriz branch.

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