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Rep-PCR characterization and biochemical selection of lactic acid bacteria isolated from the Delta area of Egypt

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ABSTRACT

Samples of raw milk and traditional dairy products were collected from different rural areas in the Delta region. 170 isolates from these products were identified using repetitive genomic element-PCR (Rep-PCR) fingerprinting. The identified isolates were tested for efficiency of biomass production and separation, acidifying activity, autolytic and aminopeptidase properties, antagonistic activities and exopolysaccharide production. The obtained results revealed that the *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus fermentum*, *Enterococcus faecium Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus plantarum* and *Lactococcus lactis* subsp. *lactis* were the predominant species in Egyptian dairy products. Two percent of *Lactococcus*, 10% of *Lactobacillus* and 1% of *Enterococcus* isolates showed fast acidifying activity. Aminopeptidase and autolytic properties were generally higher for most *Lactobacillus* strains when compared to other strains. Among these species, *lactobacillus paracasei* subsp. *paracasei* was the highest in Aminopeptidase activity and autolytic properties. Antagonistic activity was detected in 40% of *Lactococcus*, 70% of *Lactobacillus* and 50% of *Enterococcus* isolates. Some isolates produced exopolysaccharides in milk and dairy products.

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1. Introduction

Lactic acid bacteria (LAB) have a technological role that is indispensable in food processing, especially in dairy manufacturing. This unique group of microorganisms is a part of the daily diet of virtually all people all over the world. They are often added as starter cultures and occurred widely as indigenous contaminants in raw milk. They possess a large number of metabolic activities that are responsible for the acceptability of fermented milk and cheeses (El Soda, 1999). A lot of research has been directed towards the investigation of the proteolytic and autolytic systems in LAB (Awad et al., 2000). These activities represent the successful key to accelerate the cheese ripening and avoid the bitterness (El-Soda et al., 2000). In the rural areas in Egypt, the wild LAB microbiota represents a natural reservoir for microbial cultures that contains diverse genetic information. It also has characteristic strains that are highly resistant to the manufacturing environmental conditions such as high salt concentration and phage infection. In addition, their antagonistic activities allow these competitive 'wild' cultures to dominate their environment. Moreover, they contribute to produce specific characteristics such as taste, aroma and texture of traditional Egyptian dairy products.

Isolation and screening of LAB from naturally occurring process have always been the most powerful means of obtaining useful cultures for commercial purposes. Egyptian dairy products are generally produced under artisan conditions from raw milk without industrial starter cultures. The main traditional cheeses are Ras cheese (hard type), Domiati cheese (brine ripened), Karish cheese (soft type) and the main fermented milk products are Zabady and Laban Rayeb (Ayad et al., 2004). Most bacterial strains included in industrial starters have very similar physiological properties, nutritional requirements and grow under similar environmental conditions (De Urraza et al., 2000). Indeed, classical phenotypical tests employed for LAB identification can be difficult to interpret and the techniques are also time-consuming. The use of molecular-based techniques offer a rapid and specific alternative. Protocols for bacterial typing using polymerase chain reaction (PCR) techniques are becoming increasingly valuable (De Urraza et al., 2000). Among these methods, randomly amplified polymorphic DNA (RAPD PCR) has been applied to species and strains differentiation (Cocconcelli et al., 1997, Drake et al., 1996, Quiberoni et al., 1998) and classification (Tailliez et al., 1998) of several LAB. In most cases, RAPD requires more than one primer to characterize strains. Since this technique uses nonspecific amplification conditions, such as low annealing temperature, and thus the reproducibility of DNA fingerprints is low, another PCR approach uses oligonucleotides designed to match consensus sequences of genomic repetitive elements obtained from enteric bacteria {Enterobacterial Repetitive Intergenic consensus (ERIC)} or Repetitive Exteragenics Palindromic (REP) sequence or streptococci (BOX sequence) (De Urraza et al., 2000). In the present study, LAB previously isolated from Egyptian dairy products and identified by Apparatus and

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Procedure of Identification (API) system and protein profiling of whole-cells using sodium dodecylesulfate poly-acrylamide gel electrophoresis (SDS-PAGE) were studied for their genetic diversity using BOX-PCR and their technological characteristics including acidifying, aminopeptidase, autolytic activities, antagonistic activities and exopolysaccharide production.

2. Materials and methods

2.1. Bacterial strains and cultivation

The reference strains were obtained from Institute National de Recherche Agronomique (INRA), Center National de Recherches Zootechnique Jouy-en Josas, France (CNRZ) and American Type Culture Collection (ATCC). These strains had been previously characterized on the basis of microscopic examination, Gram staining and catalase reaction. Stock cultures were maintained at −80 °C in reconstituted skimmed milk plus 15% glycerol and working cultures were prepared from frozen stocks through two transfers in MRS or M_{17} broth. One hundred and seventy isolates were isolated at Laboratory of Microbial Biochemistry of dairy microorganisms (LMB), Alexandria University from 44 dairy samples (cow's and buffalo's milk, Zabady "Egyptian fermented milk", Rennet, Domiati, Kareish and Ras cheeses) which were collected from different regions in Delta area in Egypt. They had been previously identified using preliminary tests at the genus level followed by identification using Apparatus and Procedure of Identification (API) system API50CHL, API20 strep (BioMérieux, France) and sodium dodecylesulfate poly-acrylamide gel electrophoresis (SDS-PAGE) analysis of whole cell proteins as described by Pot et al. (1994).

2.2. Rep-PCR analysis

Total DNA was extracted from 1.6 ml of fresh cultures in the exponential phase using the Wizard DNA purification Kit as described with the manufacturer (Promega). The quality of DNA obtained was estimated by comparison to known standards in ethidium bromide stained 0.8% agarose gel. The DNA concentration of each sample was adjusted to 25 ng/µl in a 25 µl PCR mixture. Amplification was performed in a 25 µl reaction volume, typically containing 25 ng genomic DNA, 0.3 µM BOXAIR primer (5′-CTACGGCAAGGCGACGCTGACG-3′), puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Sweden) which included deoxynucleoside triphosphate at a concentration of 200 µM, 2.5 U of puReTaq DNA polymerase,10 mM Tris–HCl, (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$ and enough sterile deionized water to bring the volume to 25 µl. PCR amplification was performed in a Flexigene thermal cycler (Techne, UK).The PCR program described by De Urraza et al. (2000) was used. PCR products were separated by electrophoresis at 50 V on a 2% (w/v) agarose (Amersham Biosciences, Sweden) and the DNA was detected by UV transillumination after staining with ethidium bromide (10 mg/ml). The molecular sizes of the amplified DNA fragments were estimated by comparison to a 100 bp DNA ladder (Promega) and were photographed using polaroid film. The Rep profiles were processed using the Gel Compar version 5.00 software (Applied Maths, Kortrijk, Belgium).

2.3. The separation of biomass

Lactobacillus strains were grown in MRS broth (Biolife, Milano, Italy) and both *Enterococcus* and *Lactococcus* strains were grown in M_{17} broth (Biolife). Thermophilic bacteria were cultured at 42 °C, while mesophilic bacteria were grown at 30 °C. The optical density of the obtained supernatant that resulted after the centrifugation was measured at 650 nm (OD650) and used to express the biomass separation according to the method of Ayad et al. (2004). A zero reading was taken as an indication for excellent separation and the OD650 ranged from 0 to 0.1 indicated a good separation of biomass. While OD650 ranged from 0.2 to 0.3 and more than 0.3 indicated a fair and poor biomass separation, respectively.

2.4. Acidifying activity

Acidifying activity was measured by the change in pH (Δ pH) over time. At the early stationary phase, bacterial cells were harvested by centrifugation at 10,000 rpm/min. The resultant cell pellet was suspended in sterile reconstituted skim milk (12.5% w/v) supplemented with sucrose (7%) and kept overnight at 4 °C. Sterile reconstituted skim milk was inoculated with 2% of suspended cells in milk sucrose as prepared in the previous step. The change of pH was determined using a pH meter (3505, Jenway, England) during 6 h of incubation at the suitable temperature.

2.5. Aminopeptidase (AP) activity

The strains were cultivated and centrifuged as described above. The obtained pellets were washed twice in potassium phosphate buffer (10 mM^{-1}, pH 7.0), re-suspended in the same buffer and diluted to $O.D._{650}$ = 1.0. The aminopeptidase activity of the strains was tested according to the procedure described by Miozzari et al. (1978) and El-Soda and Desmazeaud (1982).

2.6. Autolytic activity

The rate of autolysis was determined according to the method described by Thiboutot et al. (1995). The autolytic activity was determined as the percentage decreases in the absorbance at 650 nm at different time intervals.

2.7. Antagonistic activity

The LAB cultures were tested against each other as described by Geis et al. (1983). The overnight cultures were spotted onto agar plates. The plates were incubated for 48 h at suitable temperature to allow producer colonies to develop. Then a suitable soft agar medium was inoculated with the indicator strain and poured onto the surface of the plate showing the colonies of the producer strain. After 18 h of incubation under anaerobic conditions, at a suitable temperature, the plates were checked for zones of inhibition surrounding the producer strain colonies. Cultures that belong to the same genus were interacted together. Each tested strain was applied as inhibitor organism while another one was taken as indicator organism.

2.8. Exopolysaccharide (EPS) production

The screening of EPS production was limited to the strains showing weak pellet after centrifugation. The procedure used consisted of revealing the presence of diffuse capsules surrounding bacteria cells. The strains were tested for capsule production using phase contrast microscopy (Zeiss.Microscope, West Germany) as described by Prescott et al. (1996). The slime production of strains was tested according to the method described by Knoshaug et al. (2000).

3. Results

3.1. Strain typing by Rep-PCR

Fifty of the isolates examined by Rep-PCR analysis were identified as *Lactobacillus plantarum*, 30 were *Lactobacillus delbrueckii* subsp. *lactis*, 16 were *Lactobacillus paracasei* subsp. *paracasei*, 15 were *Lb. delbrueckii* subsp. *bulgaricus*, 12 were *Lactobacillus fermentum*, 35 were *Enterococcus faecium* and 12 were *Lactococcus lactis* subsp. *lactis* based on DNA banding patterns using BOXAIR primer. The amplification profiles from

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