IDENTIFICATION OF MESOPHILIC LACTIC ACID BACTERIA ISOLATED FROM TRADITIONAL EGYPTIAN DAIRY PRODUCTS.

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SUMMARY

A total of 158 mesophilic lactic acid bacteria (LAB) isolated from milk and traditional dairy products from Egypt were assigned to the genera Lactococcus and Lactobacillus. using phenotypic method (API system) 147 strains were identified to the species level; the remaining 11 strains yielded unsatisfactory results with API system. The SDS-PAGE technique of whole cell proteins was evaluated as an advanced tool for the identification of LAB. Therefore, protein fingerprints were registered for these 11 strains and compared to a large number of LAB reference strains stored in database.

The identification strains were observed of the different sources of isolation, Lactococcus was the predominant genera in raw milk, while mesophilic lactobacilli were detected in high levels in different types of cheese.

Keyword: lactic acid bacteria (LAB); phenotypic method (API system); SDS-PAGE technique.

INTRODUCTION

Lactic acid bacteria are widespread in nature and predominate in the microflora of milk and its products. Many species are involved in the daily manufacturing of dairy products. *Lactococcal* strains are essential to milk fermentation, especially in the cheese making process, providing optimal conditions for curd formation and for the development of texture and flavour. It is important to the dairy industry to identify new strains of *Lactococcus lactis* subsp. *lactis* for cheese manufacture. The classification of *Lactococcus lactis* subspecies based on phenotypic characteristics is of primary importance to the dairy industry, as phenotypes directly reveal the abilities required in milk fermentation. (Nomura *et al.*, 2002).

Mesophilic lactobacilli predominate in the non-starter lactic acid bacteria (NSLAB) flora, although pediococci and micrococci may also be found (Dacre, 1958; Fitzsimons *et al.*, 1999; Fryer and Sharpe 1966). Adventitious mesophilic lactobacilli are usually present because of postpasteurization contamination, but may also constitute part of raw milk microflora and survive pasteurization (Turner *et al.*, 1986). The role of NSLAB in ripening has not been resolved satisfactorily yet, although inclusion of adjunct cultures of some strains of NSLAB during cheese manufacturing increases the level of free amino acids, peptides and free fatty acids, which leads to enhancement flavour intensity and accelerating cheese ripening (Lane and Fox, 1996; McSweeney *et al.*, 1993; Corsetti *et al.*, 1998; El Soda *et al.*, 2000). In order to better understand the relationship between the aromatic quality of the cheese and the microbiological flora, which produced it, rapid and reliable identification methods are needed.

The objectives of the present study were, therefore, to identify the mesophilic lactic acid bacteria from raw milk and traditional Egyptian dairy products, using phenotypic methods. These cultures will then be subjected to a screening process based on technological and production criteria. Strains showing distinctive characteristics will be evaluated in cheesemaking.

MATERIALS AND METHODS

C.1. Collection of the samples

The samples were collected from raw milk and the following traditional Egyptian dairy products Ras cheese, Domiati cheese, Mish, Zabady and Laban Rayeb.

C.2. Isolation of mesophilic lactobacilli and lactococci strains

Samples were transported to the laboratory at 4°C and analyzed in the same day. Milk samples were incubated at 30°C, 37°C. One gram of the cheese or 1 ml of fermented milk was cultured in 10 ml sterilized skim milk before incubation at 30°C, 37°C until coagulation, they were then plated on M_{17} agar (Biolife, Italy) to isolate lactococci strains (Terzaghi and Sandine 1975), while MRS agar (De Man *et al.*, 1960) was used to isolate lactobacilli strains under anaerobic conditions at 30°C and 37°C for 48h.

C.3. Identification of the isolates

All strains were examined microscopically by Gram staining (Harigon and MacCane 1976), tested for catalase activity and for the production of CO2 from glucose. Separated colonies of cocci were tested for growth in M17 broth at 10°C for 10 days and at 45°C for 48h. Colonies were also tested for growth in M17 containig 6.5% NaCl. Separate colonies of rods were tested for growth in MRS broth at 10°C for 10 days and at 45°C for 48h.

One hundred and fifty eight isolated strains were partially characterized and grouped in two major categories ; lactococci and mesophilic lactobacilli. The identification was carried out for all isolated strains using the API 50 CHL galleries (BioMérieux, Marcy, l'Etoile, France) for the lactobacilli and API 20 STREP (BioMérieux, Marcy, lEtoile, France) for *lactococci* as recommended by the manufacturer. The API LAB Plus version 3.2. program (BioMérieux) was used to analyze the reaction and fermentation profiles obtained with the identification strips. Strains which yielded

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unsatisfactory results, usually coding for more than one species or strains which could not be identified were subjected to sodium dodecyl sulphate polyacrylamide gel electophoresis (SDS-PAGE). For this purpose, strains were grown on MRS agar at 37°C for 48h. Cell-free extracts of approximately 100mg were prepared. Bacterial cells (wet weight), suspended in 0.9 ml of sample treatment buffer (0.062M Tris-HCl buffer pH 6.8 containing 5% v/v mercaptoethanol and 10%v/v glycerol) by sonication on ice using Ultrasonic XL2020 apparatus equipped with a needle probe tipe (Length 12.7cm. Diameter 3.8cm.) during 5 min. After lysis 0.2 ml of SDS were added to the fraction, heated at 95°C for 10 min., and cooled on ice. This denatured fraction was used for SDS-PAGE analysis.

C.4. Electrophoresis of protein samples

The SDS method described by Pot *et al.*, (1994) was used for the separation of the intracellular proteins. Registration of the protein electrophoretic patterns, normalization of the densitometric traces, grouping of strains by the Pearson product moment correlation coefficient (r) and UPGMA cluster analysis using the software package Gel Compar version 4. Identification of these strains was performed by comparison of their protein patterns to a data base containing 200 normalized protein fingerprints of reference strains from almost all known species of lactic acid bacteria (Pot *et al.*, 1993). Pattern storage and comparison were performed using the software package Gel Compar version 4.

RESULTS AND DISCUSSION

D.1. Phenotypic characterization

Seventy six rods and 82 cocci isolates were Gram positive and catalase negative. Table(1). reveal that all lactobacilli could grow at 10°C, 54% could grow at 45°C, while the remaining 46% could not grow at 45°C. These cultures were temporarily grouped as mesophilic lactobacilli. The production of gas was observed in only one strain. Eighty-two of the cocci were able to grow at 10°C and could not grow at 45°C, and were considered to be lactococci (Schleifer *et al.*, 1985). It was of interest to notice that 21% of the isolated lactococci could grow in the presence of 6.5% NaCl. In fact according to Teuber *et al.*, (1991); Schleifer *et al.*, (1985) and Williams *et al.*, (1990) *Lactococcus* could grow at 10°C, but could not grow at 45°C and in 6.5% NaCl.

Using The API 20 STREP galleries 82 *Lactococcus* were identified as indicated in table (2).

All *Lactococcus lactis* subsp. *cremoris* were negative to arginine hydrolysis. This test which is included in the API 20 STREP galleries is useful for the differentiation between *Lactococcus lactis* subsp. *cremoris*, and *Lactococcus lactis* subsp. *lactis*. *Lactococcus lactis* subsp. *cremoris* isolates produced acid from ribose except for one strains. According to Schleifer *et al.*, (1985) *Lactococcus lactis* subsp. *cremoris* do not produce acid from Ribose. The API 20 STREP system correctly identified all *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. Our results in that respect are comparable to the findings of Pot *et al.* (1996), who found that 59% of *Lactococcus lactis* subsp. *lactis* tested were correctly identified using API 20 STREP. Using API 50CHL galleries it was found that 65 lactobacilli belonged to *Lactobacillus rhamnosus* (32 strains), *Lactobacillus plantarum* (21 strains),

Lactobacillus pentosus (5 strains), *Lactobacillus paracasei* subsp. *paracasei* (6 strains) and *Lactobacillus brevis* (one strain). On the other hand, 11 *Lactobacillus* strains could not be identified with the API 50CHL galleries (Table. 2). Dellagilo (1989) and Giraffa *et al.*, (1998) found that 48% of the *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus delbruekii* subsp. *lactis* tested were identified correctly using API 50 CHL. Bill *et al.*, (1992) and Klinger *et al.*, (1992) indicated that some commercial identification system often yield good results regarding genus identification, but they were not fully adequate at the species level.

D.2. Identification of isolated using SDS-PAGE protein pattern

Using the Gel Compar software package, the protein patterns of the eleven lactobacilli that were not identified using API method were compared to a library of protein fingerprints representing most species of lactic acid bacteria. For every sub species found in the isolates

a limited number of reference patterns was selected from the database to run a numerical analysis. Figure (1) displays the different SDS-PAGE clusters obtained after numerical comparison of some of the isolated lactobacilli which yielded unsatisfactory results with the API 50 CHL. The results of API 50 CHL for 10 strains of these isolates were *Lactobacillus plantarum* & *Lactobacillus pentosus*. Five of them were found to be *Lactobacillus plantarum* using the SDS-PAGE technique while the remaining were *Lactobacillus rhamnosus*. A strain which was identified as *Lactobacillus pentosus* & *Lactobacillus brevis* when using the API system was identified as *Lactobacillus rhamnosus* by SDS-PAGE. It was of interest to notice that the SDS-PAGE results are in agreement with the pre-identification tests. In fact the strains identified as *Lactobacillus rhamnosus* grew at 10°C and 45°C. The identification by SDS-PAGE of whole cell proteins of strains of most lactic acid bacteria including the genus *Lactobacillus* is known to be reliable on the species and subspecies level (Jarvis and Wolff 1979; Tsakalidou *et al.*, 1994; Pot *et al.*, 1993; 1994 and De Angelis *et al.*, 2001)

D.3. Bacterial content of the different sources of isolation

Figure (2) described the distribution of the species in the different samples. It was not the main purpose of this paper to systematically sample the different cheeses, neither in time nor in function of the location. Therefore, the results presented in Fig (2) can only give a rough estimate of the bacterial content of the different samples isolated with the procedures and media described above. It is remarkable that lactococci was the predominant genera in raw milk, since 98% of isolated from different raw milk were *Lactococcus* . Teuber and Geis (1981) reported that raw cow's milk consistently contains *Lactococcus lactis* subsp. *lactis* and suggested that lactococci enter the milk from the exterior of the udder during milking and from the feed, which may be the primary source of inoculation. Desmasures *et al.*, (1998) found that, of 62 isolates from two samples of raw milk 61% were *Lactococcus lactis* subsp. *lactis*. Ayad *et al.*, (1999) found that all strains isolated from raw milk obtained from goats, sheep and cows milk were identified as *Lactococcus lactis* subsp. *lactis or Lactococcus lactis* subsp. *cremoris*. Our data also reveal that 30% *Lactobacillus plantarum*, 25% *Lactobacillus rhamnosus*, 5% *Lactobacillus paracasei* subsp. *paracasei* and 40% *Lactococcus lactis* subsp. *lactis* were isolated from Zabady. Sandine *et al.*, (1972) reported that the most recognized habitat for the lactococci is dairy products. Pot *et al.*, (1996) reported that lactococci are traditionally associated with the dairy environment and with foods.

On the other hand Tsakalidou *et al.*, (1994) found that, *Lactococcus lactis* subsp. *lactis* was isolated from cheeses but not from milk or yogurt. In the case of Laban Rayeb the predominant specie was *Lactobacillus plantarum* (58%), followed by *Lactobacillus rhamnosus*(21%), the remaining isolates were found to be *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus pentosus*. Little information is available in the literature on the microbiology of Laban Rayeb; *Lactococcus lactis* and *Lactobacillus casei* were found to be the predominant flora of Laban Rayeb (Demerdash, 1960; Abd-el-Malek and Demerdash, 1970; Abd Elhamid 2002). Also Abou-Donia (1991) reported the isolation of *Lactococcus lactis subsp. lactis, Lactococcus lactis subsp. cremoris and Streptococcus bovis* from Laban Rayeb.

Lactobacillus rhamnosus, Lactobacillus paracasei subsp. *paracasei* and *Lactobacillus plantarum* strains could be isolated from Domiatti cheese. These data was in agreement with Helmy (1960), who found that the most commonly found lactobacilli in Domiati cheese were *Lactobacillus casei, Lactobacillus plantarum, Lactobacillus brevis and Lactobacillus fermenti*. On the other hand, Fahmy and Youssef (1978) found that *Lactobacillus casei* and *Lactobacillus delbrueckii* subsp. *bulgaricus* were the predominant species isolated from ripened Domiatti cheese. Also El-Soda and Abd El-Salam (2002) reported that the *Lactobacillus casei, Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus delbrueckii* subsp. *bulgaricus* are the predominate lactobacilli in cheese matured in brine, while Manolopoulou *et al.*, (2003) found that the most common microorganisms found during Feta cheese ripening were *Lactobacillus plantarum, Lactobacillus pentosus* and *Lactobacillus paraplantarum*. *Lactobacillus rhamnosus* was predominant in Mish cheese and represented 75% of the isolates, one strain of *Lactobacillus plantarum* and *Lactococcus lactis* subsp. *lactis* were isolated. A complex flora was present in Ras cheese, *Lactobacillus* was the predominant genera. The following species were isolated, *Lactobacillus rhamnosus* (35%), *Lactobacillus plantarum* (30%), *Lactobacillus pentosus* (10%), *Lactobacillus paracasei* subsp. *paracasei* (5%) in addition one strain of *Lactobacillus brevis* and *Lactococcus lactis subsp. lactis* (18%). Our results in that respect are comparable to the work of several authors Tzanetakis and Litopoulou- Tzanetaki (1992) and Litopoulou- Tzanetaki and Tzanetakis (1992) found that *Lactobacillus* has been isolated in large numbers from Greek traditional cheese. It is also well known that the majority of non-starter lactic acid bacteria (NSLAB) found in most types of cheese and constituted of mesophilic lactobacilli (Demarigny *et al.*, 1996; Williams and Banks 1997; Bouton *et al.*, 1998). Also Fox *et al.*, (2000) reported that most, if not all, cheeses, whether made from raw or pasteurized milk, contain adventitious NSLAB. It was also of interest to notic that *Lactobacillus rhamnosus* was isolated from all Egyptian samples collected. On the other hand, De Angelis *et al.*, (2001) found that *Lactobacillus plantarum* and *Lactobacillus paracasei* were present in all Italian cheese types, except Fossa cheese. Also Tzanetakis and Litopoulou- Tzanetaki (1992) reported that, *Lactobacillus plantarum* was the predominant species in Feta and Telem Greek cheeses.

CONCLUSION

The identification of the different cultures isolated from milk and its products in the present study indicated that lactococci was the predominant genera in raw milk. The mesophilic lactobacilli *Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus rhamnosus* and *Lactobacillus paracasei* predominated in different types of cheese. In the light of these results, it is obvious that these bacteria are well adapted to cheese manufacturing environment and can contribute to the physicochemical and sensory characteristics of cheese. Thus the role of this group of microorganism needs further study.

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Table 1. Principal physiological and biochemical characteristics of rods and cocci isolated from Raw milk and traditional Egyptian dairy products.

Characteristic	Rods	Cocci
Gram-Stain	76/76*	82/82
Catalase	0/76	0/82
Growth at 10°C	76/76	82/82
45°C	41/76	0/82
Growth in 6.5% NaCl	N.D.	16/82
CO2 production	1/76	0/82

* Number of positive strains/Number of strains tested.
N.D. Not Determine.

Table 2. API results for isolated strains.

Identification	Number of strains
Using API 20 STREP	
Lactococcus lactis subsp. *lactis*	75
Lactococcus lactis subsp. *cremoris*	7
Using API 50 CHL	
Lactobacillus rhamnosus	32
Lactobacillus plantarum	21
Lactobacillus pentosus	5
Lactobacillus paracasei subsp. *paracasei*	6
Lactobacillus brevis	1
Not identified	11

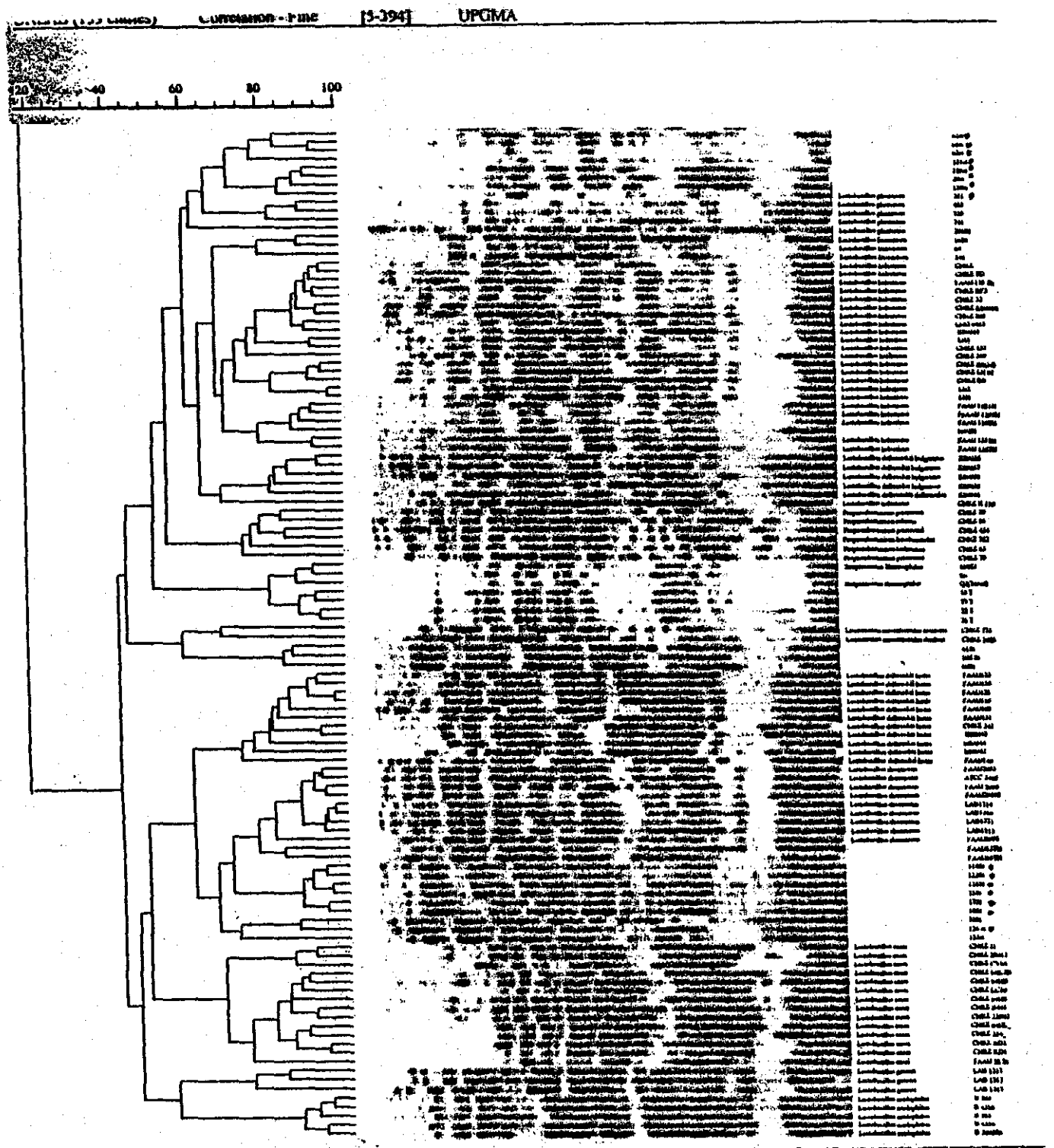
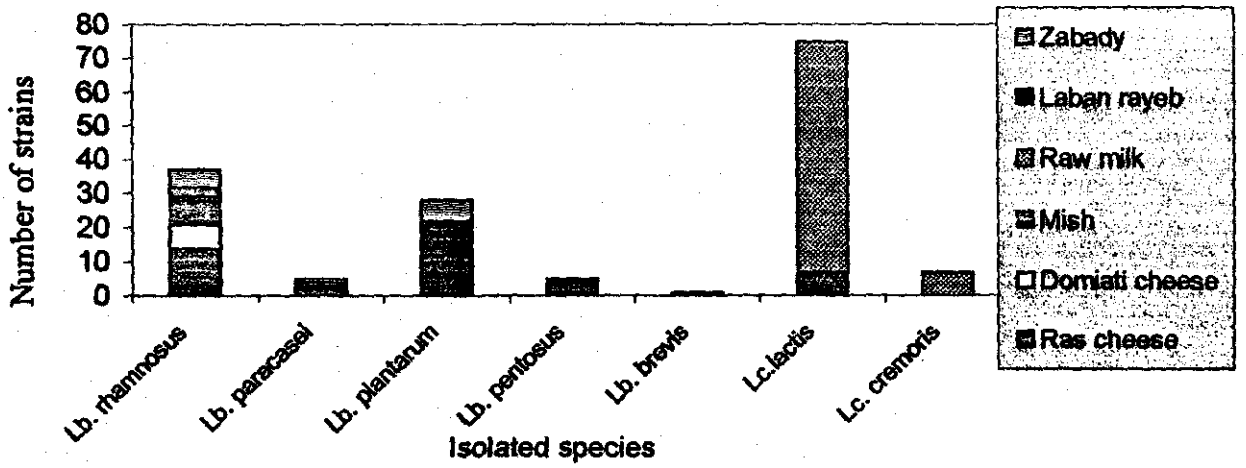


Fig. 1: Dendrogram calculated by the unweight average pair grouping method of correlaltion coefficients obtained between all pairs of one-dimensional SDS-PAGE protein patterns of strains of the unknown isolated cultures, compared to a number of reference representative strains. * are milk and milk products isolates.

Fig 2. LAB in the different Egyptian products



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الملخص العربي

التعرف علي بكتيريا حمض اللاكتيك الميزوفيلية المعزولة من منتجات الألبان التقليدية المصرية

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تم عزل ١٥٨ سلالة من بكتيريا حمض الاكتيك المحبة للحرارة المتوسطة من اللبن و منتجاته المختلفة مثل الجبن الراس و السدمياطي والمش و الألبان المتخمرة مثل اللبن الرايب و الزبادي و تم تجميع هذه العينات من مناطق مختلفة في جمهورية مصر العربية.وبعد ذلك تم التعرف علي هذه السلالات باستخدام الاختبارات المبدئية التي تعتمد علي الخصائص المورفولوجية للبكتيريا و اختبار الكتاليز و القدرة علي النمو علي درجة حرارة 10°م ، ٤٥ °م و النمو في وجود ٦,٥ % كلوريد الصوديوم و المقدرة علي انتاج ثاني أكسيد الكربون. كما تم استخدام نظامي تخمر الكربوهيدرات API system و نظام فصل البروتين بالهجرة الكهربائية SDS-PAGE Technique في التعرف علي السلالات البكتيرية المعزولة.

و قد أظهرت النتائج المتحصل عليها تمكنا من التعرف علي ١٤٧ سلالة بكتيرية باستخدام API SYSTEM

أما الاحدي عشرة سلالة المتبقية فقد تم التعرف عليها باستخدام SDS-PAGE technique