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Isolation, identification and selection of lactic acid bacteria cultures for cheesemaking

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Abstract: Lactic Acid Bacteria (LAB), which are considered to be the most useful microorganisms to society, are involved in the manufacture of thousands of fermented foods. They are also beneficial in flavoring foods, inhibiting pathogenic as well as spoilage bacteria in these products. A total of 2000 isolates of LAB were obtained from traditional Egyptian dairy products (different types of raw milk, ras, domiatti and kareish cheese, mish, cream, butter and fermented milk such as laban, rayeb and zabady) obtained from different regions in Egypt. The pre-identification tests were performed according to the morphological characteristics: catalase test, growth at 10°C and 45°C, growth in the presence of 6.5% NaCl and Co2 production. The isolates were subjected to phenotypic and cell wall protein characterization analyses. Phenotypically, 1006 strains were identified using API 50 CHL and API 20 Strep (Apparatus and procedure of identification). The SDS-PAGE technique (Sodium dodycyl sulfate) of whole cell protein was evaluated as an advanced tool for the identification of LAB. Therefore protein fingerprints were registered for 514 strains and compared to a large number of LAB reference strains stored in database format. The SDS-PAGE technique confirmed 94% of the API identification results. The identified strains were tested for acidifying activity, autolytic and aminopeptidase properties, antagonistic activities and polysaccharides production.
Selected cultures where then evaluated as starter or adjunct cultures for Ras and Domiatti cheese production in the cheese research laboratory. Higher cheese flavor scores were consistently obtained in Ras cheese made with cultures showing high peptidase and esterase activity and a high rate of autolysis. Development of free amino acids and free fatty acids in Ras cheese were always higher when compared to the cheese made using other starters.

Key words: LAB (Lactic Acid Bacteria) - Free amino acids - Free fatty acids - SDS-PAGE technique - API technique.

عزل وانتخاب سلالات بكتريا حمض اللاكتيك المستخدمة في صناعة الجبن

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ملخص: تعتبر بكتريا حمض اللاكتيك من اكثر الكائنات الحية الدقيقة نفعا للإنسان حيث نجدها ركنا أساسيا في صناعة العديد من الأغذية المتخمرة كما إنها هي المسئولة عن إنتاج الطعوم المختلفة في الأغذية التي تدخل في صناعتها كما إنها تثبط عمل البكتريا المرضية. وقد تم عزل وتنقية حوالي ٢٠٠٠ سلالة بكتيرية من اللبن الخام و منتجاته المختلفة مثل الجبن الراس و الدمياطي و القريش و المش و القشدة والزبد والألبان المتخمرة مثل اللبن الرايب والزبادي. تم تجميع هذه العينات والحصول عليها من مناطق مختلفة في جمهورية مصر العربية. وتعتمد اختبارات التعرف المبدئي علي السلالات علي الخصائص المورفولوجية للبكتريا واختبار الكتاليز والنمو علي ١٠°م و٤٥°م والنمو في وجود ٦,٥% كلوريد صوديوم وإنتاج غاز ثاني أكسيد الكربون. كما تم استخدام نظامي تخمر الكربوهيدرات API System ونظام فصل البروتين بالهجرة الكهربائية SDS-PAGE Technique في التعرف علي السلالات البكتيرية المعزولة حيث تم التعرف علي ١٠٠٦ سلالة بكتيرية بنظام الـ API System وعلي ٥١٤ سلالة بكتيرية بنظام الـ SDS-PAGE Technique وقد أكد هذا النظام ٩٤% من النتائج المتحصل عليها باستخدام نظام الـ API System . وقد تم انتخاب السلالات المتعرف عليها تبعا

للخواص التكنولوجية والإنتاجية مثل القدرة علي إنتاج الحامض والتحلل الذاتي للخلايا وتحليل البروتين و القدرة علي إنتاج مواد مثبطة و مواد لزجة. وفي نهاية الأمر يتم تقسيم السلالات المنتخبة كبادئات لانتاج الحامض او بادئات للتسوية تستخدم في صناعة الجبن الرومي او الدمياطي في معمل بحوث الجبن حيث تم تطبيق ذلك علي الجبن الراس وتم الحصول علي جبن يتميز بمذاق جيد وارتفاع الأحماض الدهنية والأمينية الحرة به.

كلمات مفتاحية: بكتيريا حمض اللاكتيك، أحماض أمينية حرة، أحماض دهنية حرة، كروما توجرافيا الفصل الكهربي، نظام التعرف على السلالات البكتيرية.

Introduction

Isolation and screening of microorganisms from naturally occurring processes have always been the most powerful means for obtaining useful cultures for scientific and commercial purposes. This is certainly true for lactic acid bacteria (LAB), which play an important role in a large number of various traditional food fermentations. Among these traditional processes, cheese and fermented milk are known to be essentially fermented by LAB, although often a functional secondary flora develops. Some properties of LAB such as flavour and texture formation are especially important to the food and feed industries because of their applicability for a large variety of products.

The dairy industry uses well-defined single strain and multiple strain starter cultures to obtain dairy products of high and constant quality. Therefore, a continuous need exists for the isolation of new strains with superior natural qualities. A high proportion of the cheese and fermented milks produced in Egypt are manufactured in the rural areas. In such products where no starter are added, fermentation occurs as a result of the wild flora present in the surrounding environment. The wild lactic acid bacterial flora represents a natural reservoir for cultures that were not exposed to any industrial selection.

Some interesting characteristics of these microorganisms are their ability to produce acid at a high and predictable rate, which is essential in cheese starter strains (Stadhouders, 1974, 1986; Sandine, 1985; Farrow, 1980).

The proteolytic activity exerted by most LAB is not only a prerequisite for growth, but also affects product texture and flavour, especially in dairy products (Law and Kolstad, 1983; Thomas and Pritchard, 1987). Proteolytic activity encompasses proteinases that degrade proteins, such as caseins, into relatively large protein fragments, and peptidases that break down protein fragments into small peptides and free amino acids. The total proteolytic activity consists of a complex mixture of a proteinase and a number of peptidases (Kamaly and Marth, 1989; Olsen, 1990)

Certain strains of LAB are able to synthesize exopolysaccharides (EPS) that are secreted into their environment, such as milk (Cerning, 1990; Ricciardi and Clementi, 2000; Sikkema and Oba, 1998). In particular for the production of yoghurt, drinking yoghurt, cheese, fermented cream and milk based desserts, EPS producing LAB are very important (Cerning, 1995; Crescenzi, 1995). EPS contribute to the texture, mouth-feel, and taste perception and stability of the final product.

The ability of LAB to produce and excrete compounds with antimicrobial activity, such as bacteriocins, is well known (Geis et al., 1983; Klaenhammer, 1988). Bacteriocins are relatively small peptides, sensitive to specific proteolytic enzymes, can be heat stable and have antimicrobial (either bacteriocidal or bacteriostatic) activity

against closely related, or in some cases a wide spectrum of microorganisms including foodborne pathogens such as *Listeria monocytogenes* and some *Clostridia*. This makes bacteriocin producers particularly for potential use in food preservation (Spelhaug and Harlander, 1989; Delves-Broughton, 1990; Marugg, 1991).

The aim of this study is to improve cheesemaking in Egypt through a selection of bacterial cultures from our environment in order to reproduce the characteristic flavour and texture of our endogenous products.

Materials and Methods

Isolation of LAB strains

The samples were collected from raw milk and traditional Egyptian dairy products such as Ras, Domiatti and Kareish cheese, mish, cream, butter, Zabady and Laban Rayeb.

The milk samples were incubated at 30°C; 37°C; 42°C, while the cheeses, fermented milk, cream and butter samples were cultured in sterilized reconstituted skim milk before incubation until coagulation. Coagulated samples were then streaked on M17 (Biolife Italy), MRS (De Man et al., 1960); Rogosa (Difco, USA); ST and SF agar media. Samples streaked on MRS and Rogosa agar, were incubated under anaerobic condition using the gas pak system (GENER box anae indicator Biomerieux) at 30°C, 37°C and 42°C for 48h. For the isolation of the strains the following media were used: M17 for *Lactococcus* and *Enterococcus* strains, M17 + Vitamin for *Streptococcus thermophilus* and MRS for *Lactobacillus, Pediococcus*, and *Leuconostoc.* The cultures were streaked on suitable media for their purification. The purified strains were stored at -20°C in sterile reconstituted skim milk (12.5% W/V) supplemented with 15% glycerol.

Identification of LAB strains

Preliminary tests

More than 2000 cultures were assigned to genus level initially by morphology and simple physiological tests following the criteria of Sharpe (1979) using morphological, phenotypic and biochemical methods. They were examined microscopically for Gram staining and catalase production (Harigon and McCane, 1976). In addition, all strains were tested for growth at 10ºC for 10 days¸ 45ºC for 48h, and CO_2 production from glucose. For cocci strains, growth on SF broth medium and in the presence of 6.5% NaCl were also considered. The cultures were classified as stated below:

Gram positive, catalase-negative rods, which grew at 45ºC and not at 10ºC, were considered as thermophilic lactobacilli, while those that didn't grow at 45ºC and grew at 10ºC were considered as mesophilic lactobacilli. Homofermentative, Gram positive, catalase negative cocci, which grew at 10ºC but not at 45ºC and 6.5% (w/v) NaCl, were considered mesophilic lactococci. Homofermentative, Gram-positive, catalase-negative cocci, which grew at 45ºC, but not at 10ºC and 6.5% NaCl, were considered as thermophilic cocci, while those which grew at 45ºC, 10ºC and 6.5% NaCl, were considered as enterococci.

API system

The API 50CH system (Biomerieux, Marcy l'Etoile France) was used for the identification of lactobacilli, lactococci, *Leuconostoc, Pediococcus*, and *S. thermophilus* strains while API 20 STREP

(Biomerieux, Marcy l'Etoile France) was used for the enzymatic and carbohydrate fermentation patterns of enterococci strains. The API system was performed according to the manufacturer's instructions. Interpretations of the fermentation profiles were facilitated by systematically comparing all results obtained for the isolates studied with information from the computer-aided database APILAB Plus V.3.2.2. in which the identification of a microorganism is accompanied by the following information: (i) The percentage of identification (%id) is an estimate of how closely the profile corresponds to the taxon relative to all the other taxa in the database. (ii) The T-index represents an estimate of how closely the profile corresponds to the most typical set of reactions for each taxon. Its value varies between 0 and 1, and is inversely proportional to the number of atypical tests. (iii) Comments on the quality of identification derived from the % id and the T-index of the selected taxon (excellent identification % id > 99.9 and T> 0.75).

SDS-PAGE of the whole-cell proteins

Five hundred and fourteen from 1006 strains previously identified from their phenotypic characteristics were submitted to SDS-PAGE of the whole-cell proteins to confirm the API results. Lactobacilli, *Leuconostoc*, *Pediococcus*, and *S. thermophilus* strains were inoculated with 2% of 24h overnight culture and the cells were collected after 16h incubation by centrifugation (7000g, 10min, 4°C). The cell pellet was washed twice with sodium phosphate buffer (0.01M; pH 7.3) containing 0.08% NaCl. On the other hand, lactococci and enterococci strains were plated on the M17 agar and incubated for 48h. Bacterial cells were collected and suspended in eppendorf tubes containing 1ml phosphate buffer (0.01M; pH 7.3). After centrifugation, the resulting pellet was washed with the same buffer. The cell pellet was resuspended in 0.9ml sample treatment buffer (0.062M Tris HCl buffer containing 5%V/V mercaptoethanol and 10 % glycerol; pH 6.8). The ice cooled cell suspension was then treated with an Ultrasonic XL 2020 apparatus using a needle probe tip and mixed by vortex. 0.1ml of SDS (20%) was added in this suspension. The mixture was heated at 95°C for 10min and cooled in ice. The whole cell protein extracts were obtained by centrifugation (7000g, 10min, 4°C) of the suspension. The 1D electrophoresis of these protein extracts was run as previously described by Pot et al., (1994). Registration of the protein electrophoretic patterns, normalization of densitometric traces, grouping of strains by the Pearson product moment correlation coefficient (r) and UPGMA (Unweighted Pair Group Method Using Averages Linkages) cluster analysis were performed by the techniques described by Pot et al., (1994) using the software package Gelcompar (Applied Maths, Belgium; 4.0). Identification of the isolates was performed by comparison of their protein patterns to the fingerprints of reference strains of LAB in the database of the Laboratory of Microbial Biochemistry of the University of Alexandria obtained from different culture collections. The reproducibility of the electrophoretic system was investigated by inclusion of reference bacterial protein extracts in each slab gel as well as repetition of the electrophoretic runs or the duplication of the injection of the same samples.

Performance tests

Acidifying activity

Seventy ml of the suitable broth media were inoculated with 10% 24h overnight, activated subcultures of LAB strains. The growth of cultures in broth media was monitored at 650nm using a spectrophotometer (Pharmacia LKB. NOVASPEC II) during 6h. At the early stationary growth phase, bacterial cells were harvested by centrifugation of 30ml of the culture (4000g, 10min, 4°C). The cell pellet was washed twice with 10ml ringer solution. The resulting cell pellet was suspended in 35ml sterile reconstituted skim milk (12.5% W/V) supplemented with sucrose (7%), 5ml of this mixture was stored in ice at 4°C for 24h. Sterile reconstituted skim milk (12.5% W/V) was inoculated with 2% of the cell pellet suspended in milk-sucrose. The change of pH was determined using a pH meter (Microcomputer pH-vision, model 05669-20) during six hours incubation at the suitable temperature.

Aminopeptidase activity (AP)

Intracellular and extracellular aminopeptidase activities (AP) were measured according to the slightly modified procedure described by Miozzari et al., (1978) using L-Leucine para nitroanilide (Leu-pNA) as substrate (El Soda and Desmazeaud, 1982). In fact, cells of different cultures were harvested in the early stationary growth phase by centrifugation at 7000g for 10min, at 4°C. The cell pellet was washed twice and the resulting pellet was then resuspended in 1/10 of the initial volume of the growth medium.

A portion of cell suspension was added to 50ml of potassium phosphate buffer (pH7, 0.01M) to obtain the cell optical density of 0.9 to 1 at 650nm. The resulting suspension was divided in two portions and each portion was centrifuged for 10min at 7000g at 4°C. After centrifugation, the cell pellets were weighed and resuspended in the potassium phosphate buffer to obtain a final concentration of cell (100mg of wet cell pellet/ml buffer). To extract intracellular enzyme, one of the portions was treated with toluene (1000mg of wet cell pellet/ml toluene). After mixing by vortex, these suspensions were frozen at –20°C for 24h. The frozen cell suspensions were thawed at room temperature and centrifuged at 7000g for 20min at 4°C. The free cell supernatant of each portion was collected and used as enzyme extract.

The aminopeptidase activity was then tested using 50µl of Leu-pNA (Biomedicals, INC.) (6mg/ml methanol); 1.8ml of potassium phosphate buffer (pH 7; 0.01M) and 0.2ml of enzyme extract. The mixture was incubated at a suitable temperature. The variation of the optical density was measured at 410nm after the changing of the colour with a spectrophotometer (Pharmacia lKB, NOVASPEC II). In these experimental conditions, the enzyme activity corresponding to $O.D._{650nm}$ (0.9-1) of cell suspension was expressed as the variation of the optical density at 410nm per minute per milliliter of enzyme extract.

Measurement of the rate of autolysis

The rate of cell autolysis was measured according to the method of Thiboutot et al., (1995) which could be summarized as follows: cells of different cultures were harvested by centrifugation and washed twice. The resulting bacterial pellet was then resuspended in 1/10 of the initial volume of the growth medium, and then frozen at –20°C. The frozen cell suspension was thawed at 37°C. A portion of cell suspension

was added to potassium phosphate buffer (pH 5.5, 0.01M) containing sodium chloride to obtain an optical density of 0.9 to 1 at 650nm. This suspension was then incubated at 40°C and cell lysis was followed by absorbance measurement at 650nm on a spectrophotometer (Pharmacia LKB. NOVASPEC II) after 6h, 24h and 48h. Cell autolysis was characterized using the percentage of cell lysis as defined by Boutrou et al., (1998).

Exopolysaccharides (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 strain producing capsules were also tested for slime formation. For these purposes, on a clean slide, a loop of broth culture was mixed with a drop of India ink, covered with a cover glass and examined under a microscope with phase contrast (ZEISS Microscope, West Germany) (Prescott, et al., 1996). For slime production, strains were streaked on the suitable media and incubated at the optimum growth temperature for 24h. Ropiness of colonies on agar surfaces was tested with a loop to observe the formation of slimy filaments.

Antagonistic effects

The LAB cultures were tested for antagonistic activity against each other as follows: The overnight cultures were spotted onto agar plates. The plates were incubated for 48h 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 18h of incubation under anaerobic conditions, at a suitable temperature, the plates were checked for zones of inhibition surrounding the producer strain colonies (Geis et al., 1983).

Enhancement cheese flavour using different starter / adjunct combinations

Cell cultivation and freeze shocking

For the cultivation of cells, MRS broth was inoculated with an active culture of the adjunct culture in the same medium. At the early stationary phase, cells were harvested by centrifugation at 4000 rpm for 10 min at 4°C. The pellet was washed twice with 0.01 Mpotassium phosphate buffer, pH.7 and resuspended in the same buffer. The cell suspension was frozen at –20°C for 24h. and thawed in a water bath at 40°C prior to use.

Preparation of cheese curd for use in cheese-making

Many experiments were performed using commercial starters and selected adjunct cultures isolated from Egyptian dairy products to make Ras cheese. These mixtures were used in order to select the best starter for making-cheese. Cheeses were manufactured from standardized pasteurized milk in 11L vats. A commercial starter culture in addition to selected and commercial adjuncts were added to milk at 32°C. Calcium chloride was added before renneting with a chymosin preparation (2% w/v, Chymax, Chr.Hansen's Lab). The coagulum was cut and cooked to 45°C for 105 min. After whey drainage, the curd was molded, pressed over-night and ripened at 15-20°C. An automatic cheese pilot plant facility was used which provided conditions similar to those used on the commercial scale.

Physico - Chemical analysis of cheese

Samples of the cheese were analyzed for titrable acidity as recommended by the Association of Official Analytical Chemists (A.O.A.C) (1960), pH determination was measured using a glass electrode (Cole – Parmer LCD model 5994-10), fat content was determined by the Gerber method as described by Ling, (1952), moisture was determined by using the moisture analyzer (Mettler Toledo Model HR73), total protein was determined according to the A.O.A.C., and salt content was determined using Chloride Meter Model PCLM3, Jenway, England.

Proteolysis in cheese was assessed by measuring the concentration of free amino groups by the Cd-ninhydrin method as described by Folkertsma and Fox, (1991). Free fatty acids (FFA) were determined according to the method described by Godinho and Fox (1981).

Results and Discussion

Identification of various lactic cultures

More than 2000 cultures were isolated from various sources. The first screening revealed the presence of 1451 cocci and 656 rods which were further identified using classical techniques and allocated to the following groups (Table 1).

From all isolated strains, 1006 strains were identified using the API system. Nine hundred and seventy nine strains were satisfactorily identified as shown in Table 2; while for the remaining 27 strains a doubtful identification was obtained. Bill et al., (1992) and Klinger (1992) indicated that some commercial identification systems often yield good results regarding genus identification but they were not fully adequate at the species level.

Table 1. Identification of isolated strains using classical techniques.

Genus	Number
Lactococcus	237 (11%)
Enterococcus	1021 (48%)
Streptococcus thermophilus	170 (8%)
Pediococcus	5 (0.2%)
Leuconostoc	18 (0.8%)
A. Obligately Homofermentative lactobacilli	130 (7%)
B. Facultatively Heterofermentative lactobacilli	278 (13%)
C. Obligately Heterofermentative lactobacilli	248 (12%)

Table 2 reveals the presence of relatively high number of enterococci in the Egyptian dairy products, 32% of the identified cultures were *Enterococcus faecium.* They produce acid from mannitol and arabinose (Durlu-Ozkaya et al., 2001). Ninety three percent of identified *Lactococcus* strains were *L. lactis* subsp. *lactis*, they hydrolyse esculin and formed acid from lactose, maltose, salicine and ribose but acid production from mannitol, sucrose and D-xylose was strain dependent (Sharpe, 1979; Schleifer et al., 1985; Balows et al., 1991). The two *S. thermophilus* strains ferment lactose and sucrose while one of them forms acid from glucose.

Identified *Lactobacillus* strains were classified into 3 groups (Kandler and Weiss, 1986) as shown in Table 2. *L. delbrueckii* subsp. *lactis* was the predominant species in group A. *L. plantarum* and *L. fermentum* strains represented the highest numbers of lactobacilli in group B and group C, respectively.

Table 2. API results for the isolated strains.

Species identified using API system	Number
Lactococcus lactis subsp. *lactis*	172 (17.5%)
Lactococcus lactis subsp. *cremoris*	13 (1.3%)
Streptococcus hermophilus	2 (0.2%)
Leuconostoc mesentroids subsp *mesentroids / dextranicum*	14 (1.4%)
Leuconostoc lactis	2 (0.2%)
Pediococcus pentosaceus	2 (0.2%)
Enterococcus faecium	312 (31.8%)
Enterococcus durans	72 (7.4%)
Enterococcus faecalis	27 (2.8%)
Enterococcus avium	5 (0.5%)
Enterococcus casseliflavus	8 (0.8%)
Obligately Homofermentative lactobacilli (G.A)	
Lactobacillus delbrueckii subsp *bulgaricus*	30 (3.2%)
Lactobacillus delbrueckii subsp *lactis*	58 (5.6%)
Lactobacillus delbrueckii subsp. *delbrueckii*	2 (0.2%)
Lactobacillus helveticus	6 (0.6%)
Lactobacillus acidophilus	4 (0.4%)
Lactobacillus salivarius	5 (0.5%)
Facultatively Heterofermentative lactobacilli (G.B)	
Lactobacillus rhamnosus	47 (4.8%)
Lactobacillus paracasei subsp. *paracasei*	20 (2.5%)
Lactobacillus plantarum	70 (7.2%)
Lactobacillus pentosus	6 (0.6%)
Lactobacillus curvatus	3 (0.3%)
Obligately Heterofermentative lactobacilli (G.C)	
Lactobacillus fermentum	88 (8.9%)
Lactobacillus brevis	7 (0.7%)
Lactobacillus collinoides	1 (0.1%)
Lactobacillus fructivorans	1 (0.1%)
Lactobacillus viridescens	1 (0.1%)
Lactobacillus cellobiosus	1 (0.1%)

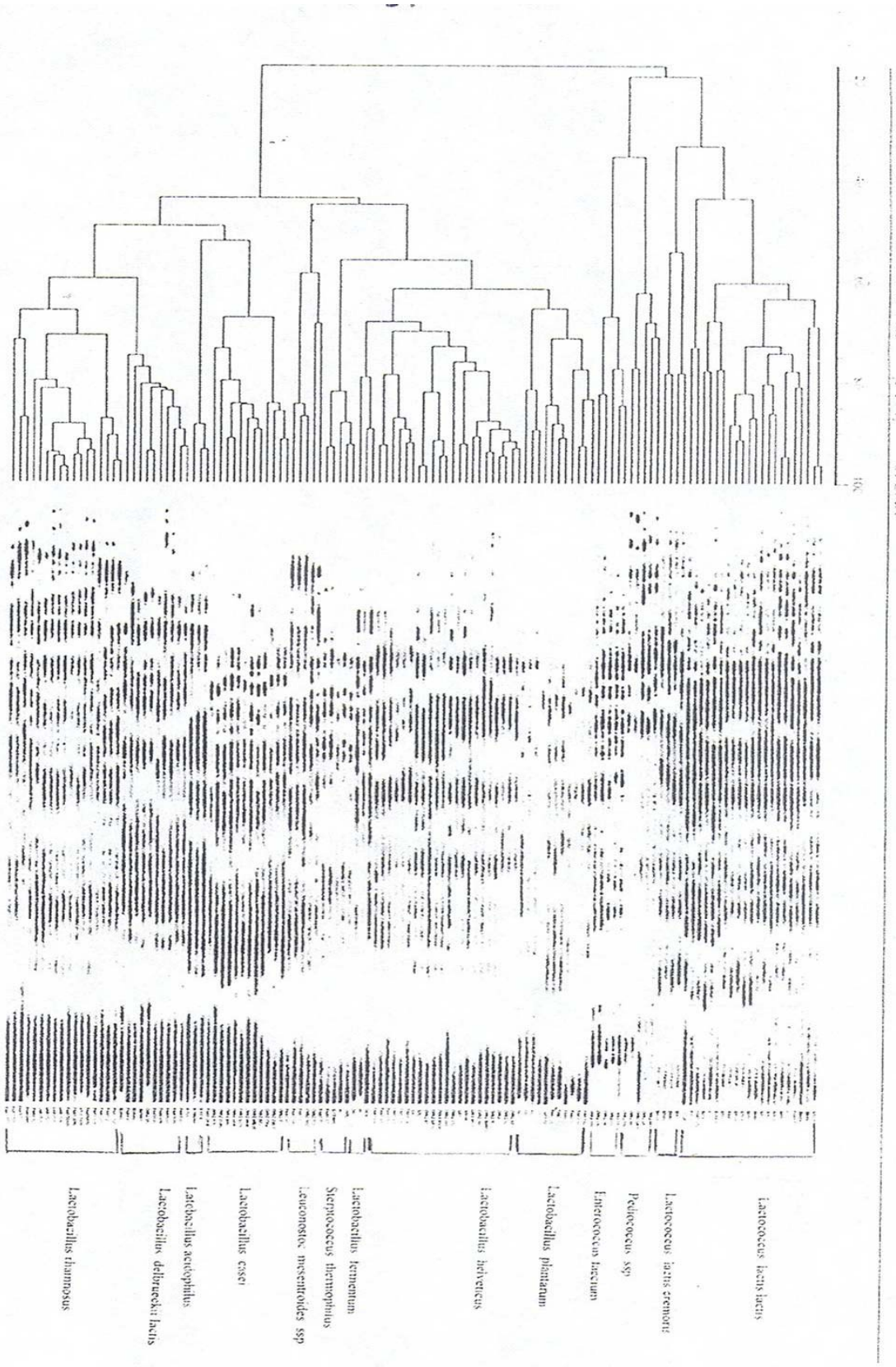

Figure 1: Dendrogram calculated by the unweight average pair grouping method of the correlation coefficients obtained between all pairs of one-dimensional SDS-PAGE protein patterns of strains of the unknown isolated cultures, compared to a number of reference representative strains.

Using the gel compar software package (version 4), 514 protein patterns were compared with protein fingerprints of reference strains including the genera *Lactobacillus, Pediococcus, Enterococcus, Lactococcus,* and *Leuconostoc*, and *Streptococcus thermophilus* (Pot and Janssens, 1993). The resulting dendrogram is shown in Figure 1. According to the SDS-PAGE results, the phenotypic characterization was confirmed for all *L. lactis* subsp. *lactis* and *S. thermophilus* strains. On the other hand, there were differences between the API system and SDS-PAGE technique results for some lactobacilli. Cultures identified as *L. delbrueckii* subsp. *lactis or L. helveticus* using the API system were classified by SDS-PAGE as *L. helveticus* (similarity 70%). Seven strains phenotypically characterized as *L. delbrueckii* subsp. *lactis* or *L. helveticus* were identified by the SDS-PAGE technique as *L. delbrueckii* subsp. *lactis.* The results of SDS-PAGE technique, confirmed 94% of the API results.

Selection of the identified cultures.

In this study, all the cultures that were satisfactorily identified were further selected according to technological criteria.

Acidifying activity

The obtained results revealed that the acidifying activity of *Lactococcus* strains was significantly higher than the activity of the other species. Figure 2 shows a typical example of three cultures with different acidifying rates. The obtained results (not shown) revealed that none of the *Enterococcus* strains can be characterized as fast, as they didn't reach a ΔpH of 0.4 in 3h. at the optimum growth temperature. The results showed that 10% of lactobacilli were fast in the acidifying rate and 66% were slow, as they didn't reach ΔpH of 0.4 after 6h. These results are in agreement with Durlu-Ozkaya et al., (2001) who reported that *Lactobacillus* strains differed in their ability to reduce the pH of milk initially and there were strains that didn't change the pH of milk after 6h.

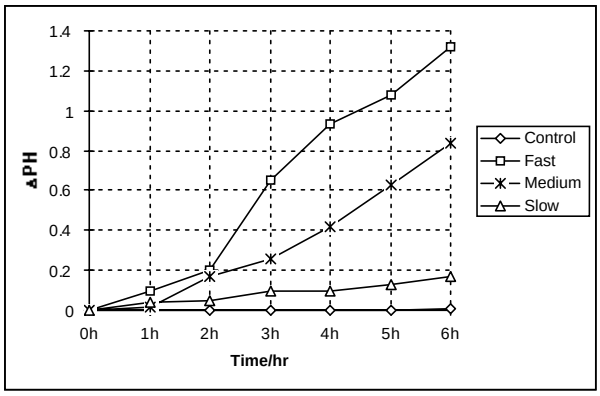

Figure 2. Acid production of different *Lactococcus lactis* subsp *lactis* strains.

Proteolytic activity

The data reported in Figure 3 revealed the presence of active AP on leucyl paranitroanilide (Leu-PNA) as substrate for lactobacilli. Aminopeptidase activity of lactobacilli was higher when compared to lactococci and enterococci. Strain FAAU 155 can be distinguished from the other strains by its higher AP activity.

The data reported here on proteolytic activity suggested that there was no relationship between AP and acidifying activities of the strains as also suggested by Durlu-Ozkaya et al., (2001); Fortina et al., (1998). Thus strain FAAU 85 revealing good acidifying activity didn't exhibit high AP, while FAAU 115 strain with good acidifying activity showed high AP activity. Some of the cultures exhibited relatively high aminopeptidase activity. They may play a role in reducing the levels of bitterness in cheese (Ardo et al., 1989; Bartles et al., 1987 a; b; El Abboudi et al., 1991; Baankreis, 1992) and they may also play an important role in the enhancement of cheese flavour.

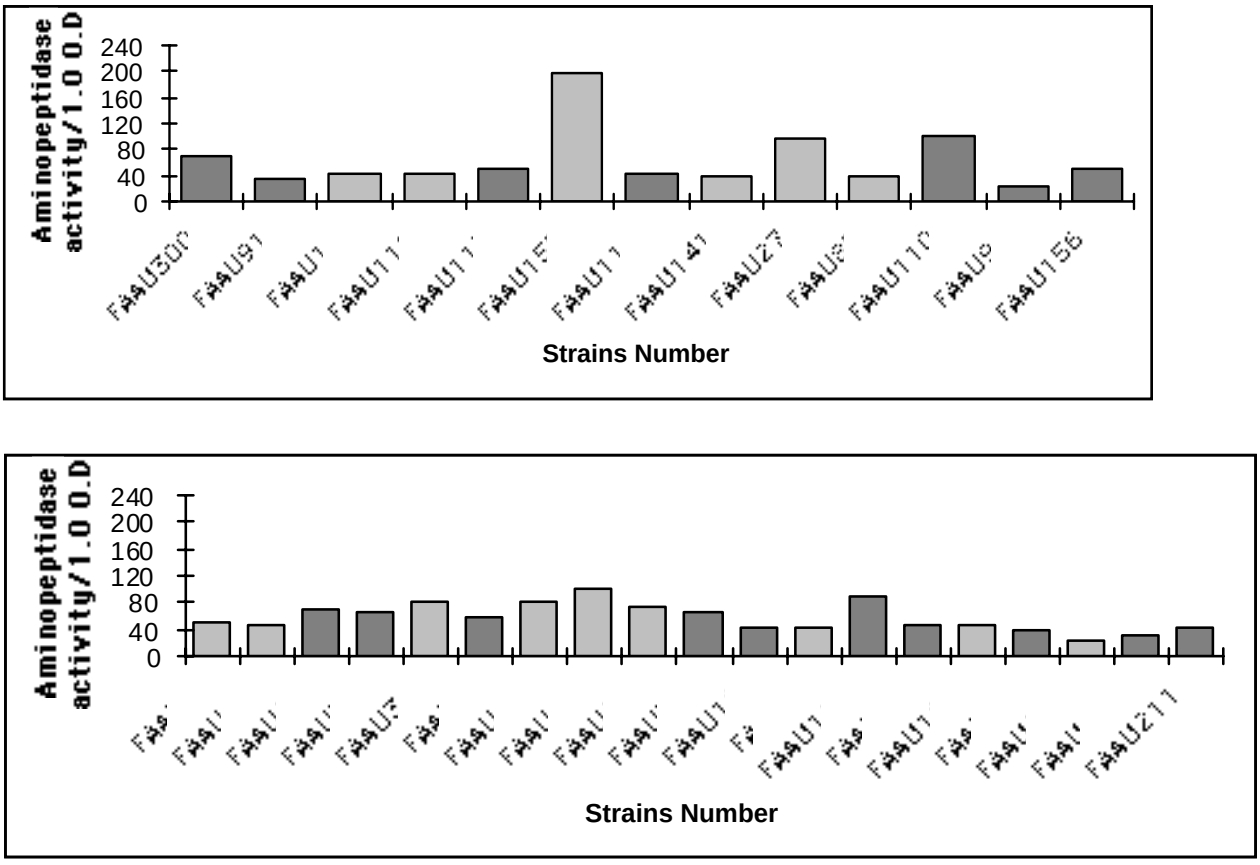

Figure 3. Aminopeptidase activity of different *Lactobacillus* cultures.

Autolytic activity

Cultures were classified to their autolytic activity into 3 groups: The highly autolytic cultures *L. rhamnosus* FAUU110, *L. paracasei* subsp. *paracasei* FAAU27, 155 which exhibited are autolysis rate ranging from 94 to 73%. The intermediate group *L. rhamnosus* FAAU23,34,55,115 showed levels of autolysis ranging from 69 to 40%. The poor autolysis cultures *L. rhamnosus* FAAU141, *L. paracasei* subsp. *paracasei* FAAU92 and *L. plantarum* FAAU20 showed an autolytic rate ranging from 39 to 4%. Highly autolytic cultures can be of interest during cheese manufacture because of the faster release of their intercellular proteolytic and lipolytic enzymes which will contribute in flavour development during cheese ripening (El Soda et al., 1995, 2000b; El Soda 1997; Klein and Lortal, 1999)

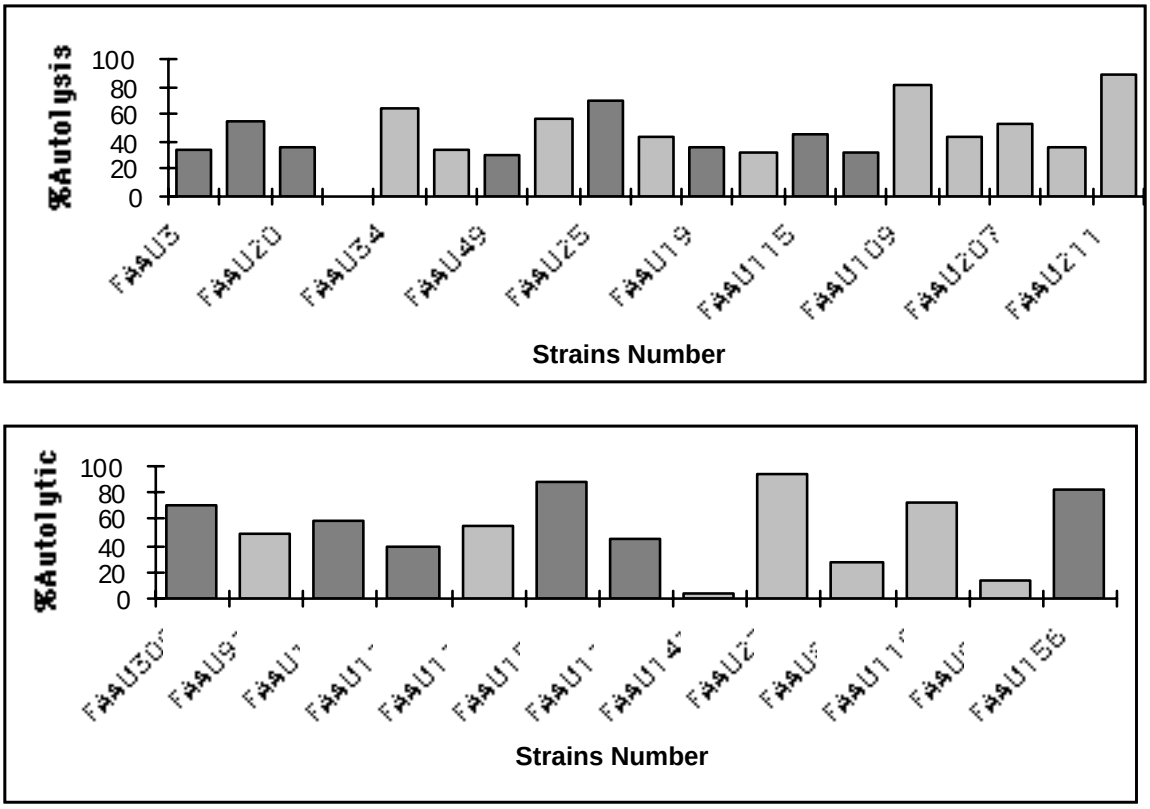

Figure 4. Autolytic activity of different *Lactobacillus* culture.

Exopolysaccharides (EPS) production

From Table 3 showing the ability of 190 strains to produce EPS, it appears that 26% of lactococci, 55% of enterococci, 42% of lactobacilli were able to produce EPS. These cultures will be evaluated for their ability to improve the texture of cheese (Oberg et al., 1998) and fermented milks (Macura and Townsley, 1984; Marshall and Rawson, 1999).

Table 3. Results of exopolysaccharides of LAB strains.

Genus	Number of strain tested	Positive results
Lactococcus	62	16
Enterococcus	45	25
Leuconostoc	2	1
Lactobacillus	81	34

Antagonistic activities

Seven hundred and eighty nine strains were screened for antagonistic activities. From the results, it could be noticed that none of *Pediococcus*, *Leuconostoc* and *S. thermophilus* strains showed antagonistic activity against each other. Among the *Lactococcus* strains, 44% showed inhibition zons against other lactococci strains. Also, it was shown that 42% of *Enterococcus* strains and 23% of *Lactobacillus* strains showed small inhibition zones against other enterococci and lactobacilli, respectively. Most lactic acid bacterial species can produce bacteriocins which are active against the lactic acid flora itself (Piard and Desmazeaud, 1992). This work is important in the preparation of starter containing multiple strains to avoid the inhibition role of them against each other.

Cheese analysis

Gross composition

The mean composition of control (cheese without adjunct) and adjunct-treated cheeses is shown in Table (4). Gross chemical composition after four months was found to be within an average of: pH: 5±0.1, Acidity %: 2.2±0.2%, Fat %: 38±2%, Moisture %: 32±1%, Protein %: 29±2%, and Salt %: 4.5±0.2%, and the means were not significantly different for all of the control and adjunct-treated cheeses.

The time required to reach the milling pH was almost identical in both control and cheese with added adjunct cultures. After overnight pressing, cheese made with added adjunct had lower pH values than did the control. This may indicate that adjunct could ferment residual lactose in cheese curd to produce lactic acid.

From these data, it can be concluded that the type of starter or adjunct did not influence the gross composition of the cheese. These results are in agreement with the data reported by Madkor et al., (2000a).

Table 4. Gross composition of Ras cheese.

Starter / adjunct combination		pH	% Fat	% Moisture	% Protein	% Salt
Control cheese	Zero Time	5.20	32.0	45.0	17.0	2.5
	Two months	5.12	38.0	35.0	22.0	4.2
	Four months	5.06	40.0	32.0	22.5	4.7
Adjunct Mix1	Zero Time	4.98	33.0	43.5	18.2	2.6
	Two months	4.95	39.0	31.6	22.6	4.6
	Four months	4.88	40.5	30.5	23.0	4.9
Adjunct Mix2	Zero Time	5.04	33.0	43.2	17.9	2.5
	Two months	4.96	39.0	31.9	21.8	4.4
	Four months	4.94	40.5	30.6	22.8	5.1
Commercial adjunct	Zero Time	5.12	33.0	43.5	18.1	2.4
	Two months	5.06	39.5	31.8	21.9	4.5
	Four months	4.94	41.0	30.7	23.1	5.1
Adjunct Mix3	Zero Time	5.01	33.5	43.0	17.7	2.5
	Two months	4.99	40.5	32.1	21.6	4.5
	Four months	4.95	41.0	30.5	23.3	5.0

Proteolysis

The levels of free amino groups in cheese as measured by the Cd-ninhydrin method showed that the adjunct-treated cheese contained significantly higher concentrations of free amino groups than did control cheese by the end of ripening (Fig. 5). The significant increase in free amino group content of cheese made with adjunct may have been due to their high peptidolytic activity and high rate of cell autolysis. Thus, depending on the extent of intracellular peptidase release and the effectiveness of peptidase activities of selected adjunct added to cheese, the water-soluble peptides can be degraded rapidly to smaller peptides and elevated levels of free amino groups. Actually, enhanced autolysis and high aminopeptidase activities of adjunct can be a limiting factor in the rapid formation of flavour constituents during ripening. (El Abboudi et al., 1991; Drake et al., 1998 and Madkor et al., 2000b).

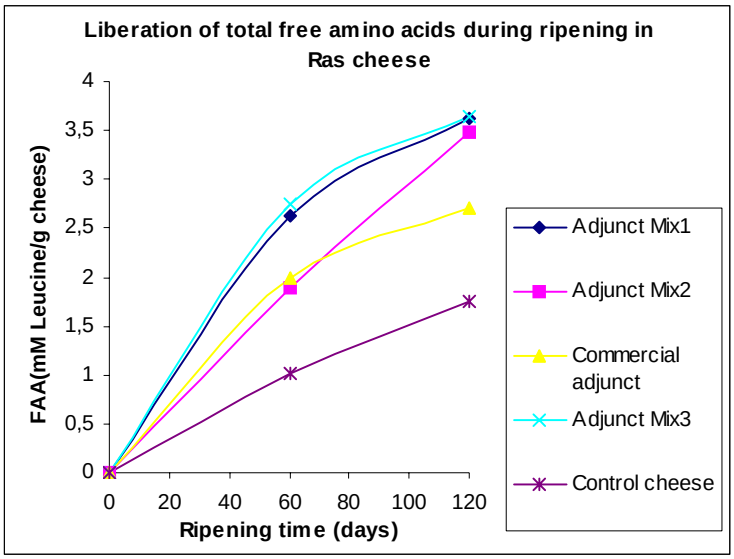

Figure 5 Evaluation of free amino acids during ripening in Ras cheese.

Lipolysis

Lipolysis in cheese during ripening, as indicated by total free fatty acids (FFA) (measured as ml alcohol NaOH N/100g cheese), is shown in Fig. 6. The adjunct-treated cheeses in general exhibited significantly higher levels of FFA liberation compared with control. In the progress of ripening compared with control, the total FFA increased considerably with time and was higher for all cheese at four months of ripening. Cheese made with adjunct formed the highest level of FFA compared with control cheese. These results indicated that the adjunct contributes to lipolysis in cheese, and different adjuncts have different lipolytic activity. (Khalid et al., 1990; Urtach et al., 1997; Madkor et al., 1999; Collins et al., 2003).

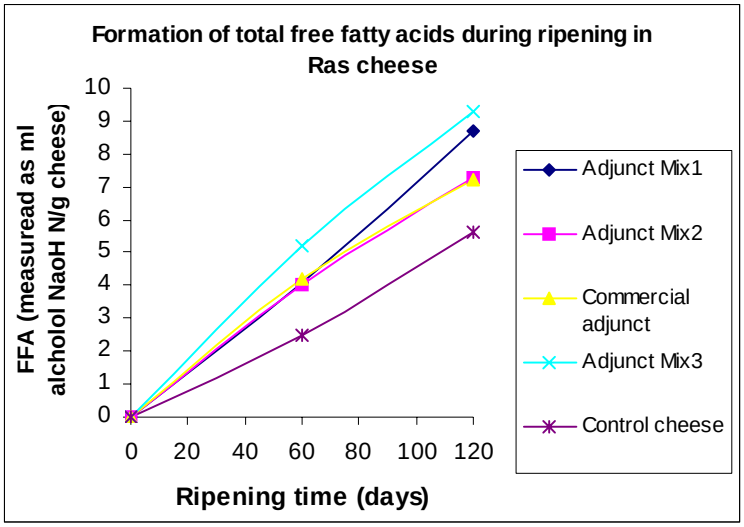

Figure 6 Evaluation of free fatty acids during ripening in Ras cheese.

Sensory characteristics

The sensory scores of cheese are summarized in Table 5. Adjunct-treated cheeses had mean organoleptic evaluation scores higher than control at each evaluation period during ripening. Other reported studies, Aly et al., (1994); Brome et al., (1990); Drake et al., (1998); Fox et al., (1998), revealed that adjunct cultures positively influenced flavour during ripening through their peptidolytic which can be linked to the high levels of free amino groups in cheese made with adjunct. Also, high FFA liberation in adjunct-treated cheeses seems to promote flavour balance. The intensity of flavour increases with time and reached its maximum after four months of ripening. Consequently, cheese maturity can be enhanced considerably depending on the type of adjunct culture used and the specific cheese-making method. (El-Soda et al., 2000a,b Katsiari et al., 2002).

Table 5. Organoleptic evaluation of Ras cheese.

Starters	After two months of ripening		After four months of ripening	
	Flavour	Texture	Flavour	Texture
Control cheese	Not acceptable flavour	Good texture	Not acceptable flavour, not close to Ras cheese	Good texture
Adjunct Mix1	Medium flavour	Good texture	Good flavour, closer to Ras than the other treatment	Good texture
Adjunct Mix2	Medium flavour	Good texture	Good flavour	Good texture
Commercial adjunct	Medium flavour	Good texture	Good flavour	Good texture
Adjunct Mix3	Medium flavour	Good texture	Good flavour, closer to Ras than the other treatment	Good texture

Conclusion

The results obtained in this study revealed the presence of a wide variety of LAB in the Egyptian environments. Some of the isolated and identified LAB show outstanding performances that were similar and in some cases higher if compared to commercially available cultures, as the production of high quality cheeses requires close attention to characterization, differentiation and maintenance of starter culture strains.

A relatively high number of enterococci was detected in Egyptian dairy products; such a situation has also been reported in a wide variety of artisanal cheeses produced in southern Europe (Portugal, Spain, Italy and Greece). Enterococci have beneficial effects in cheesemaking such as hydrolysing milk fat by esterases (Tsakalidou et al., 1993) and producing typical flavour components such as acetaldehyde, acetoin and diacetyl (Centeno et al., 1996; Travalelliet et al., 1987). The cheese results indicated that maturation indices and flavour quality can be enhanced by using a combination of commercial starter culture and selected cultures isolated from the Egyptian environments, which were used as adjuncts.

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