J. Food and Dairy Sci., Mansoura Univ., Vol. 3 (2): 75 - 84, 2012

ISOLATION AND IDENTIFICATION OF *Enterococcus* STRAINS FROM OLD EGYPTIAN HARD CHEESE.

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ABSTRACT

Six bacterial strains were isolated from old Egyptian hard cheese, and identified by rapid ID 32 strep V2.0 as *Enterococcus durans* with 99.9 % identity. The strains were tested for growth under different stress conditions such as different temperatures (42, 46, 50, 55°C), antibiotic (Erythromycin EM) and different salt concentrations (1, 2, 3, 4, 5 % NaCl). One strain, which appeared to be rather resistant to the different stress conditions, was selected for determination the characteristics of the plasmid harboured by the strain. To study the biological functions of the plasmid, wild type and plasmid-cured derivatives were compared i) for resistance against different antibiotics (ampcillin AMP, chloramphenicol C, cefotaxime CTX, EM, gentamycin CN, kanamycin K, lincomycin MY, methicillin MET, neomycin N, ofloxacin OFX, rifampicin RD, streptomycin S, tetracycline TE, vancomycin VA), ii) resistance against high temperature and iii) for ability to grow at high salt concentrations. Results showed that there was no difference in antibiotics resistance for wild type and cured strain, except for S and VA (cured strain was less sensitive than the wild-type) . Determination of growth rates of both strains in medium containing EM showed that the cured strain grew better than the wild type at 42°C, however, in the absence of EM the wild type strain grew better than the cured one at 42, 46 and 50°C. No significant differences between both strains were seen when they were grown at different NaCl-concentrations of 1, 2, 3, 4 and 5 %.
Keywords: *Enterococcus durans*, plasmid, antibiotics resistance, NaCl resistance

INTRODUCTION

Bacteria of the genus *Enterococcus* are an important group of lactic acid bacteria (LAB), which have a predominant habitat in the gastrointestinal tract of humans and animals. They also persist in the extra-enteral environment and can colonize diverse niches due to their high heat tolerance and ability to survive under adverse environmental conditions. Thus, enterococci occur in large numbers in foods, especially those of animal origin, such as fermented sausages and cheeses. It is well known, that enterococci are an important part of the bacterial population of several cheeses, such as Mozzarella (Coppola *et al*., 1998), Feta and other's (Psoni *et al*., 2006).

According to results obtained by Psoni *et al*., (2003) on the lactic microflora of Batzos cheese from raw caprine milk throughout the lactation season, enterococci were found to be abundant in cheese made in winter and their frequency increased with ripening and storage. *Enterococcus durans* was the most frequently isolated species. It was also found among the predominant lactic microflora of the cheese made in spring. These results suggest a possible significant role of enterococci for ripening of the cheese. A reliable identification of enterococci present in traditional foods to the species

and strain level might be of great importance for the dairy industry. DNA-based methods allow discrimination of the isolates at the species or strain level. Proteolytic activity, acidity and bacteriocin production are important traits for the ripening of cheese and different strains within each species might exhibit different activities.

Although some authors have claimed in the past that high levels of contaminating enterococci could lead to deterioration of sensory properties in some cheeses (Thompson and Marth, 1986; Lo´pez-Diaz *et al*., 1995), other literature reports discuss the beneficial role of the presence of enterococci in cheese. Enterococci contribute to the ripening and aroma development of these products due to their proteolytic and esterolytic activities, as well as the production of diacetyl and other important volatile compounds. Furthermore, enterococci seem to play an important role in improving flavor development and quality, not only of cheese, but also of other traditional fermented foods, such as vegetables and sausages (Sarantinopoulos *et al*., 2001a,b).

Antibiotic resistance encompasses both natural (intrinsic) resistance and acquired (transferable) resistance. Enterococci possess a broad spectrum of antibiotic resistances within these two types (Klare *et al*., 2001). Examples of intrinsic resistance are vancomycin (VanC type) resistance in *E. gallinarum*, and resistance towards streptogramins in *E. faecalis*, as well as resistance to isoxazolyl penicillins, cephalosporins, monobactams, aminoglycosides (low level), lincosamides (mostly), and polymyxins. The resistance to ampicillin (especially in *E. faecium*), tetracyclines, macrolides, aminoglycosides (high level), chloramphenicol, trimethoprim / sulfamethozaxole , quinolones, and streptogramins (in *E. faecium* and related species) are acquired, as well as resistance towards glycopeptides (vancomycin). Vancomycin resistant enterococci (VRE) possess vanA, vanB, vanD, and vanE type resistance genes (Klare *et al*., 2001). Ampicillin, vancomycin and gentamicin are the clinically most relevant antibiotics to cure infections with multiple VRE strains. It should be noted that the extensive use of vancomycin has steadily raised the number of VRE over the past two decades and therefore the percentage of invasive nosocomial enterococci displaying high-level vancomycin resistance (Endtz *et al*., 1999).

This work aimed at investigating the presence of enterococcal strains in Egyptian stored hard cheese, selecting the most resistant strain, and searching for the presence of a plasmid to study the biological functions of the plasmid harboured by the strain.

MATERIALS AND METHODS

Strains isolation:

An extract of traditionally stored Egyptian Ras cheese manufactured from raw milk was plated on M17 medium. Six colonies were isolated from the plates at random.

Bacterial strain identification:

Identification was done with the system rapid ID 32 strep V2.0, as described by Freney *et al.* (1992). This system includes 32 wells containing

various biochemical tests in dehydrated form: seven -osidase reactions (,ß-glucosidase, 0 glucuronidase, two different substrates for P-galactosidase, a-galactosidase, N-acetyl-1-glucosaminidase, and 1- mannosidase), 17 carbon substrate fermentations (ribose, mannitol, sorbitol, lactose, trehalose, raffinose, sucrose, L-arabinose, D-arabitol, cyclodextrin, glycogen, pullulan, maltose, melibiose, melezitose, methyl-3-D-glucopyranoside, and tagatose), three arylamidase reactions (alanylphenylalanyl- proline, pyroglutamic acid, and glycyltryptophan), One phosphatase (alkaline phosphatase), and four classical biochemical tests (arginine dihydrolase, acetoin production, hippurate hydrolysis, and urease). After growth on sheep blood agar medium, a bacterial suspension was prepared in 2 ml of sterile water and adjusted to a McFarland standard 4 turbidity. Fifty-five microliters of the suspension was deposited into each well. After 4-h incubation at 37°C, a reading was taken with an ATB 1520 reader (API-bioMerieux) linked to an ATB 1545 computer. Identification was obtained with ATB Plus software.

Bacterial growth:

The strains were tested for growth in MRS medium under different stress conditions such as different temperatures (42, 46, 50, 55°C), antibiotic (Erythromycin Em) and different salt concentrations (1, 2, 3, 4, 5% NaCl) Growth was determined by measuring turbidity by using a DU-65 spectrophotometer.

Plasmid isolation from Gram-positive bacteria:

The extra chromosomal DNA of the test strains was extracted as described by Anderson and McKay (1983) modified by El Demerdash (2003). The plasmids were separated by agarose gel electrophoresis (0.8% agarose gels in 40-mM Tris–acetate, 1-mM EDTA, pH 8.0 buffer) at 100V. A marker (λ-*sty*1) was used in order to determine the plasmid sizes.

Plasmid curing:

For plasmid curing the protoplast method of Gasson (1980) was used with some modifications (El Demerdash, *et al.* 2003). *Enterococcus durans* cells, grown overnight at 37°C were inoculated into MRS medium supplemented with 40 mM DL-threonine. They were incubated at 37°C until an optical density at 620 nm (OD620) of 0.6 to 0.7 was reached. Then, 4 ml of the cells were harvested by centrifugation at 14,000 x *g* for 1 min. The cell sediment was washed with protoplast buffer (40 mM ammonium acetate, 0.5 M sucrose, 0.25% gelatin ; pH 6.5) and resuspended in 0.5 ml of the same buffer containing 2.5 mg of lysozyme and 1 mg of mutanolysin. Incubation was at 37°C for 45 to 60 min. When the protoplasts had formed, as followed by phase-contrast microscopy, they were harvested by centrifugation, washed with protoplast buffer, resuspended in 1 ml of the same buffer, and serially diluted in the same buffer by a factor of up to 10^7. Dilutions were plated onto MRS agar and then incubated at 37°C for regeneration. Colonies appearing after about 4 days were checked for the absence of plasmid.

Antibiotics resistance test:

The following types of antibiotics and their corresponding concentrations were used to compare the antibiotic resistance of both cured and wild-type cells.

Ampicillin	AMP	10µg
Chloramphenicol	C	30µg
Cefotaxime	CTX	30µg
Erythromycin	Em	15µg
Gentamycin	CN	10µg
Kanamycin	K	30µg
Lincomycin	MY	15µg
Methicillin	MET	5µg
Neomycin	N	30µg
Ofloxacin	OFX	5µg
Rifampicin	RD	30µg
Streptomycin	S	10µg
Tetracycline	TE	30µg
Vancomycin	VA	30µg

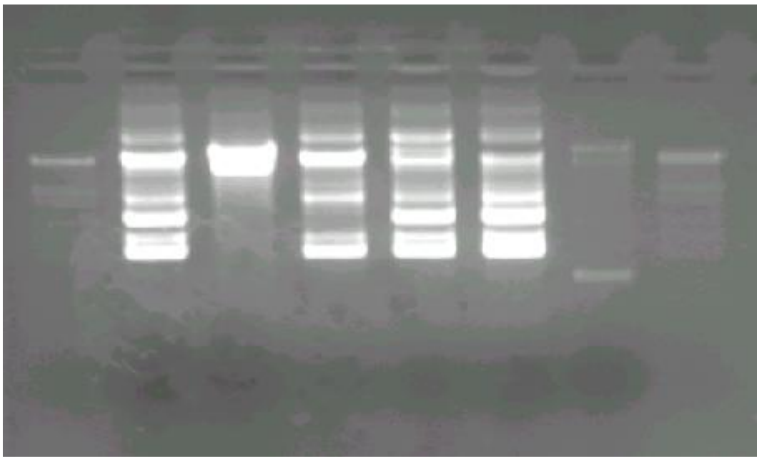
RESULTS AND DISCUSSION

Identification for the different strain with the system rapid ID 32 strep V2.0:

The cells were identified by rapid ID 32 strep V2.0 as *Enterococcus durans* with a very good identification of 99.9%.

Plasmid isolation:

The isolated strains were subjected to plasmid isolation to investigate the presence of plasmids. Results represented in Figure (1) showed that, the six strains carried different types of plasmids. Strain number 2 carried only one plasmid, while the other strains carried more than one plasmid.



λ- *sty*1 1 2 3 4 5 6 λ- *sty*1

Figure (1): Gel electrophoresis for plasmids isolated form the six strains

Evaluation of the isolated strains for growth under multiple stress conditions:

All the isolated strains were tested for their ability to grow under different stress conditions such as high t temperatures(42, 46, 50, 55°C), antibiotic (Em) and different salt concentrations (1, 2, 3, 4, 5% NaCl).The results obtained indicated that strain number 2 showed the best response to these stress conditions (data not present).

As shown in Figure (2), in the presence of Em the strain grown better at 46°C than at 42°C. However, the optical density reached the same value after 5 hours of incubation. At higher temperatures the strain slower and reached only about 0.4 OD.

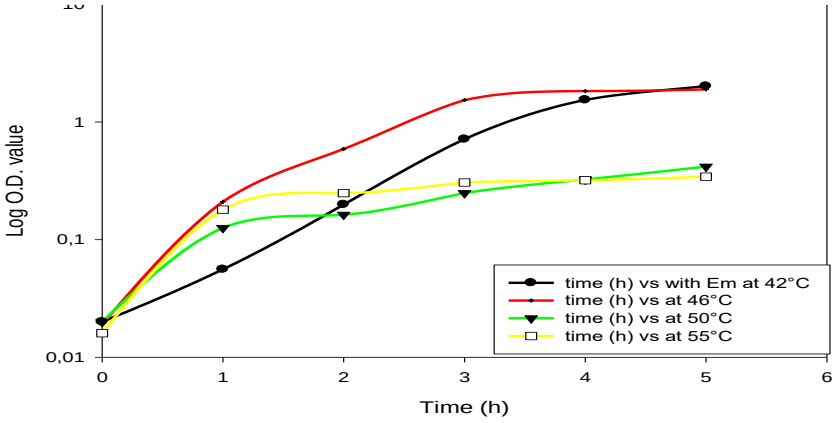


Figure (2): Growth curve for strain 2 at different temperatures in the presence of erythromycin.

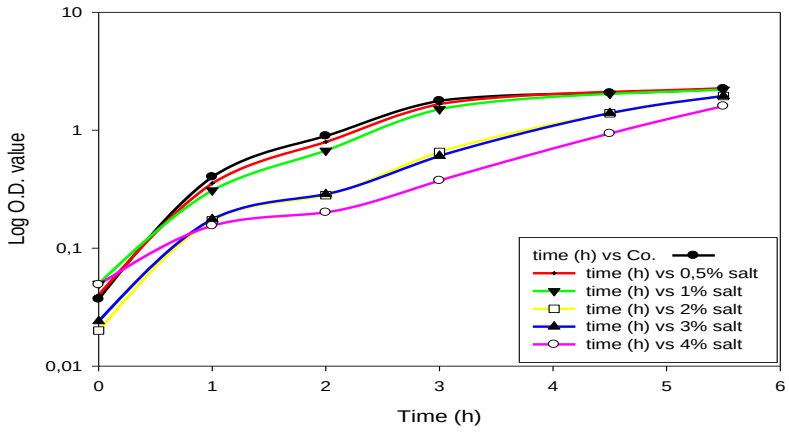


Figure (3): Growth curve for strain 2 at different NaCl concentrations at 42°C.

Figure (3) shows that growth decreased with increasing NaCl concentration, however, even at 4% NaCl acceptable growth was observed. At the end of the incubation period (after 5 hours) no significant differences in growth rates were observed at all tested salt concentrations.

Curing of plasmid from strain 2:

Protoplast formation and regeneration method was applied to cure the plasmid present in strain number 2. About 20 colonies were randomly picked and subjected to plasmid isolation. The percentage of cured cells was found to be about 19.0%, as shown in Figure (4).

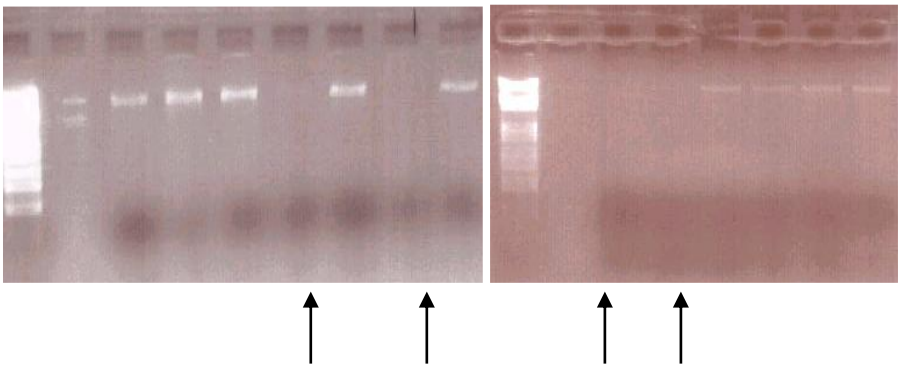


Figure (4): gel electrophoresis pattern for plasmid isolation of cured cells.

Comparison between the stress response of both cured and wild-type strains:

Sensitivities of both cured and wild type strains to different antibiotics is shown in Table (1). Both strains showed comparable resistance or sensitivity for all tested antibiotics except for S and VA: the cured cells being more resistant than the wild type.

Table (1): Antibiotic resistance for wild-type and cured strains.

Antibiotic	AMP	C	CTX	E	CN	K	MY	MET	N	OFX	RD	S	TE	VA
Wild type	30	25	6	15	18	6	12	10	18	20	25	10	30	20
Plasmid cured	30	25	6	15	18	6	12	10	18	20	25	6	30	18

Numbers = clear zone in mm **<10 = resistant**
10-15 = intermediate **< 15 = sensitive**

Effect of high temperature on wild type and cured strain:

The results showed that the cured strain grew better in the presence of Em than the wild type. This may be attributed to the presence of a resistance gene on the plasmid. Also the result showed that the cured strain grew better in the presence of Em than in the absence. Best growth was observed for the wild type without Em at 46 ºC.

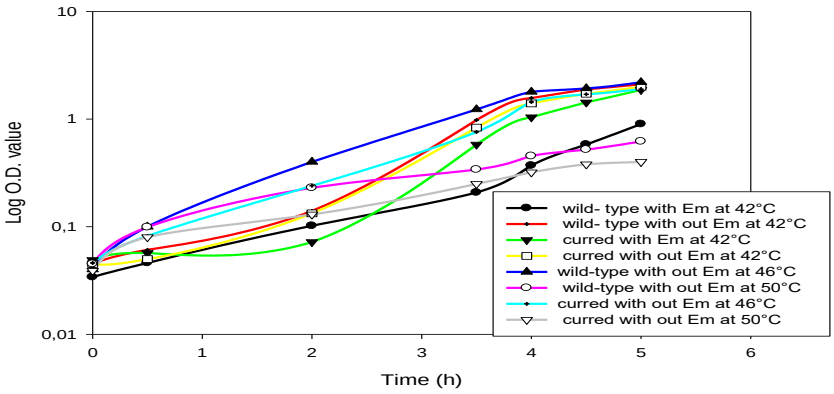


Figure (5): Growth curves of cured and wild- type strains at different temperatures with or without Em.

Effetc of salt on cured and wild - type strain:

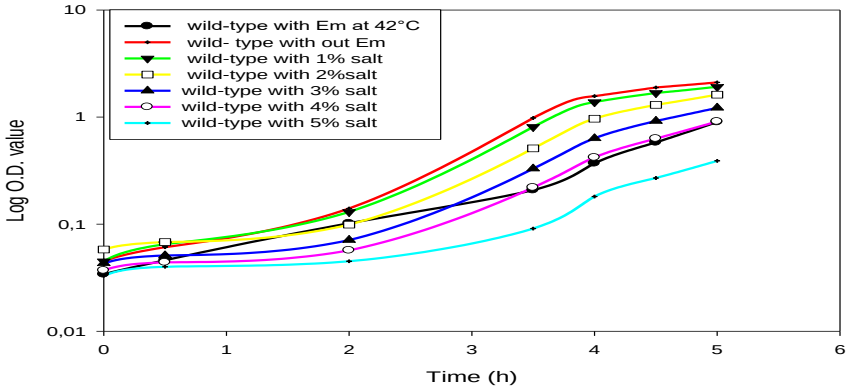


Figure (6): Growth curves of wild- type strain at different NaCl concentrations with or without Em.

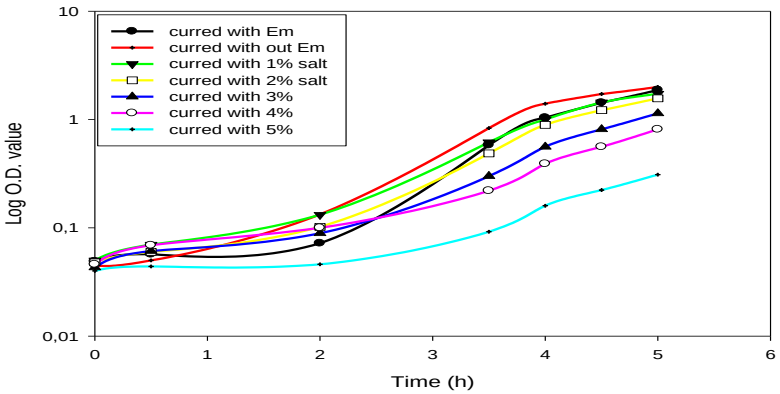


Figure (7): Growth curves of curried strain at different NaCl concentrations with or without Em.

Growth of both wild-type and curried strains was almost similar except for better growth of the cured strain in the presence of Em. (Figures 6, 7).

Survival of bacteria under adverse conditions is assured by many mechanisms. Different responses to different stress treatments (e.g. heat, low pH, osmotic shock, etc.) have been studied extensively. Bacteria have evolved stress-sensing systems and defenses against stress, which allow them to withstand harsh conditions and sudden environmental changes (Van de Guchte *et al*., 2002).

Thermophilic lactic acid bacteria (T-LAB) are traditionally used for the manufacture of yoghurt and hard 'cooked' cheeses (Emmental, Gruyère, Parmigiano, Grana etc.), which all require an incubation of the milk or curd at a relatively high temperature (45°C or above) during their production process. Not many LAB strains are able to grow or survive at such high temperatures (Delcour *et al.*, 2000.). They are subjected to stressful conditions during preproduction, production, and postproduction phases of dairy food fermentations. Stress may be induced by lyophilizition or concentration, extreme temperatures (heat, cold), salt treatment (NaCl), rising lactic acid concentration, with concomitant pH shift, and nutrient deprivation. These and possibly other types of stress induced by production parameters may impact on the survival of LAB cultures and the performance of biochemical activities at a desired level. In recent years interest has grown in stress response phenomenon of LAB species, since understanding the mechanism of stress response may lead to the development of cultures with improved capacity to survive and function under industrial conditions (Somkuti and Steinberg, 1999). In Greek cheeses, enterococci can reach numbers of up to 10^6–10^8 CFU/g and may play an acknowledged role in development of the organoleptic characteristics during ripening. Due to their proteolytic and lipolytic activities as well as their ability to metabolize citrate they may contribute to the sensorial and textural properties of the cheese (Psoni *et al*., 2006).

In conclusion, our results indicated that the *Enterococcus durans* strain tested shows good stress responses against different stressful conditions occurring in industrial dairy products. This should qualify the strain for application in cheese production.

REFERENCES

Anderson, D. G.; and McKay, L. L. (1983). Simple and rapid method for isolating plasmid DNA from lactic streptococci. Appl. Environ. Microbiol.,46:549-552.

Coppola, T.M., Parente, J.E., Dumontet, S. And La Peccerella, A., 1998. The microflora of natural whey cultures utilized as starters in the manufacture of Mozzarella cheese from water buffalo milk. Lait 68, 295–310.

Delcour, J.; Thierry, F. and Pascal, H. (2000). Advances in the genetics of thermophilic lactic acid bacteria. Current Opinion in Biotechnology 2000, 11:497–504.

El Demerdash, H. A. M. (2002). New Techniques for food biotechnology: development of Food – Grade Vectors for Improvement of thermophilic Starter Culture Performance. Ph.D thesis, CAU, Kiel

El Demerdash, H.A.M., Heller, K.J. and Geis, A. (2003) Application of the *shsp* Gene, Encoding a Small Heat Shock Protein, as a Food-Grade Selection Marker for Lactic Acid Bacteria Applied and Environmental Microbiology, p. 4408–4412 Vol. 69, No. 8

Endtz, H.P., van den Braak, N., Verbrugh, H.A. and van Belkum, A., (1999). Vancomycin resistance: status quo and quo vadis. European Journal of Clinical Microbiology and Infectious Diseases 18, 683– 690.

Freney J., S. Bland, J. Etienne, M. Desmonceaux, J. M. Boeufgras, and J. Fleurette (1992) Description and Evaluation of the Semiautomated 4-Hour Rapid ID 32 Strep Method for Identification of Streptococci and Members of Related Genera J. of Clinical Microbiology. 30, 2657-2661.

Gasson, M. J. (1980). Production, regeneration and fusion of protoplasts in lactic streptococci. FEMS Microbiol. Lett. **9:**99–102.

Klare, I., Werner, G. and Witte, W., (2001). Enterococci. Habitats, infections, virulence factors, resistances to antibiotics, transfer of resistance determinants. Contributions to Microbiology 8, 108– 122.

Lo´pez-Diaz, T.M., Santos, J.A., Gonzalez, C.J., Moreno, B. and Garcia, M.L., (1995). Bacteriological quality of traditional Spanish blue cheese. Milchwissenschaft 50, 503–505.

Psoni L. , C. Kotzamanides , C. Andrighetto , A. Lombardi , N. Tzanetakis and E. Litopoulou-Tzanetaki (2006) Genotypic and phenotypic heterogeneity in Enterococcus isolates from Batzos, a raw goat milk cheese International Journal of Food Microbiology 109 ,109–120

Psoni, L., Tzanetakis, N. and Litopoulou-Tzanetaki, E., (2003). Microbiological characteristics of Batzos, a traditional Greek cheese from raw goat's milk. Food Microbiology 20, 575–582.

Sarantinopoulos, P., Andrighetto, C., Georgalaki, M.D., Rea, M.C., Lombardi, A., Cogan, T.M., Kalantzopoulos, G. and Tsakalidou, E., (2001a). Biochemical properties of enterococci relevant to their technological performance. International Dairy Journal 11, 621– 647.

Sarantinopoulos, P., Kalantzopoulos, G. and Tsakalidou, E., (2001b). Citrate metabolism by Enterococcus faecalis FAIR-E 229. Applied and Environmental Microbiology 67, 5482–5487.

Somkuti, G. A. and Steinberg D. H. (1999). Distribution of plasmidborne stress protein genes in *Streptococcus thermophilus* and other lactic acid bacteria. Curr Microbiol, 38:43-47.

Thompson, T.L. and Marth, E.H., (1986). Changes in Parmesan cheese during ripening: microflora-coliforms, enterococci, anaerobs, propionibacteria and staphylococci. Milchwissenschaft 41, 201–204.

Van de Guchte, M.; Serror, P.; Chervaux,C.; Smokvina, T.; Ehrlich, S. D. and Mauin, E. (2002). Stress response in lactic acid bacteria. Antonie Leeuwenhoek, 82 : 187-216.

عزل وتعريف سلالات بكتريا الانتيروكوكاى من الجبن المصرية الجافة القديمة

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تم عزل ستة سلالات بكتيرية من عينات الجبن المصرية الجافة القديمة أو المخزنة (المسواة) . وتم تعريف تلك السلالات بنسبة ٩٩,٩% على أنها تتبع النوع *Enterococcus durans* وذلك باستخدام تكنيك التعريف ID32 strep V2.0 .

تم أختبار قدرة السلالات البكتيرية الستة على النمو تحت ظروف قاسية مختلفة مثل النمو على درجات حرارة مرتفعه (٤٢ ، ٤٦، ٥٠ ، ٥٥°م) تركيزات متدرجة من ملح الطعام (١ ، ٢ ، ٣ ، ٤ ، ٥%) .

فى المرحلة التالية من البحث تم أخذ سلالة واحدة والتي أظهرت أعلى مقاومة للظروف القاسية السابقة لتقدير تتابع الينوكليوتيدات للبلازميد المعزول منها. ولدراسة ارتباط الوظائف البيولوجية بوجود هذا البلازميد بالخلية تم تحضير خلايا منزوعة البلازميد من خلايا تلك السلالة الأصلية وأجريت عمليات نمو للخلايا الأصلية وكذلك الخلايا منزوعة البلازميد المشتقة منهما لمعرفة مدى مقاومة كل منها لمختلف ظروف النمو غير المناسبة (القاسية) مثل :-

١- التعرض لأنواع متعددة من المضادات الحيوية مثل : أمبسيللين ، كلورامفينكول ، سيفوتاكسيم ، إريثروميسين ، جنتاميسين ، كاناميسين، استربتوميسين ، تتراسيكلين ، فانكوميسين ،لينكوميسين، ميثيسيلين ، نيوميسين ، أوفلوزاسين ، ريفامبيسين .

٢- مقاومتهما لدرجات الحرارة العالية .

٣- قدرتها على النمو في وجود تركيزات مرتفعه من ملح الطعام وقد أظهرت النتائج عدم وجود اختلافات بين الخلايا الاصيلة والخلايا منزوعة البلازميد فى مقاومتها لوجود المضادات الحيوية فيما عدا الإستربتومايسين والفانكوميسين حيث كانت الخلايا الأصلية أكثر مقاومة للأول وأكثر حساسية للثانى عن الخلايا منزوعة البلازميد .

بدراسة التأثير المشترك لوجود عاملى ضغط مجتمعين على معدلات النمو لكلا النوعين من الخلايا عند تنميتهما فى وجود أو فى غياب المضاد الحيوى الاريثروميسين . ففي وجود الاريثروميسين نمت الخلايا الخالية من البلازميد بمعدلات أفضل عند درجة حرارة ٤٢°م فقط بينما فى غياب هذا المضاد الحيوى نمت الخلايا الأصلية بمعدلات أفضل عند جميع درجات الحرارة المستخدمه كذلك لم تظهر اية فروق معنوية بين معدلات نمو النوعين من الخلايا فى وجود تركيزات مختلفة من ملح الطعام .

قام بتحكيم البحث

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