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STUDIES ON ASEPTIC RAS CHEESE SLURRY

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ABSTRACT

The proteolytic and autolytic activities in buffer system of six strains of lactic acid bacteria isolated from Egyptian dairy products were examined. The selected strains (*Lactococcus lactis* sub sp. *lactis* 64RM, *Lactococcus lactis* sub sp. *lactis* 608N, *Lactobacillus salivarius* 194KC, *Lactobacillus rhamnosus* 56R, *Enterococcus faecium* 151D and *Enterococcus faecium* 174RM) were blended into freshly made starter–free Ras cheese (acidified with glucono- δ –lactone, GDL) to form slurries that were ripened anaerobically at 30°C for 5 days. Results showed relatively constant values of moisture and fat contents in slurries, while pH values decline after five days of ripening. The highest release of free fatty acids was noticed in four slurries (*Ent. faecium* 174RM, *Lb. salivarious* 194KC, *Lb. rhamnosus* 56R and *Lc. lactis* subsp. *lactis* 608N). on the contrary, the mixture of strains in slurries showed a moderate release of free fatty acids. Peptidase activities in the proteolytic system of the tested Ras cheese slurries have been demonstrated from the Cadmium- ninhydrine (Cd-ninhydrine) reaction of water-soluble extract. The highest values of free amino acids (FAA) were noticed in the individual tested lactobacillus strains. Whereas, slurry containing mixed strains showed a moderate release of FAA (*Lb. salivarious* 194KC + *Lc. lactis* subsp. *lactis* 64RM). Proteolysis of studied slurries was also qualitatively followed by using Urea- polyacrylamide gel electrophoresis technique. Casein degradation arises quickly in slurries during 5 days of ripening. α_{s1}-casein hydrolysed to small weight peptides, while, the β- casein is apparently not degraded. The organoleptic evaluations were correlated with the release of free amino acids and aminopeptidase activity. So, the present investigation emphasizes the importance of proteolytic system of selected strains in improving the flavour and texture of cheese.
Keywords: Aseptic Ras cheese slurry, Proteolysis, Lactic acid bacteria, Ripening.

INTRODUCTION

Investigation of factors involved in flavour development in hard cheeses such as Ras cheese is a time and money consuming process because these cheese varieties require 4-12 months to develop full flavour. This severely limits the number of investigations that can be completed in a given time. Cheese curd slurry systems which are prepared by mixing fresh cheese curd with water, and ripened at elevated temperatures to accelerate the cheese ripening process offers a solution to this problem.

Use of cheese curd slurry system to obtain rapid flavour development is not a new concept. Early work on slurry systems was aimed at providing an intensely flavoured product for use by the prepared food industry (Kristoffersen *et al.*, 1966, Sutherland *et al.*, 1975). Harper and Kristoffersen (1970) who demonstrated that the slurry systems replicated the ripening processes in natural cheeses, obtained flavour development in Cheddar and Swiss cheese slurries in only a few days of ripening. Their work created an interest in employing slurries as model systems for investigating the cheese ripening process (Thakar and Upadhyay, 1992). Similarly, Awad *et al.*, (2001) demonstrated the efficiency of proteolytic systems in *Lactococcus*,

Lactobacillus and *Enterococcus* strains for more trials in real cheese systems. This study, aimed to investigate the possible role of such culture for the acceleration of cheese ripening and enhancement of cheese flavour.This paper describes the development of an aseptic system for preparation of cheese curd slurries which remain free of contamination during 5 days of ripening at 30°C.This method can be applied in the manufacture of processed cheese as a simple and rapid method for selecting flavour producing starter bacteria for studying biochemical processes in processed cheese ripening.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactococcus lactis 64 RM, and 608N; *Enterococcus faecium*174RM and 151D ; *Lactobacillus salivarius*194KC and *Lactobacillus rhamnosus* 56R were isolated from different dairy products and identified in the laboratory of the biochemistry of dairy microorganisms, Department of Dairy Science and Technology at the Faculty of Agriculture, Alexandria University (table 1).
Lactococcus lactis 64 RM, and 608N; *Enterococcus* 174RM and 151D were cultivated in M17 medium, while *Lactobacillus* 194KC and 56R were grown in MRS medium. The optimum temperature for *Lactococcus* strains was 30°C, while for the remaining tested strains was 42°C. Cells were harvested at early stationary phase by centrifugation at 10000 xg for 20 min at 4°C. The pellets were then washed twice with 0.01M potassium phosphate buffer pH 7.0, and stored at -20°C.

Intracellular enzyme assays

Determination of proteolytic activity

The method of Miozzari *et al.*, (1978) was used for the permeabilization to evaluate the proteolytic activity in the previous obtained pellets. The substrate used for the determination of aminopeptidase activity was L-leucyl paranitroanilide (Leu-*p*NA) as described in the method of El Soda and Desmazeaud (1982). One unit of enzymatic activity was defined as the amount of enzyme producing a change of 0.01 unit/min of absorbance at A_{410}.

Measurement of the rate of autolysis

A portion of cell suspension was added to 0.01M phosphate buffer pH 5.5 containing 1M sodium chloride to obtain an optical density of 0.9-1.0 at 650 nm and incubated at 30°C and 42°C for *Lactococcus lactis* and *Lactabacillus* and *Enterococcus*, respectively. After different time intervals, the percentage decrease in Optical Density was measured and expressed as % autolysis (Thiboutot *et al.*, 1995).

Design of experiment

After obtaining the previous results, the six single strains were used to prepare three mixtures:

1- *Lb. salivarius* 194KC + *Ent.faecium* 174RM (lactobacilli strain with high aminopeptidase (A.P) and autolytic activities and cocci strain with a weak aminopeptidase activity and a moderate autolytic activity).

2- *Lb. salivarius* 194KC + *Lc. Lactis* subsp. *lactis* 64RM (lactobacilli strain with high aminopeptidase (A.P) and autolytic activities and cocci strain with weak aminopeptidase and autolytic activities).
3- *Lc. Lactis* subsp. *lactis* 608N+ *Lb. rhamnosus* 56R (cocci strain with high aminopeptidase and autolytic activities and a bacilli strain with moderate aminopeptidase and autolytic activities).

The six single, three previous mixed strains and control were used in the preparation of Ras cheese slurries.

Strain preparation for slurries

The cultures were sub cultured onto M17 and MRS as described previously. The cells were harvested when their growth reach to the early stationary phase by centrifugation at 10000 xg for 15min at 4°C. The obtained pellets were washed twice by 0.01M potassium phosphate buffer pH 7.0 and suspended in the same buffer for obtaining ≈ 1 Optical Density. The suspension was stored at -20°C before use. Each LAB suspension contained ~$1x10^8$ cfu.

Manufacture of starter free cheese

Starter-free stirred –curd Ras type cheese was made essentially as described by Hofi et al., (1970). To 30 L of warm (30 °C) pasteurized milk (72 °C for 16s), containing 2.8% fat , and 101.53g of glucono- δ –lactone, GDL (Roquette Frères, Gurnee, IL) dissolved in 394.74 ml deionized water. Single-strength calf rennet (Chr. Hansen's Laboratory, Milwaukee, WI) was used to coagulate the cheese milk after brief stirring for 30 min under quiescent conditions. The coagulum was cut and cooked at 45°C for 30 min and held at this temperature for 15 min. Whey was drained at pH 5.5 and the unsalted curds used to prepare cheese slurries.

Preparation of cheese slurry

Cheese slurries were prepared by a modification of the method of Kristoffersen *et al.*,. (1966). Unsalted curd (170g), LAB suspension (3ml), NaCl (6g), and sterile distilled water (20ml) were blended into slurry in a sterile blender jar. The slurry was transferred aseptically into a sterile wide mouth bottle which was capped loosely and incubated at 30°C / 5days under anaerobic conditions using BBL™Gas Pack 100™ anaerobic system (Bection Dickinson and company, Sparks, USA) A control slurry was prepared without the bacterial cell suspension. To study the effect of mixed cultures, one cocci was mixed with a bacilli tested strain. All experiments were carried out in duplicates.

Chemical analysis

Cheese slurries were analyzed for fat by Gerber method; moisture by using the moisture analyzer (Mettler Toledo Model HR73, Switzerland) and pH was measured using a glass electrode (pH model 3900-010, Precisa, Switzerland).The concentration of free fatty acids were determined by the method of Deeth et al., (1975) and expressed as mM oleic acid/g cheese. The concentration of free amino acids in the water soluble extracts were determined in duplicate using the method of Doi et al., (1981) and modified by Folkertsma and Fox (1992) using Cd-ninhydrin. Because the most abundant free amino acids present in cheese are leucine and glutamic acid (Thomas and Prichard, 1987), The A_{507} was converted to mM leucine from a

standard curve prepared with leucine. Samples were analysed by urea polyacrylamide gel electrophoresis (PAGE) using a plateau II vertical slab gel unit (Bio-Rad Laboratories, USA). Electrophoresis was performed according to the method of (Andrews 1983), cheese fat layer was removed by acetone. Twenty milligrams of cheese proteins was dissolved in 1 ml of sample buffer containing 8 M urea, 2% 2-mercaptoethanol and 0.01% Bromophenol, the gels were stained directly with Commassie brilliant blue G250 and 10µl of sample was applied to the gel.

Organoleptic evaluation of cheese slurries

The cheese slurries were organolypticaly tested by 10 panelists at the Faculty of Agriculture, Department of Dairy Science and Technology. The assessment flavour was carried with maximum score of 20 points.

RESULTS AND DISSUCTION

Intracel ular enzyme assays

Results from figure (1) reveal different values of aminopeptidase (A.P) and autolytic activities. *Lb. salivarius* 194KC showed the highest proteolytic and autolytic activities
(46 unit/1O.D and 74%, respectively). The highest A.P activity in *Lactobacillus* strains was previously mentioned by El soda and Desmazeaud (1982) and Awad et al., (2001). On the other hand, aminopeptidase activity in both tested *enterococcus* strains (8unit/1O.D) was lower than those of remaining tested strains. However, the autolytic activity in *Ent. faecium* 174RM was higher (53%) than the other tested enterococci strain (27%). Concerning the *Lactococcus* strains, *Lc. Lactis* subsp. *lactis* 608N a moderate aminopeptidase activity (30.4unit/1O.D) and a high autolytic activity (61%) were observed

Composition of slurry

The percentage of moisture and fat were relatively constant in slurries while pH decrease compared to the zero time (Table 2). After five days of ripening, moisture ranged from 38.72 to 42.53% and pH values ranged from 5.13 to 5.32 in the slurries. Moistures were higher than those found typically in Ras cheese >36% (Abou Donia, 2002). This can be explained as a result of addition 20ml distilled water throughout the preparation of cheese slurry. Higher moisture content in slurries could increase the microbial growth and subsequently increase their enzyme activities (Fox, 1989). These reactions with elevated ripening temperature promote the acceleration of cheese ripening. Analysis of proteolytic products of slurries will provide information on contributions of the tested strains and allow for judgments to be made on their usefulness in cheese ripening (Muehlenkamp-Ulateand Warthesen, 1999). Fat contents of slurries were lower than those previously reported by Abou Donia, (2002) for type of Ras cheeses. Slurries pH values were lower when compared with the results obtained by El Soda and Abou-Donia, (1978) and Attia and Gooda, (1993), as they found the pH values of Ras cheese samples were 5.78 and 5.57, respectively. These results indicated that the moisture and the ripening temperature facilitate rapid utilization of residual lactose in slurries. No

significant, differences in pH were found between the control slurries and those with added *Ent. faecium* 174RM and *Ent. faecium* 174RM +*Lb. salivarius* 194KC throughout ripening, this indicating that these strains could not contribute significant acid production within the slurries.

Liberation of Free Fatty Acids

The release of free fatty acids (FFA) increased visibly throughout the five days of ripening (Fig. 2). The analysis of slurries individually showed a similar higher value of free fatty acids (3.95 mM oleic acid/g cheese) released by the lipolytic activities of the following four strains (*Ent. faecium* 174RM, *Lb.salivarious* 194KC, *Lb. rhamnosus* 56R and *Lc. lactis* subsp. *lactis* 608N). Slurries containing mixed strains (*Lb. rhamnosus* 56R + *Lc. lactis* subsp. *lactis* 608N and *Lb. salivarious* 194KC + *Lc. lactis* subsp. *lactis* 64RM) showed a moderate release of FFA (3.67 and 3.38 mM oleic acid /g cheese, respectively).

The liberation of FFA in cheese contributes in general in the development of flavour. Cheeses containing adjunct cultures and having high levels of free fatty acids were reported by Madkour et al., (1999) and were attributed to the release of intracellular esterases and lipases.

Liberation of Free Amino Acids

The release of Free Amino Acids (FAA) increased visibly throughout the five days of ripening as compared with the zero time. Data in figure (3) shows the concentration of free amino acids of the various WSN extracts after reaction with Cd-ninhydrin. Since Cd-ninhydrin reacts specially with free amino acids (FAA) in the WSN extract, the A_{507} is proportional to the concentration of free amino acids present. The highest level of free amino acids (0.156 and 0.147 mM leucine equivalents) was obtained in the WSN extracts from the *Lb. salivarious* 194KC and *Lb. rhamnosus* 56R slurries, respectively. This suggests higher peptidase activity in the lactobacilli strains compared to the remained tested strains in slurries and might significantly contribute to liberation of amino acids during cheese maturation. Similarly, Hickey et al., (1983) and Awad et al., (2001) found that lactobacilli were more proteolytic and released more amino acids than did lactococci. Also, Gomez et al.,., (1996a,b) and El Soda et al., (1999) reported that the *Lactobacillus* showed a higher affinity towards small MW peptides, and could easily remove all the hydrophobic peptides and produce a high level of free amino acids. The lowest level of FAA (0.104 mM leucine equivalents) was noticed in the control slurry. Mean concentrations of FAA in the WSN extracts from slurries containing *Lc. lactis* subsp. *lactis* 64RM and *Lb. salivarious* 194KC+ *Ent. faecium* 174RM were nearly similar values to the control slurry (0.112 and 0.113 mM leucine equivalents respectively), indicating that, these strains have a very weakly A.P activities. In addition, the moderate concentration of FAA was observed in slurries containing *Lc. lactis* subsp. *lactis* 608N and *Lb. salivarious* 194KC + *Lc. lactis* subsp. *lactis* 64RM (0.14 and 0.139 mM leucine equivalents respectively). The results of the ripening indices of the cheese slurry illustrated in figure 3 confirm the foregoing findings described in figure (1). In consequence, higher levels of protein breakdown can be measured in cheese slurries after 5 days of ripening. These present data are in agreement with those of El Soda et al., (1999).

Urea - Polyacrylamide gel electrophoresis (PAGE)

Electrophoretograms of the tested slurries proteins at zero and 5 days of ripening are presented in figure (4). Generally, the degradation patterns of Ras cheese slurries after 5 days of ripening (B) were observed apparently when compared with the Ras cheese slurries at zero time of ripening (A). The ripened slurries showed an extensive hydrolysed of α_{s1}–casein than β- casein. Fox et al.,. (1993) reported the similar results in Dutch and Cheddar cheeses. In cheese, β- casein could not be hydrolysed by chymosin possibly due to the hydrophobic interactions between C-terminal regions of β- casein which makes the preferred cleavage sites unavailable (Fox and Stepaniak, 1993). Also, the conditions of cheese (high concentration of salt and protein) assumed a little presence of monomeric β-casein (Creamer, 1976). Whereas, the degradation of β-casein depends on its degree of association, monomeric β-casein being the only substrate susceptible to proteolysis by rennet.

The α_{s1}-casein was hydrolyzed initially to α_{s1}-I-casein and subsequently to other peptides faster in their electrophoretic mobilities than that of α_{s1}-I-casein. So, it is apparent that α_{s1}-I-casein is hydrolysed and is the main source of peptides in cheese. Electrophretograms shown in figure (4B) revealed the intensity of the bands corresponding to small molecular weight peptides in all the tested slurries and there were slight qualitative differences among them. A high peptide concentration of high mobilities was found in slurries of *Lb. salivarius* 194KC+ *Ent. faecium* 174RM, *Ent. faecium* 151D and control indicating that, these strains have weak proteolytic system for dgradation such peptides to free amino acids.

Organoleptic evaluation

The sensory evaluation in cheese slurries are shown in table (3). These results can be well correlated with the activity of aminopeptidase (Fig. 1) and the release of free amino acids (Table 3). The cheese slurry containing *Lb. salivarious* 194KC + *Lc. lactis* subsp. *lactis* 64RM had a high score of flavour compared to the remained tested slurries and was closest properties to the ripened Ras cheese (14 and 16 score respectively). On the other hand, the control slurry has no cheesy aroma.

Table (1): Isolated starter strains present in cheese slurries.

Stains	source	Method of identification
Lactococcus lactis sub sp. *lactis* 64RM	Milk	API 20 or 50CH carbohydrate fermentation strips (BioMerieux, Hazelwood, MO)
Lactococcus lactis sub sp. *lactis* 608N	yogurt	SDS-PAGE technique
Lactobacillus salivarius 194KC	Karish cheese	API 50CH carbohydrate fermentation strips (BioMerieux, Hazelwood, MO) and SDS-PAGE technique
Lactobacillus rhamnosus 56R	Ras cheese	API 50CH carbohydrate fermentation strips (BioMerieux, Hazelwood, MO)
Enterococcus faecium 151D	Domiatti cheese	SDS-PAGE technique
Enterococcus faecium 174RM	Milk	API 20CH carbohydrate fermentation strips (BioMerieux, Hazelwood, MO)

So, the cheese slurries could be a good model system to study the contribution of the proteolytic systems of selected strains and thus improved the cheese flavour and quality.

Table (2): Chemical composition of slurries at zero time and after 5 days.

Type of slurry	PH		Moisture%		Fat%	
	0 days	5 days	0 days	5 days	0 days	5 days
C	5.57	5.13	42.21	42.07	20.5	20.5
Ent. faecium 174RM	5.6	5.16	44.6	38.72	25	25
Ent. faecium 151D	5.6	5.19	42.94	40.3	25.5	24
Lb. salivarius 194KC	5.57	5.21	44.77	42.53	25	24
Lb. rhamnosus 56R	5.56	5.32	43.63	38.74	21	20
Lc. lactis sub sp. *lactis* 608N	5.65	5.23	42.24	39.64	24	23
Lc. lactis sub sp. *lactis* 64RM	5.64	5.22	44.22	40.42	25	23
Lb. salivarius 194KC+ *Lc. lactis* sub sp. *lactis* 64RM	5.66	5.26	41.22	39.29	26	26
Lb. rhamnosus 56R+ *Lc. lactis* sub sp. *lactis* 608N	5.64	5.28	42.9	40.56	24.5	25
Lb. salivarius 194KC+ *Ent. faecium* 174RM	5.65	5.14	43.43	40.99	24.5	23

Table (3): Organoleptic evaluation of cheese.

Slurries	Score of flavour
C	9.11
Ent. faecium 174RM	12.7
Ent. faecium 151D	12.6
Lb. salivarius 194KC	14
Lb. rhamnosus 56R	10.89
Lc. lactis sub sp. *lactis* 608N	13.89
Lc. lactis sub sp. *lactis* 64RM	13.67
Lb. salivarius 194KC+ *Lc. lactis* sub sp. *lactis* 64RM	14.11
Lb. rhamnosus 56R+ *Lc. lactis* sub sp. *lactis* 608N	12.89
Lb. salivarius 194KC+ *Ent. faecium* 174RM	13.33
Ripened Ras cheese	16

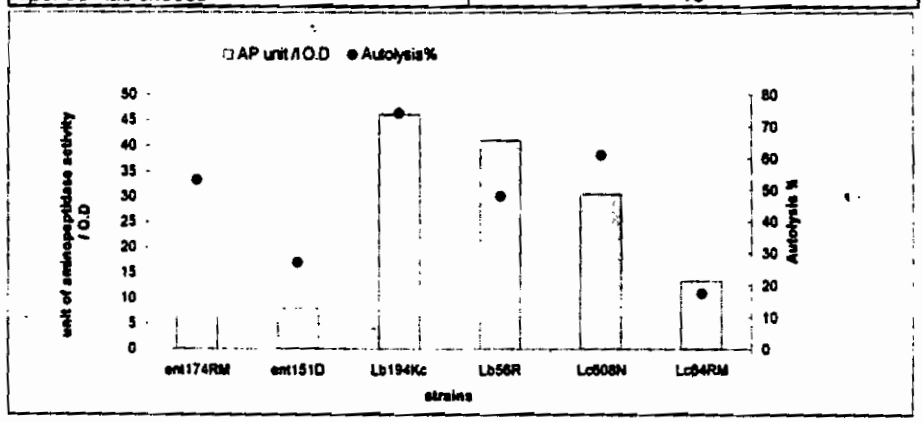

Figure (1): Aminopeptidase and Autolytic activities in tested strains.

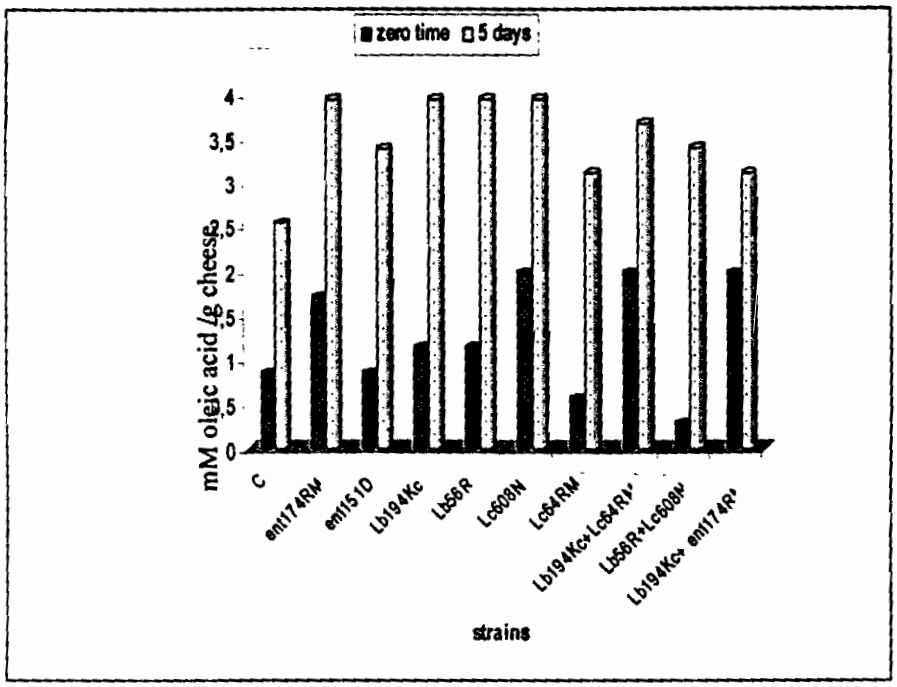

Figure (2): Liberation of Free Fatty acids from slurries cheese; ■ zero time; □after five days of incubation.

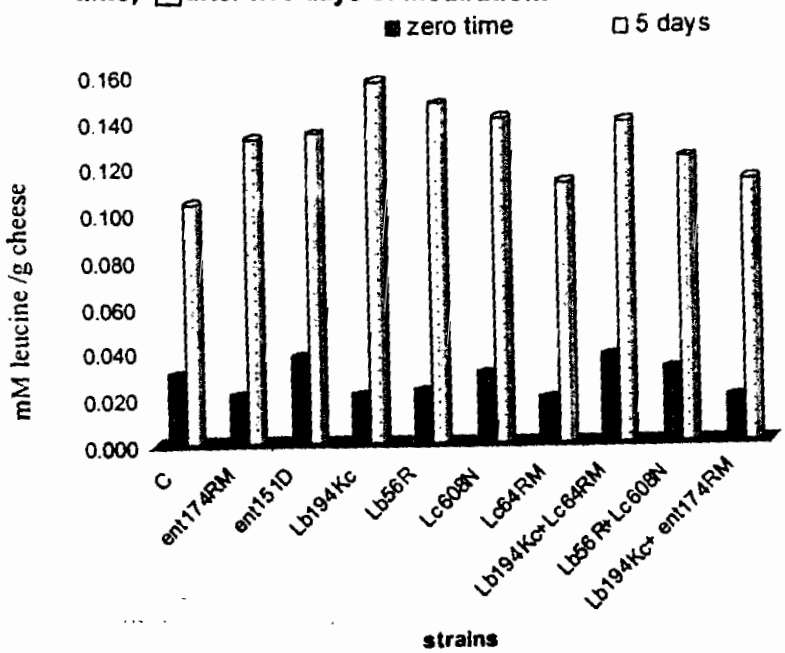

Figure (3): Liberation of Free Amino acids from slurries cheese; ■ zero time; ⊠ after five days of incubation.

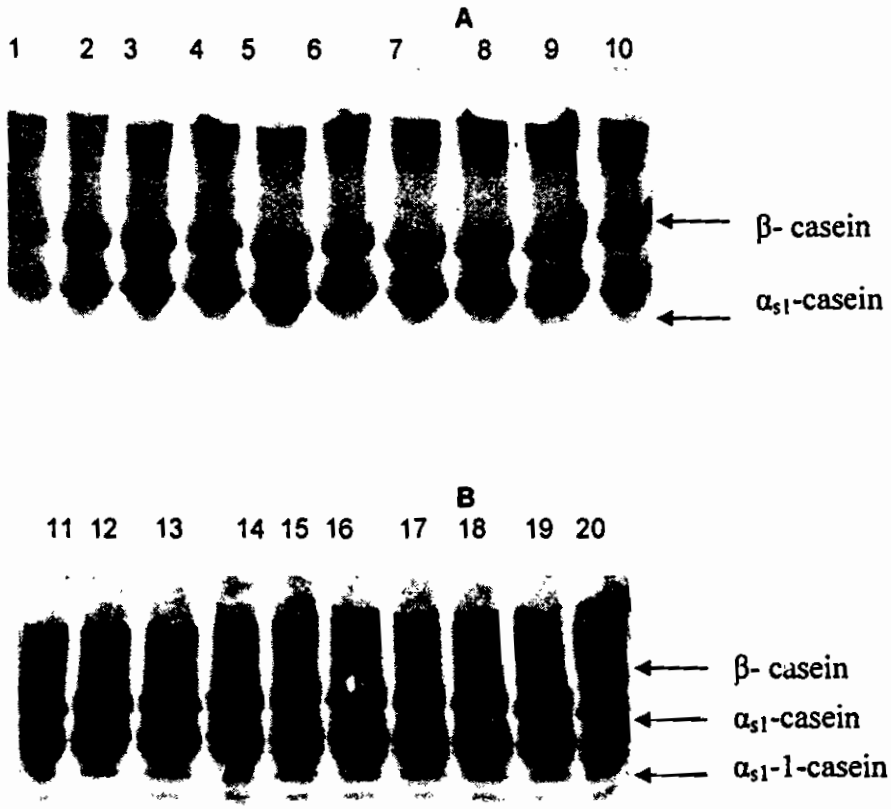

Figure (4): Urea-polyacrylamide gel electrophoretograms of Ras cheese slurry proteins during zero (A) and 5 days (B) of ripening at 30°C.

Control Ras cheese slurry (lanes 1 and 11), Ras cheese slurry made with *Lb. rhamnosus* 56R (lanes 2 and 12), Ras cheese slurry made with *Lc. lactis* sub sp. *lactis* 64RM (lanes 3 and 13), Ras cheese slurry made with *Lb. salivarius* 194KC (lanes 4 and 14), Ras cheese slurry made with *Lc. lactis* sub sp. *lactis* 608N (lanes 5 and 15), Ras cheese slurry made with *Ent. faecium* 174RM (lanes 6 and 16), Ras cheese slurry made with *Ent. faecium* 151D (lanes 7 and 17), Ras cheese slurry made with *Lb. salivarius* 194KC+ *Ent. faecium* 174RM (lanes 8 and 18), Ras cheese slurry made with *Lb. salivarius* 194KC+ *Lc. lactis* sub sp. *lactis* 64RM (lanes 9 and 19), Ras cheese slurry made with *Lb. rhamnosus* 56R+ *Lc. lactis* sub sp. *lactis* 608N (lanes 10 and 20).

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دراسات على معلق جبن الراس المعقم

عيشه العطار

قسم علوم و تكنولوجيا الالبان – كلية الزراعه – جامعة الاسكندريه

تمت دراسة التحلل الذاتي والنشاط التحللي للبروتين في البفر لست سلالات من بكتريا حمض الاكتيك المعزولة من منتجات الألبان المصرية وكانت السلالات المعزولة هي *Lactococcus lactis* sub sp. *lactis* 64RM, *Lactococcus lactis* sub sp. *lactis* 608N, *Lactobacillus salivarius* 194KC, *Lactobacillus rhamnosus* 56R, *Enterococcus faecium* 174R M.و*Enterococcus faecium* 151D
تم خلط تلك السلالات مع خثرة الجبن الراس الطازجة الخالية من أي محتوي ميكروبي و تم تحضير معلق خثرة الجبن بواسطة التحميض بالجلوكونو دلتا لاكتون و تمت التسوية في ظل ظروف لا هوائية علي درجة حرارة ٣٠ °م لمدة ٥ أيام.
أوضحت النتائج ثبات نسبي في قي قيم المحتوي الرطوبي و الدهن داخل معلق الخثرة طوال فترة التسوية، بينما كان هناك أنخفاض في قيم رقم الحموضة بعد ٥ ايام من التسوية.
كما بينت النتائج ان أعلي أنتاج للأحماض الدهنية الطيارة كان في أربعة معلقات من خثرة الجبن
(*Ent. faecium* 174RM, *Lb.salivarious* 194KC, *Lb. rhamnosus* 56R and *Lc. lactis* subsp. *lactis* 608N).
في حين ان استخدام خليط من السلالات داخل معلق خثرة الجبن ادي لانطلاق متوسط للأحماض الدهنية الطيارة. تمت دراسة النظام التحللي للبروتين في داخل معلق الخثرة بأستخدام تفاعل النينهيدرين للمستخلص الذائب في الماء. اوضحت النتائج ان أعلي أنتاج للأحاض الأمينية الحرة في معلق خثرة الجبن كان في حالة السلالات المعوية الفردية. بينما معلق الخثرة المحتوى علي خليط من السلالات أدي لأنطلاق متوسط للأحماض الأمينية الحرة كما في حالة (*Lb. salivarious* 194KC + *Lc. lactis* subsp. *lactis* 64RM).
تم في هذه الدراسة أيضا تتبع التحلل البروتيوليتي في معلق الخثرة بأستخدام طريقة اليوريا بولي أكريل أميد جيل الكتروفوريسيس خلال فترة خمسة أيام من التسوية. و قد بينت النتائج حدوث تحلل سريع للكازين حيث وجد ان ألفا أس واحد يتحلل ألي ببتيدات صغيرة في الوزن الجزيئي، بينما لم يحدث اي تحلل ظاهر للبيتا كازين .
كما وجدت هناك علاقة واضحة ما بين أنطلاق الأحماض الأمينية الحرة و النشاط التحللي لانزيمات ا للأمينوببتيداز مع الخواص الحسية و العضوية لمعلق الخثرة. وتوضح الدراسة اهمية تتبع النظام التحللي البروتيوليتي لبكتريا البادىء المستخدمة في تحسين الخواص العضوية والحسية علي قوام و تركيب الجبن.