

PRODUCING NOVEL ANTIFUNGAL LACTIC ACID BACTERIA (LAB) WITH POTENTIAL FOR PROLONG SHELF-LIFE OF CHEESES.

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ABSTRACT

Of 108 lactic acid bacteria (LAB) isolates, 9 isolates had a broad antifungal activity against *Penicillium Spp.* were isolated from naturally fermented milk products. The isolates were processed under cheese manufacturing conditions to study its relation with prolonging the shelf-life of cheese. The technological properties of these isolates has been determined by studying the acidifying and proteolysis activity. Three isolates with moderate or low acidifying and proteolysis activity were selected for further studies. The factors affecting the rate of growth of these selected strains, i.e. pH, NaCl and temperature were evaluated. The results indicated that, strains grown well at pH 5.5 – 6.6, tolerate salt concentration at 3 – 5% and showed good growth rate at 10 and 37°C. These properties gave these strains potentiality for use as starter-like or as adjunct cultures. When these strains used as adjunct culture in Ras cheese manufacture, the cheeses had composition resembling normal composition of Ras cheese with positive effect on cheese quality. The cheeses had prolonged shelf-life against fungi spoilage.

INTRODUCTION

Various food commodities can be contaminated by wide spectrum of filamentous fungi, leading to important economic losses and harmful effects on human and animal health. Prevention of fungal growth in various food commodities is the best method of protection from the harmful effects on human and animal health. Many physical and chemical methods have been developed in inhibition of fungi, but some moulds acquired the ability to resist chemical treatment and some preservatives. The preserving capacity of bacteria naturally occurring in food has gained increased interest during the recent years, due to the consumers demand for reduced chemical preservatives.

The public necessity for high quality food, without addition of chemical preservatives, with extend shelf life, determined the search for new strains of lactic acid bacteria that are able to control the fungal growth of cheese-spoilage and mycotoxigenic species.

In the present study, new strains of antifungal LAB isolated from naturally fermented milk products in our laboratory were studied. Further study to evaluate the performance activity of these antifungal-LAB is needed to detect whether the isolated culture were able to grow in cheese milk and to survive cheese manufacturing conditions and to select performant strains as biopreservatives for Ras cheese.

MATERIALS AND METHODS

Materials:

Strains of lactic acid bacteria (LAB):

Nine identified strains of lactic acid bacteria (LAB) isolated from traditional milk products, had a broad spectrum of antifungal activity against a wide range of fungi isolated from the surface of cheeses, has been collected in our laboratory by Maha et. al., (2015).

Methods:

Technological properties of antifungal LAB:

Acidifying activity:

Reconstituted (10% skim milk powder) autoclaved at 121°C for 15 min. was inoculated with 10%, 24h overnight activated subculture of identified antifungal-LAB strains. The acidifying activity and pH were monitored after 24h incubation at 37°C.

Proteolysis activity:

The proteolysis activity of antifungal-LAB was measured using procedure of Moore and Stein (1954). The extent of proteolysis was measured at OD₅₇₀.

Effect of different fermentation conditions:

Effect of pH on the growth of antifungal LAB:

The pH of suitable broth media was adjusted to 2.5, 3.5, 4.5, 5.0, 5.5 and 6.0 using 1M HCl or NaOH. The broth was autoclaved at 121°C for 15 min, cooled to room temperature and inoculated with 1% (v/v) anti-fungi strains and incubated at 32°C for 20 h. OD₆₀₀ was measured. The pH of MRS was plotted on abscissa vs. OD₆₀₀ value was used as ordinate to determine growth curve at different pH values.

Effect of salt on growth of antifungal LAB:

In cheese industry salt-in-moisture (S/M) is an important quality criterion. NaCl was added to suitable broth media at concentrations of 2, 3, 4, 4.5, 5.0 and 5.5% (w/v). The broth was autoclaved at 121°C for 15 min., cooled to room temperature and inoculated with 1% (v/v) antifungal strains and incubated at 32°C for 20 h. The OD₆₀₀ was measured and growth curve determined by plotting salt concentration vs. OD₆₀₀.

Effect of temperature on growth of antifungal LAB.

Inoculated anti-fungi strains in broth (1% v/v inoculum) were incubated at different temperatures, 10, 37 and 45°C for 20 h. OD₆₀₀ was measured after incubation at each temperature and graphed to determine growth curve. Blank MRS was used.

Cheese making:-

Ras-type cheeses were manufactured in dairy lab according to Adb-el-tawab 1963 and Osama 2005, divided into 4 portions as follows; control (without antifungal strain), cheese with added antifungal LAB strain as adjunct cultures, *Lactococcus lactis* ssp *lactis*, Z8 (strain 1) or *Pediococcus acidilactis*, R18 (strain 2) or *Lactobacillus paracasei* ssp *paracasei*, K58 (strain 3) the ripened at 13-14°C and 85-90% moisture.

Cheese analysis:

Chemical composition

The pH was measured using a pH meter (model SA 720, USA), moisture content and total nitrogen content were determined according to Ling (1963).

Assessment of cheese proteolysis

Protein degradation in cheese was followed by the determination of water soluble nitrogen (WSN) according to Kuchroo and Fox (1982), 5% Phospho-tungstic acid soluble nitrogen (PTA-SN) as determined according to Jarrett *et al.* (1982). Non protein nitrogen (NPN) was determined in 12% TCA filtrate.

Antifungal activity of LAB on fungi growth in cheese was followed during cheese ripening. The strains of antifungal LAB used were added to cheese milk as adjunct culture, and their inhibition effect against airborne-mould growth were observed throughout ripening period(60 day).

RESULTS AND DISCUSSION

In this study the technological properties of antifungal-LAB isolated from naturally fermented milk products (Laban Zeer, Laban Rayeb and Kariesh cheese) and factors affecting their growth conditions in cheese were determined. The potential for use as novel dairy starter and/or protective culture was also studied.

Technological performance of Antifungal LAB:

In this study, strains of Antifungal LAB listed in Table (1) were used

pH and acidifying activity:

Results in Table (2) and Figs (1, 2) indicated the acidifying activity and changes in pH of LAB strains in milk. The % of acidity of different strains at the end of incubation time were ranged between 0.176 to 0.473 compared to 0.14 for control, while the pH range were 6.5 to 5.00 for experimental strains compared to pH 6.7 for control. The results indicated that the rate of changes in acidity and pH were strain dependent. Strains of *Lactobacillus paracasei ssp paracasei* (K66) and *Lactococcus lactis ssp lactis* (K17) showed the highest acid production compared with strains of *Pediococcus acidilactis* (R18) and *Lactococcus lactis ssp lactis* (Z8) which gave lowest acidity production..

Table (1): strains of Antifungal LAB.

Isolates	Identification
K17	<i>Lactococcus lactis ssp lactis</i>
K18	<i>Lactococcus lactis ssp.lactis</i>
Z8	<i>Lactococcus lactis ssp lactis</i>
R18	<i>Pediococcus acidilactis</i>
K58	<i>Lactobacillus paracasei ssp paracasei</i>
K66	<i>Lactobacillus paracasei ssp paracasei</i>
K67	<i>Lactobacillus acidophilus</i>
K44	Enterococci spp
K45	Enterococci spp

Table (2): pH, acid development and proteolytic activity of antifungal LAB in skim milk after 24 hrs:

Isolated number	pH	Acidity %	Proteolytic activity
C	6.7	0.14	0.092
K17	5.12	0.432	0.136
K18	5.52	0.364	0.139
K44	6.5	0.194	0.116
K45	6.43	0.225	0.169
K58	5.68	0.315	0.134
K66	5.00	0.473	0.129
K67	6.37	0.226	0.169
Z8	6.35	0.189	0.104
R18	6.43	0.176	0.134

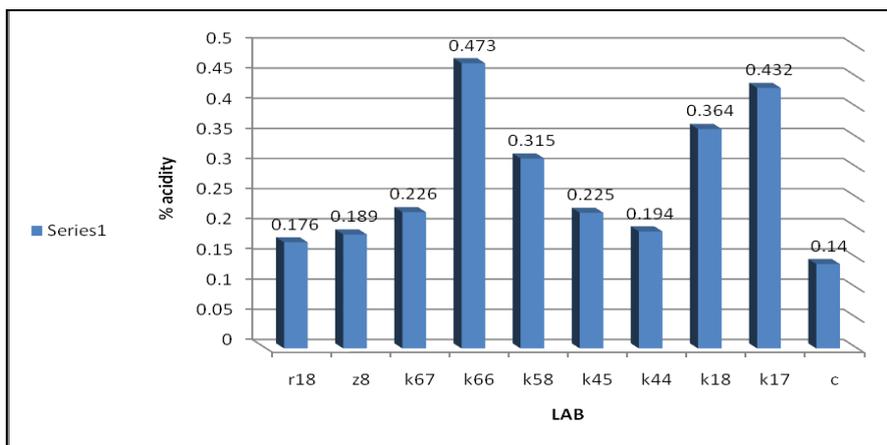


Fig (1): acidifying activity of LAB

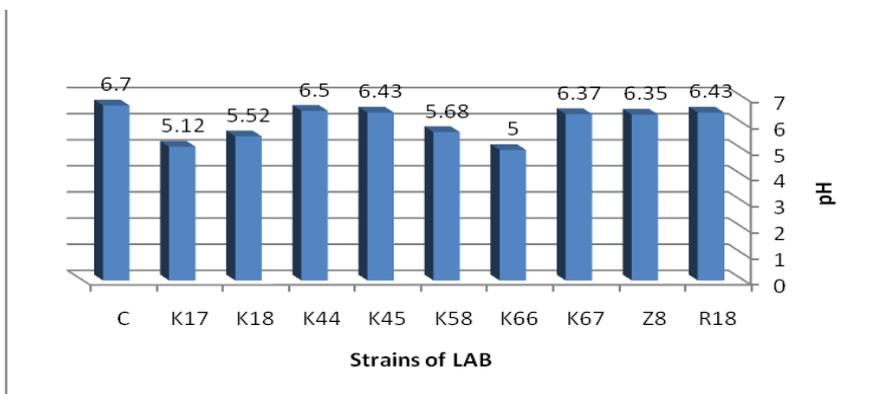


Fig (2): PH of LAB

Proteolytic activity:

The proteolytic activity of antifungal LAB strains was assessed by measuring the O.D. values at 570 after 24h incubation at 37°C. The results in Table (2) and Fig (3) indicated that the rate of proteolysis was strain dependent. The rate of proteolysis was varied greatly between the strains, as the strains of Lactobacilli (K67) and Lactococci (K45) gave the highest rate of proteolysis , i.e. 0.169, some strains – representing the same genus - (K58 and Z8) showed low proteolysis activity.

From these preliminary tests, the results indicated the differential ability of these strains for acid production and protein proteolysis. According to technological criteria, the strains with the highest acid production and proteolysis activity were excluded, and three species with low or moderate acid and proteolysis activity, represented the different bacterial genus and different milk products (Z8, R18 and K58), were selected for further technological studies.

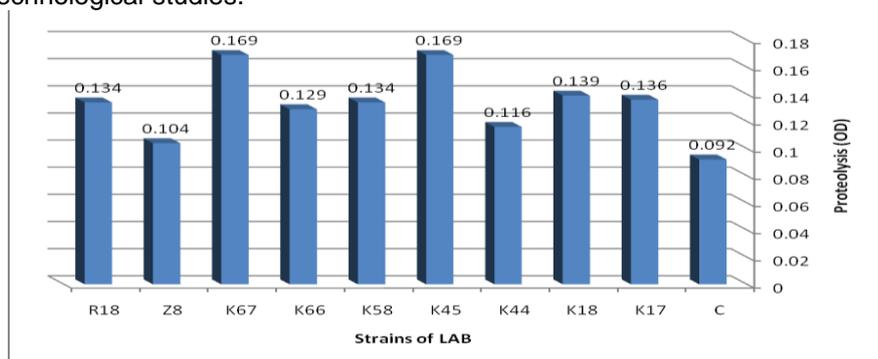


Fig (3): proteolytic activity of LAB strains

Effect of cheese manufacturing conditions on the growth and activity of selected antifungal-LAB:

Three strains of antifungal-LAB, i.e. *Lactococcus lactis ssp lactis*, Z8; *Pediococcus acidilactis*, R18 and *Lactobacillus paracasei ssp paracasei* K58, were selected for further technological studies. To determine their ability to survive within the cheese manufacturing and ripening conditions, i.e. growth at different pH, salt and temperature.

Effect of pH:

The growth of the three antifungal-LAB that grown in suitable broth media adjusted at pH 2.5 to 6.6 and incubated for 72 h at 32°C was monitored. The effect of changes in pH of cheeses during manufacturing process, between 6.6 for cheese milk to pH 5.0 or 5.5 at whey drainage stage, on the growth antifungal LAB was determined.

Result in Table (3) show the rate of growth of these three strains as determined by OD values. A little decrease in the growth of strains R18 and K58 was observed, otherwise the strain Z8 showed an increase in the rate of growth with the decrease in pH from pH 6.6 to pH 5.5.

This behavior of growth for these three strains at this range of pH (6.6 – 5.5) gave them the preferentiality for use as starter-like or as adjunct starter in hard cheese making.

Table (3): Effect of pH on the rate of growth of LAB (Z8, R18 and K58) isolates

pH	Z8	R18	K58
2.5	0.785	0.564	0.362
3.5	0.801	0.682	0.420
4.5	0.852	0.722	0.595
5.0	0.902	0.943	0.922
5.5	1.132	0.993	1.010
6.0	1.72	1.023	1.053
6.6	0.903	1.082	1.087

Effect of NaCl on the rate of growth of LAB (Z8, R18 and K58) isolates:

NaCl is an inhibitory substance which may inhibit growth of certain types of bacteria. Because some LAB was sensitive to NaCl then it would not be able to show its activity in the presence of NaCl, it was essential to test the NaCl tolerance of these LAB isolates. In this study the NaCl tolerance test for growth of these three isolates was determined up to 5.5 NaCl concentrations. Although the rate of growth was decreased with the increase in NaCl concentration, all isolates exhibited salt tolerance at 3 to 5% NaCl as shown in Table (4), although sharp decrease was observed at higher or lower NaCl concentrations.

Table (4): Effect of NaCl on the rate of growth of LAB (Z8, R18 and K58) isolates

NaCl	Z8	R18	K58
0	1.930	1.719	0.964
2	1.835	1.071	0.750
3	0.993	1.014	0.742
4	0.964	0.974	0.633
4.5	0.8.57	0.871	0.572
5.0	0.551	0.461	0.330
5.5	0.433	0.406	0.300

Effect of temperature on the rate of growth of antifungal-LAB.

The temperature is an important factor which can dramatically affect the bacterial growth. The reason for choosing this range of temperature was to detect whether the isolated cultures would favour the growth within the range of manufacturing and ripening temperature. In this study, the three antifungal-LAB isolates were grown at 10, 37 and 45°C in appropriate broth media for 20 h. The rate of growth was followed based on measurement of optical density (OD) in the culture broth. Curves in Figs 6,7 and 8 indicated that the rate of growth seemed to be more temperature dependent, since they did not perform well at 45°C. Concerning the growth

rate of isolates at 10°C, it is time dependent, as the rate of growth increased with the increase in incubation time, the OD values after 10 hrs were 2.5, 2.33 and 1.07 for isolates of Z8, K58 and R18 respectively. On the other hand, the maximum growth rate at 37°C showed the same trend but the exponential phase was decreased fastly compared with the time consumed for the growth curve at 10°C. These results revealed that these isolates are more tolerant for growth at 10°C and have good ability to grow well at ripening temperature.

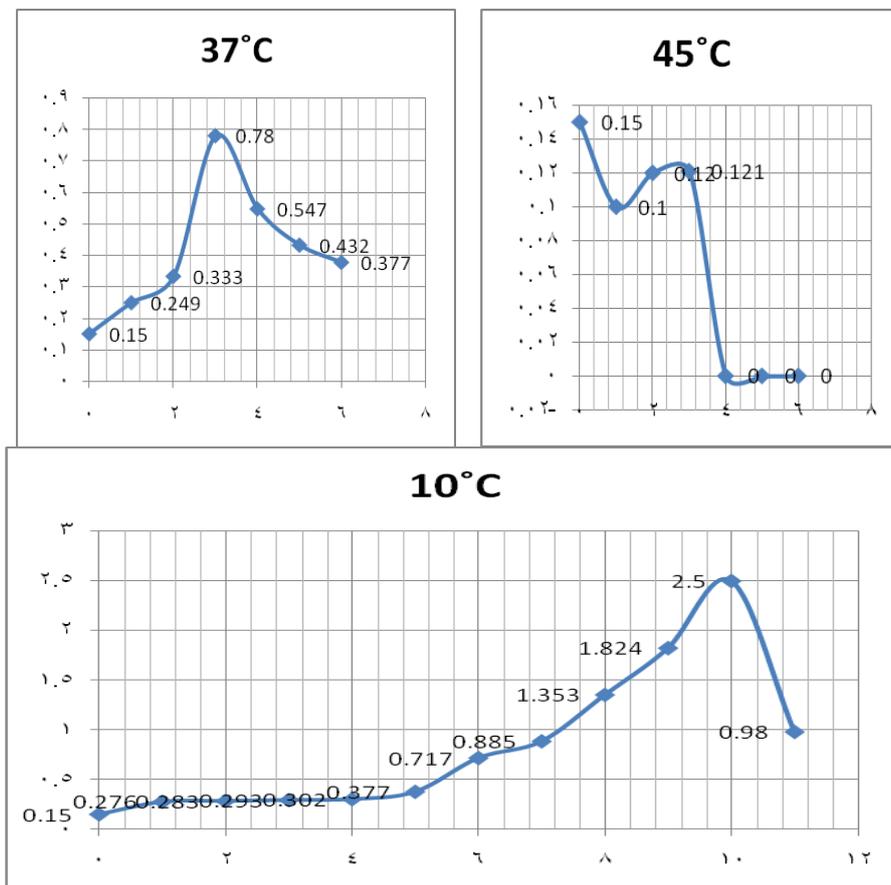


Fig (6): Effect of incubation time at 10, 37, 45°C on the rate of growth of Z8 strain

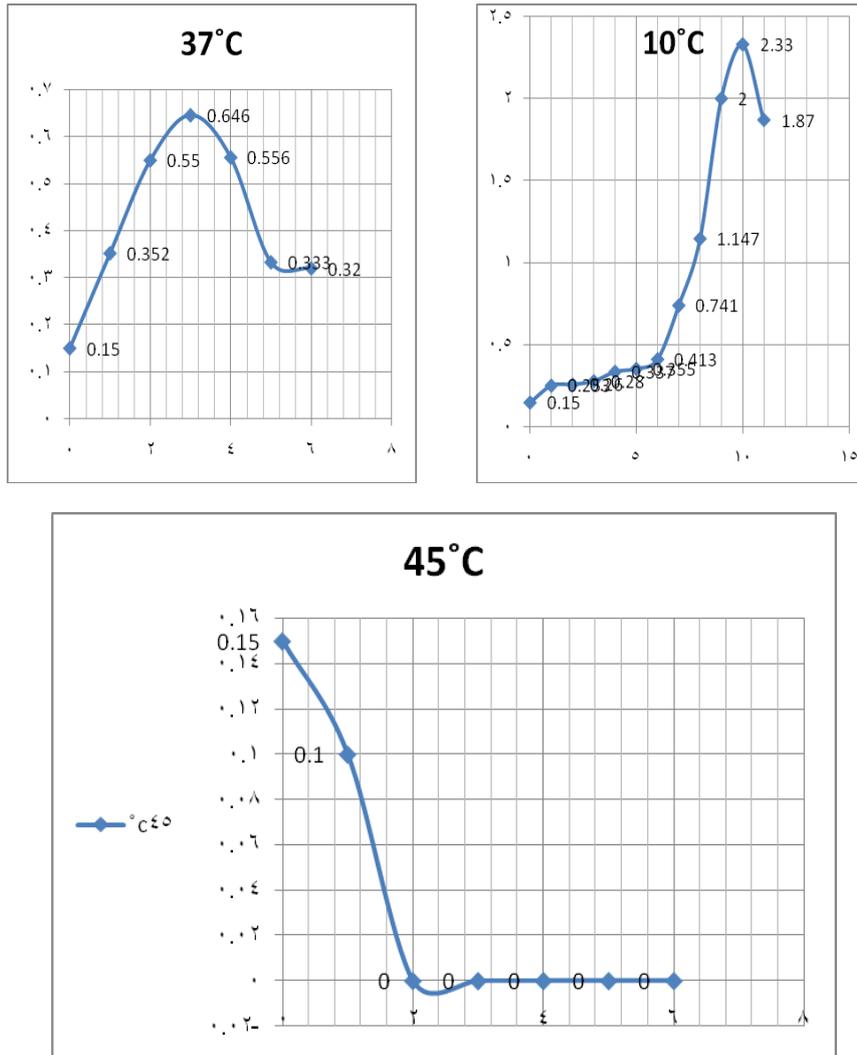


Fig (7): Effect of incubation time at 10, 37, 45°C on the rate of growth of k 58 strain

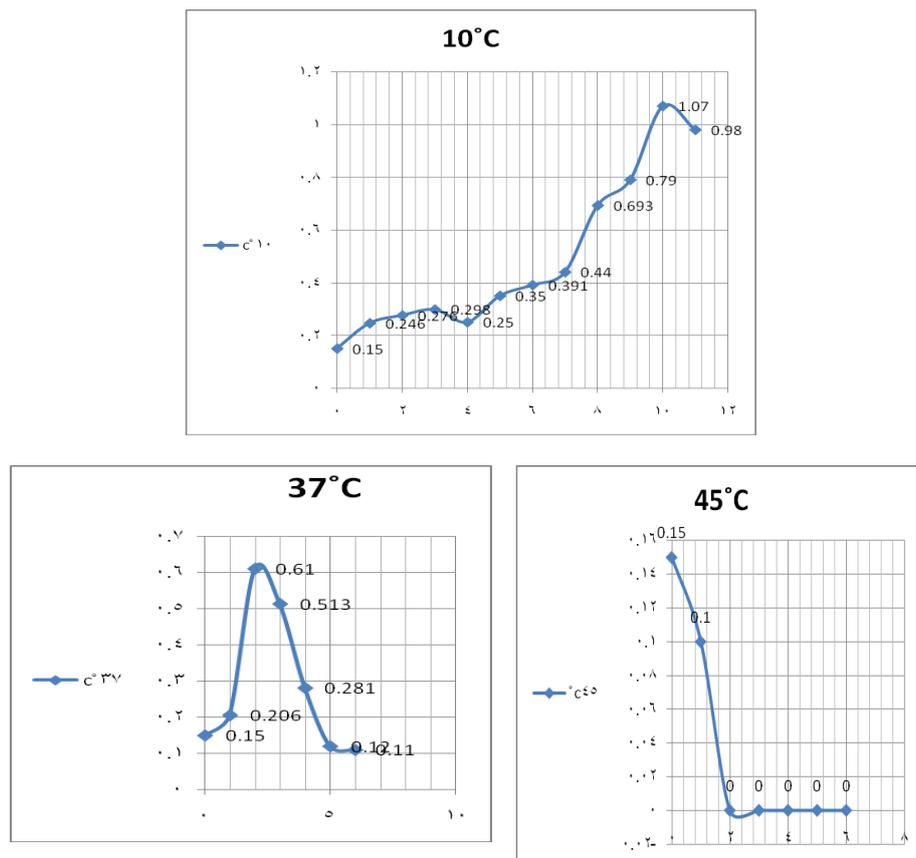


Fig (8): Effect of incubation time at 10, 37, 45°C on the rate of growth of R18 strain

Effect of antifungal LAB on the shelf life and chemical composition of Ras Cheese:

Compositional analysis of cheeses:

The compositional analysis of all cheeses, both with added adjunct cultures and control cheese was presented in Table (5). Cheeses made with adjunct cultures had higher level of moisture than control cheese. The moisture content of adjunct cultured cheeses ranged between 38.41 to 40.66 % compared to 31.975 % for control cheese. These variations in moisture content between control and added adjunct cheeses was also observed with cheddar cheese with added *L. Plantarum* strain and *L. amylovorus* strain as adjunct culture (Ciocia, 2010 and Pawlowska, 2013).

The protein content of all cheeses approximately was the same in all cheeses; it was 30.55 % for control cheese and 29.541 – 31.038% for treatments.

However, the pH of adjunct cultured cheeses showed different trend between cheeses. The pH of cheeses with strain Z8 and R18 treatments was lower compared to pH of control and strain 3 (k58) cheeses. El Soda et al., (2003) reported that the acidifying ability of *Lactococcus* strains was significantly higher than the activity of other species. While Durlu-Ozkaya et al., (2001) reported that *Lactococcus* 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 6 h.

In general, the cheeses were within the range of Ras cheese composition although little effects on the composition and pH of cheeses were observed with adjunct antifungal LAB strains .

Table (5): Effect of antifungal LAB on the chemical composition of Ras cheese:

Ripening period	Control			Z8			R18			K58		
	pH	Mois	Prot	pH	Mois	Prot	pH	Mois	Prot	pH	Mois	Prot
0 day	5.09	31.975	30.55	4.88	40.66	30.815	4.66	39.34	31.038	5.55	38.41	29.541
15 days	5.11	31.270		4.87	38.532		4.60	39.17		5.52	37.25	
30 days	5.17	30.460		4.67	36.586		5.46	38.026		4.98	35.87	

Moi=Moisture

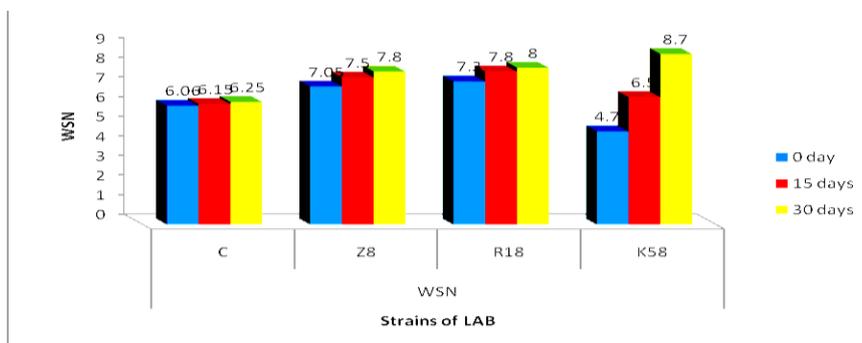
Pro= Total Protein

Effect of adjunct cultures on proteolysis of cheeses:

The rate of proteolysis of control and adjunct cultured cheeses was monitored by determining WSN, NPN and PTA-SN.

Water Soluble Nitrogen (WSN):

The level of water-soluble N (WSN) is a widely-used index of cheese ripening (Fox et al, 1995b). Results in the present study (Table 6 and Fig 9) indicated that, the WSN content in all cheeses increased with the extending of ripening period. Water-insoluble peptides in Cheddar cheese have been studied by McSweeney et al (1994), who found that the principal peptides in this fraction were produced from α s1-casein by chymosin or pepsin or from β -casein by plasmin and therefore that the complementary primary water-soluble peptides must be produced via the action of these enzymes..



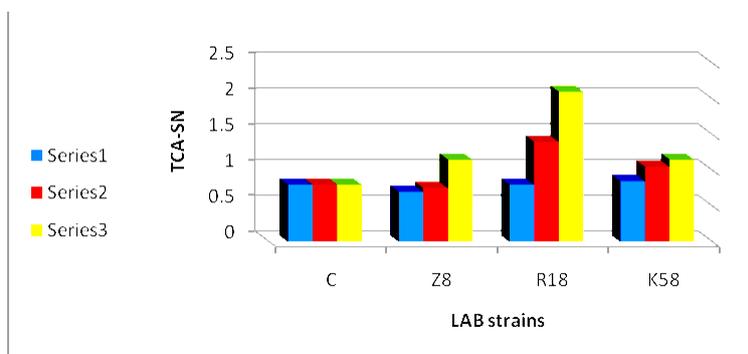
Fig(9) :effect of antifungal LAB on WSN content

At the beginning of ripening period, variable values of WSN were recorded between adjunct cultured cheeses and control (without adjunct culture). A higher WSN was observed for cheeses with added adjunct cultures (strains 1 and 2) compared with the levels of WSN in control and cheese with strain of *Lactobacilli* (strain 3). These variations of WSN of cheeses might be related to the variations in pH of cheeses at this period of ripening (Table 5). Results in Tables 6 showed that, cheeses with lower pH values were the higher in WSN content.

With the progress in ripening period an increase in WSN was observed. However, after 30 day ripening, cheese with added adjunct *Lactbacillus* strain 3 showed the highest WSN content compared to the others. El-Soda et al., (2003) related this to the higher aminopeptidase activity of *Lactobacilli* compared to *Lactococci*.

Table (6): Effect of Antifungal LAB on the rate of proteolysis during cheese ripening.

Ripening period (days)	WSN				TCA-SN				PTA-SN			
	C	Z8	R18	K58	C	Z8	R18	K58	C	Z8	R18	K58
0	6.06	7.05	7.3	4.75	0.8	0.7	0.8	0.85	0.7	0.7	0.7	0.85
15	6.15	7.5	7.8	6.5	0.8	0.75	1.4	1.05	0.7	0.8	0.8	0.8
30	6.25	7.8	8.0	8.7	0.8	1.15	2.1	1.15	0.8	0.7	0.8	0.9



Fig(10): Effect of antifungal LAB on TCA-SN content

Trichloroacetic acid soluble nitrogen (TCA-SN):

TCA-NS fraction indicates the intensity degree of proteolysis in cheeses and is a better index of maturity than WSN (Venema et al., (1987). Results in Table (6) and Fig (10) showed that the mean values of TCA-SN during 30 days of ripening were similar for adjunct cultured cheeses. The variability found is predictable due to the diversity of LAB and rennet enzymes of each particular cheese, which are responsible of the gradual breakdown of high and medium molecular mass peptides and caseins into lower molecular mass peptides and amino acids.

The nitrogen content of the larger peptide underwent a great increase during ripening showing values higher in the cheese after 30 days of ripening than those at the beginning of the manufacturing process. This high content

at the end of the ripening time of the cheeses, an important factor in the development of cheese texture, makes evident the contribution of the rennet activity to this fraction.

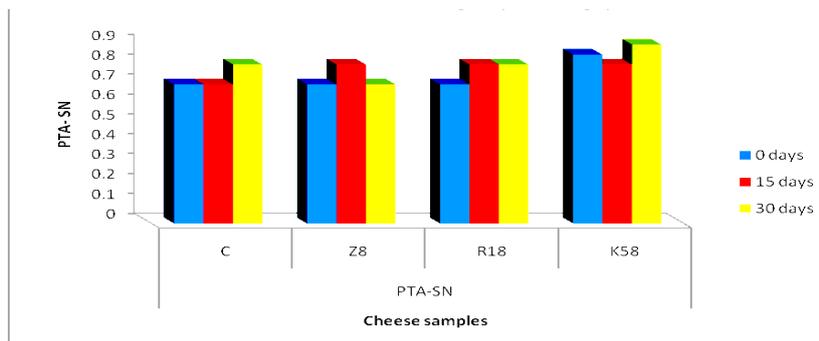


Fig (11): Effect of antifungal LAB on PTA-SN cont on cheese during period

Phosphotungstic acid soluble nitrogen (PTA-SN):

Phosphotungstic acid is a very discriminating protein precipitant; only free amino acids (apart from lysine and arginine), and peptides less than about 600 Da are soluble in 5% PTA (Jarrett et al, 1982). PTA -soluble N has been used widely as an index of free amino acids in cheese (Wilkinson et al, 1992; Guinee et al, 1995).

The development of 5% PTA-soluble N in Ras cheeses of the present study is shown in Table (6) and Fig.(11). Experimental cheeses made with adjunct antifungal cultures (*Pediococcus acidilactis*, R18) and *Lactobacillus paracasei ssp paracasei* (K58) demonstrated slightly higher PTA-SN content than the control cheese. This trend may be due either to increase peptidase activity in cheeses made with adjunct or reduced utilization of free amino acids (FAA) by adjunct cultures (Lynch et al., 1996)

Taken into account, these results suggest that the adjuncts of the three strains of antifungal-LAB, i.e. *Lactococcus* (*Lactococcus lactis ssp lactis*, Z8), *Pediococcus* (*Pediococcus acidilactis*, R18) and *Lactobacillus paracasei ssp paracasei* (K58) cultures had positive impact on cheese quality.

Effectiveness of selected anti-fungal LAB on mould growth on cheese.

This part of study evaluate the potential of the antifungal activity of selected LAB strains (*Lactococcus lactis ssp lactis*, Z8; *Pediococcus acidilactis*, R18 and *Lactobacillus paracasei ssp paracasei* (K58) as cheese preservatives against airborne moulds on Ras cheese during 2-months ripening period. The design of the study was demonstrated in section of Materials and Methods.

Antifungal activity of LAB against air borne moulds:

It has been previously reported that *Lactococcus lactis ssp lactis*, Z8; *Pediococcus acidilactis*, R18 and *Lactobacillus paracasei ssp paracasei* K58 showed antifungal activity against all indicator moulds (Maha et al., 2015). In current study, antifungal activity of selected strains against airborne mould contaminations of cheeses was examined. The current study shows that

antifungal LAB (Z8, R18 and K58) exhibited antifungal activity against airborne mould contaminations. Comparing with the spoilage rate of the control cheese, addition of antifungal LAB as adjunct culture in cheese manufacturing retarded, to varying degrees, the outgrowth of airborne fungi (Table 7). When these strains were included as adjunct cultures in cheese, the fungi growth on cheese was retarded up to the end of the ripening period (2 months) which was 45 days longer than the control cheese (containing no adjunct). In the control cheese, the fungi was observed first after 15 days and the intensity of fungi growth increased with the progress in ripening period. Many studies demonstrated to the antifungal effect against mould growth in cheese. Pawlowska (2013) revealed that, the shelf life of cheddar cheese contained *L. amylovorus* DSM 19280 strain when challenged against airborne moulds, was up to 18 days while control cheese spoiled after 12 days. Similarly, the same observation was found by Zhao (2011) who used some strains of Lactobacilli (NB, DC2 and SDR) as antifungal against the growth of moulds on Cheddar cheeses. They found that, cheeses with added antifungal strains extended the shelf life between 2 to 4 days compared to the spoilage time of the control cheese. Also, Cheong et al., (2014) indicated potential for the control of spoilage mould in cheese products. The cottage cheese inoculated with antifungal LAB, *Lb. plantarum* isolates did not show sign of mould growth until at least day 18 with some cheese not showing any visible mould at day 29. Of note are two *Lb. plantarum* strains which are able to prevent visible mould growth beyond day 29, the last time-point of experiment.

Table (7): Inhibition of airborne growth by antifungal LAB in Ras cheese.

Cheeses	Ripening period (days)			
	0	15	30	60
Control	-	1 spot	3 spot	5 spot
Z8	-	-	-	1 spot
R18	-	-	-	5 spot
K58	-	-	-	2 spot

CONCLUSION

These results revealed to the importance of traditional fermented dairy products as a conserve for LAB. The biodiversity of LAB in traditional fermented milks make researchers to isolate new species of LAB that have technological and health effects, also with high feasibility as bioprotective agent against microbial spoilage of dairy food.

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استنباط سلالات من بكتريا حمض اللاكتيك مقاومة لنمو الفطريات على اسطح الجبن.

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تم عزل ٩ سلالات بكتيرية من بكتريا حمض اللاكتيك ذات المقاومة الواسعة لنمو الفطريات على اسطح الجبن، واجريت بعض المعاملات على هذه السلالات تحت الظروف التصنيعية للجبن بغرض استخدامها في زيادة القدرة الحفظية للجبن. تم اختيار ثلاث سلالات من هذه البكتريا من حيث قدرتها على انتاج حمض اللاكتيك وتحلل البروتين بالإضافة الى قدرتها العالية لمقاومة نمو الفطر على اسطح الجبن. وقد وجد أن هذه البكتريا ذات قدرة متوسط على انتاج حمض اللاكتيك وتحلل البروتين مما يمكن من استخدامها كبادئات أو ميكروبات مضافة مع البادئ (مساعدته). كما تم دراسة تأثير الحموضة والملح والحرارة على نمو هذه السلالات، ووجد أن هذه السلالات تنمو بدرجة جيدة عند 5.5 – pH 6، ٣- ٥% ملح ودرجة حرارة ١٠ – ٣٧ °م.

وقد استخدمت هذه السلالات في صناعة الجبن الراس ووجد أن الجبن الناتجة ذات تركيب مشابه للتركيب الطبيعي للجبن الراس وأدى استخدام هذه السلالات الى تحسين صفات التسوية للجبن. كما ادى استخدام هذه السلالات الى تأخر نمو الفطر على اسطح الجبن لمدة ٦٠ يوما مقارنة ب ١٥ يوما في الجبن الكنترول.