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THE PROTECTIVE ACTION OF BACTERIOCINS PRODUCED BY SELECTED STRAINS OF LACTIC ACID BACTERIA AGAINST COMMON PATHOGENIC BACTERIA IN DOMIATI CHEESE

Abd El-Rahman A. Ali1, Ahmed M. Metwally[a], Hadil F. Atia[b]*, Amal H. Mahmoud[b], Hisham M. El Shishtawy[c]

[a] *Dairy Department, Faculty of Agriculture, Cairo University, Giza, Egypt.*

[b] *Special Food and Nutrition Research Department, Food Technology Research Institute, Agricultural Research Center, Giza, Egypt.*

[c] *Microbial Molecular biology department, AGERI, Agricultural Research Center, Giza, Egypt.*

Abstract

Bacteriocins are ribosomal-synthesized peptides produced by Lactic acid bacteria which can kill other bacteria. The present study aimed to study the effect of bacteriocin produced by three different Lactobacillus strains, (*Lactobacillus helveticus*, *Lactobacillus rhamnousus* and *Lactobacillus acidophilus*) as a natural bio preservative in Domiati cheese. The chemical composition, antioxidant and antibacterial activity was determined during room temperature storage for 3 months which affected the quality of cheese and accelerated its spoilage. The highest antioxidant and antibacterial activity produced by *Lb. helveticus* followed by *Lb. rhamnousus*. The moisture content of all cheese treatments showed a significantly decreased ($p < 0.05$) during ripening due to the biochemical changes after 3 months. All cheese treatments exhibited higher acidity than the control. Fat content in all treatments significantly increased throughout the ripening period due to the decrease in the moisture content and there were no significant differences between treatments. Water-soluble Nitrogen (WSN) content significantly increased by adding Lactobacillus strains after 3months of storage. Hence, the purified Cell - Free Supernatant (CFS) from *Lb.helveticus* at a concentration of 2.5µg/ml, which was more effective than other strains, would be used as a protective culture to delay the growth of pathogenic bacteria, particularly *S. typhimurium and L. monocytogenes* in Domiati cheese.

Keywords: Domiati cheese; Bacteriocin; Lactobacillus strains; Pathogenic bacteria; Bio preservation.

1. Introduction

Dairy products are considered one of the most important media suitable for the growth of most pathogenic microorganisms. Staphylococcus aureus, Salmonella spp., Listeria monocytogenes and Escherichia coli (E. coli) are the most detected pathogens associated with milk and dairy products. Cheese is one of the most common foods over the world. Outbreaks due to consumption of cheese contaminated with pathogenic bacteria and their toxins have the most importance of public health and economic consequences [1].

Cheese has been considered as a better probiotic carrier compare to other fermented milk products. Cheese characteristics like pH, higher content of fat and solid consistency matrix offer greater protection to these cultures in the gastrointestinal tract (GIT) [2]. Lactic acid bacteria (LAB) have been used in the fermentation of foods, for flavor and texture, and also for their ability to inhibit the growth of pathogenic bacteria. The preservation effect of cheese results from the metabolites, such as lactic acid and hydrogen peroxide, that produced by LAB

Corresponding author.E-mail: fouad.hadil@gmail.com (Hadil F. Atia)

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as well as the antimicrobial action of bacteriocins [3].

Most of the LAB are Generally Recognized as Safe (GRAS), granted by the American Food and Drug Agency (FDA). The European Food Safety Authority (EFSA) also granted the Qualified Presumption of Safety (QPS) status to most of the LAB genera. Bacteriocins, produced by the LAB have been proposed and even successfully applied as a natural antimicrobial alternative in food preservation [3].

Bacteriocins are ribosomal synthesized and extracellularly released as bioactive peptide or peptide complexes, which have a bactericidal or bacteriostatic effect on other species. Bacteriocins may have a narrow spectrum, by inhibiting bacteria taxonomically close, or a broad spectrum, by inhibiting wide variety of bacteria. The use of bacteriocins as natural food preservatives fulfills consumer demands for high quality and safe foods without the use of chemical preservatives [4].

Listeria monocytogenes is a psychrotrophic microorganism that can grow at temperatures ranging from 1 to 45 °C. It is highly salt-tolerant and can initiate growth at a relatively low pH. These characteristics make L. monocytogenes particularly difficult to control in food, and its presence leads to a high-risk factor. Consumption of contaminated food was directly linked to several cases of listeriosis [5]. Domiati cheese is the most popular soft white pickled cheese in Egypt. It is consumed fresh or after 3 - 6 months of ripening period in pickling solution [6]. Traditional Domiati cheese had been made from raw milk for a long time; thus, different groups of microorganisms are present in the cheese, some of which participate in flavor and texture development and some of which may be pathogenic or cause defects in cheese. Pasteurization of milk is recommended before cheese making to improve the hygienic quality of cheese. This has a negative effect on the natural flora present in raw milk and changes some of the physicochemical properties of the milk. [7] studied the addition of starter culture on texture and flavor of Domiati cheese during pickling.

Fresh cheeses are particularly sensitive to colonization by *L.monocytogenes* through post-process contamination. Due to the relatively high pH and water activity allowing the growth of this microorganism during cheese storage at 4°C, fresh cheese deserves particular attention from a hygienic/ safety perspective. One of the approaches used to prevent the growth of undesirable microorganisms in food is the use of bacteriocins or bacteriocin producing lactic acid bacteria (LAB). A large number of bacteriocins possess an anti-listerial activity and many of them have been applied to the control the growth of *L.monocytogenes* in cheeses [8].

Therefore, the objective of this research was to study the protective action of selected bacteriocins from some Lactic Acid Bacteria strains in Domiati cheese during the storage period.

2. Experimental

2.1. Materials

Buffalo's milk and buffalo's skim milk were obtained from Animal Production Research Institute, Agricultural Research Center (ARC), Ministry of Agriculture, Dokki, Egypt.

Lactobacillus helveticus CH 5 were obtained from Chr. Hansen, while *Lactobacillus acidophilus* 20552 ATCC and *Lactobacillus rhamonusus* GG (ATCC 53103), *Bacillus cereus* ATCC 6538, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 9027, *Escherichia coli* ATCC 2592, *Listeria monocytogenes* (ATCC 7644) were obtained from Cairo Microbiological Resources Centre (Cairo, MIRCEN), Faculty of Agriculture, Ain Shams University -Egypt.

Nutrient agar and broth were obtained from Difico, Sparks, Maryland (USA). and MRS agar and broth was purchased from Biolife- Itali

2.2 Methods

2.2.1 Manufacture of Domiati cheese.

Dommati cheese was manufactured from standardized buffaloes' milk (4%fat) according to [1] as follows: Standardized buffaloes' milk (4%fat) is heated to 80°C, then cooling to 40°C and the salt is added (7%). 0.02% of Calcium chloride was added. The milk was divided into 7 parts: the first part was a control (without starters) the other parts each one was inoculated with 2% for litre of prepared starter culture as follows: T1 (*Lb. rhamonusus*. (a), T2 (*Lb. acidophilus* (b), T3 (*Lb. helveticus*. (c), T4 (a+b), T5 (a+c) and T6 (b+c). inoculated milk was held for 45 min before the addition of rennet (0.2g/L). leave it until coagulating. Filling the obtained cheese in plastic containers for whey drainage. Then cutting into cubes and packaging in plastic bags in its pasteurized whey then kept at room temperature for 90 days. All cheese treatments were sampled and analysed when fresh and after one, two, and three months.

Cheese was stored for three months. All cheese treatments were sampled and analysed when fresh and after one, two, and three months.

2.2.2 Preparation of the Water-soluble extract of Domiati cheese.

The water-soluble extract was obtained from cheese by extraction according to [2] 10 g of a cheese sample was homogenized in 10 mL in distilled water, then the

homogenate was centrifugated at 6000 g/20 min at 5ºC. The soluble extract was filtered through a Whatman 42 and the resultant extract was kept at – 20 ºC.

2.2.3 Cell-free culture supernatant preparation

Cell-free supernatant (CFS) was obtained by centrifugation of water-soluble extract at 8000 ×g for 20min at 4 °C. The supernatant was then sterilized by filtration through a 0.2-µm syringe filter (Millipore, Bedford, MA, USA).

2.2.4 Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) is the lowest *Lactobacillus* spp. concentration that shows no visible growth. The MIC test was conducted using broth-dilution technique as per standard ISO 10932/IDF 233. According to [3].

2.2.5 **Chemical composition of cheese.**

Cheese moisture, fat, total solids (T.S), total protein content (TP), and titratable acidity (T.A %) were determined according to [4] Water soluble nitrogen (WSN) and total nitrogen (TN) were determined according to [4].

2.2.6. Sensory evaluation of cheese.

Organoleptic properties of cheese samples were assessed by 10 members of Dairy Department Fac. of Agri., Cairo Univ. and Special Foods Department, Agric. Res. Center, Ministry of Agric., Egypt according to [5].

2.2.7. Measurement of antioxidant activity

DPPH radical assay

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity of WSE of samples either fresh or during storage was determined according to [6] with some modifications**.** 1.5 mL of sample (at concentration of 2mg/ml) was mixed with 1.5 mL of 0.1 mM DPPH in ethanol. The mixture was left in the dark for 30 min at room temperature then the absorbance at 517 nm was measured.

Radical scavenging activity% = (A0 - A1 / A0) *100

Where A0 is the absorbance of the control and A1 is the absorbance of the water-soluble extract containing bacteriocin.

2.2.8. The study of antibacterial properties

Antibacterial analysis of bacteriocin.

The inhibitory effects of partially purified CFS were studied by microscale optical density assay (MODA). According to [7] and [8]). The % inhibition were calculating using the following formula

$$\% \text{ inhibition} = \frac{\text{control OD - sample OD}}{\text{control OD}} \times 100$$

2.2.9. Study of Molecular weight of bacteriocin.

Sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE)

An overnight culture was precipitated. After centrifugation (10000 rpm, 10 min, 4°C), the pellet was air-dried. *Lactobacillus* proteins were analysed by (SDS-PAGE) according to [9].

2.3. Statistical analysis

Duncan´s Multiple Range test (SPSS statistics 26) were used to analyse all the data.

3. Results and discussion

3.1 Chemical composition

Moisture content is presented in table 1. The moisture content of all samples significantly decreased by the storage period, this is due to the biochemical changes after 3 months of storage [10] are in an agreement with these results where they are found that all cheese samples showed a gradual loss of moisture over the ripening period. There were significant differences among cheese treatments during storage, the moisture content varies based on the Lactobacillus strain used. This decrease was caused by the acidity development during the storage period.

Changes in titratable acidity of cheese made with different lactobacillus strains are shown in Table (1). It is noted that, TA gradually increased during ripening periods. All cheese treatments showed higher acidity values than the control cheese. There were slight differences among fresh cheese treatments, and became significant after 8 weeks. Significant differences between cheese treatment in acidity were related to the growth rate of the added *Lactobacillus* strains and their abilities to ferment lactose during the ripening period. These results are in agreement with [11] and [12] where they showed that acidity of all cheeses increase with ripening.

Table (1) shows cheese fat content in all treatments. These results showed that fat content increased in all cheese treatments as the percentage of moisture decreases during the ripening period. There were no significant differences between treatments. These results agree with [11] and [12]

Data of the WSN of cheese treatments is shown on Table (1). WSN of fresh cheese samples ranged from 0.17 to 0.25%. WSN increased in all cheese treatments during the pickling period. This was related to the rate of proteolysis throughout the storage period. The WSN content significantly increased by adding Lactobacillus strains after 90 days of storage especially T1, T5 and T6. This increase could be due to the activity of peptidases and proteinases released from *Lactobacillus* strains. These results agreed with [11]

Table 1.
Chemical composition of Domiati cheese as affected by different *lactobacillus* strains and storage period

	Moisture %	Titratable Acidity%	Fat	WSN%
Storage period (days) Fresh				
Cont	72.0 ^{m,n} ± 2.8	0.077^{a,b}±0. 006	10.1^{a,b}±0. 5	0.176^{c}±0 .001
T1	71.76 ^{m} ±2.3	0.14 ^{b,c,d,e}±0.015	9.7^{a}±0.34	0.12 ^{b} ±0.001
T2	63.4^{g,h,I,j,k} ±3.2	0.13 ^{a,b,c,d,e} ±0.01	10.5 ^{d,e} ±0.3	0.175 ^{c} ±0.005
T3	62.5^{j,k,l} ±3.06	0.136 ^{b,c,d,e} ±0.015	10.3 ^{f,g} ±0.3	0.27^{f}±0. 03
T4	69.9^{k,l,m}±1 .12	0.06^{a}±0.01 5	10.1 ^{a,b} ±0.6	0.2 ^{e,f} 5±0.015
T5	63.5 ^{g,h,I,j,k} ±2.7	0.15 ^{c,d,e} ±0.03	10.6 ^{b,c} ±0.2	0.24 ^{e,f} ±0.035
T6	66.06 ^{j,k,l} ±0.58	0.16 ^{c,d,e,f,g} ±0.01	11.1^{c,d}±0. 5	0.187 ^{c,d} ±0.01
Storage period (30 days)				
Cont	66.06 ^{e,f,g,h} ±0.5	0.067^{a}±0.0 15	11.3^{d}±0.3	0.085^{a}±0 .002
T1	64.5^{h,I,j,k,l} ±1.3	0.156 ^{c,d,e} ±0.0153	12.5^{f,g}±0. 3	0.156 ^{b,c} ±0.0015
T2	60.6^{e,f,g,h,}± 4.6	0.16 ^{c,d,e,f} ±0.006	12.4 ^{f,g} ±0.2	0.2^{d,e}±0. 01
T3	60.8^{h,I,j,k,l}± 4.55	0.15 ^{c,d,e} ±0.012	12.5 ^{f,g} ±0.2	0.19 ^{c,d} ±0.017
T4	61.06^{h,I,j,k} ±2.9	0.1 ^{a,b,c,d} ±0.015	12.1^{f}±0.3	0.19 ^{c,d} ±0.018
T5	54.3 ^{a,b,c} ±1.1	0.1 ^{a,b,c} ±0.02	12.03 ^{e,f} ±0.5	0.187 ^{c,d} ±0.01
T6	65.47^{i,j,k,l} ±1.3	0.28^{i}±0.1	11.4^{d}±0.5	0.68^{j}±0. 03
Storage period (60 days)				
Cont	60.6^{c,d,e,f,g} ±0.76	0.15 ^{c,d,e} ±0.012	12.9 ^{g,h,I,j} ±0.5	0.176^{c}±0 .001
T1	62.6^{b,c,d,e,f} ±2.6	0.28^{i}±0.10 4	12.8^{g,h}±0. 3	0.525 ^{h} ±0.003
T2	56.3^{b,c,d,e} ±5.12	0.2^{f,g,h,i} ±0.025	12.4 ^{f,g} ±0.8	0.5^{h}±0.0 4
T3	55.16^{a,b,c,d} ±4.38	0.17 ^{e,f,g,h} ±0.005	13.3 ^{h,I,j} ±0.2	0.25 ^{e,f} ±0.016
T4	56.4^{a,b}±1. 05	0.14 ^{c,d,e} ±0.015	12.3 ^{f,g} ±0.12	0.25 ^{e,f} ±0.018
T5	51.8 ^{a,b} ±1.2	0.2 ^{h,i} ±0.03	12.4 ^{f,g} ±0.15	0.34^{g}±0. 015
T6	56.72^{b,c,d}± 3.7	0.25^{i}±0.04	12.4 ^{f,g} ±0.12	0.5^{h}±0.0 08
Storage period (90 days)				
Cont	58.6^{l,m,n} ±0.76	0.17^{d,e,f,g,h}± 0.005	13.2 ^{h,I,j} ±0.2	0.23 ^{d,e} ±0.02
T1	59.8 ^{d,e,f,g} ±0.9	0.24^{i}±0.02 5	13.5^{i,j}±0. 3	0.54^{h}±0. 017
T2	50.86^{a,b,c}± 1.16	0.24^{i}±0.01 5	13.6^{j}±0.2	0.6^{i}±0.0 25
T3	57.33^{c,d,e,f} ±1.05	0.2^{f,g,h,i} ±0.025	13.4 ^{h,I,j} ±0.3	0.25^{e,f}±0. 04
T4	52.5^{a,b,c} ±1.69	0.2 ^{f,g,h,i} ±0.03	12.8 ^{g,h,i} ±0.3	0.36^{g}±0. 018
T5	53.03^{a,b,c,}± 0.72	0.2 ^{g,h,i} ±0.05	13.4 ^{h,I,j} ±0.	0.25 ^{e,f} ±0.02
T6	50.3 ^{a,b} ±1.006	0.29^{i}±0.0 3	13.3^{h,I,j}± 0.12	0.59^{i}±0. 046

The data values are expresed as mean±SD and cells superscript with different letters are significantly different ($p < 0.05$).
C: control cheese made without Lb.

T1: cheese made with 1% *Lb. rhamonusus*
T2: cheese made with 1%*Lb. acidophilus*
T3: cheese made with 1%*Lb. helveticus*
T4: cheese made with *Lb. rhamonusus* and *Lb. acidophilus*
T5: cheese made with *Lb. rhamonusus* and *Lb. helveticus*
T6: cheese made with *Lb. acidophilus* and *Lb. helveticus*

3.2 Antioxidant activity

Results of antioxidant properties of cheese treatments are shown in Fig. (1). The antioxidant activity (as radial scavenging activity %) was measured by using the DPPH method that it is ranged from 33.6% to 75.3%. Addition of Lactobacillus strains had a significant effect on the scavenging activity of DPPH radical of cheeses during ripening which was higher than that of control cheese.

The antioxidant activity of *L. helveticus* CH5 had the highest DPPH scavenging activity, followed by *L. acidophilus* 20552 ATCC and *L. rhamnosus* GG. The highest value of antioxidant was obtained from *Lactobacillus helveticus* strain. The activity of antioxidant increased significantly with the increase of storage period. The effect of *Lactobacillus helveticus* strain on antioxidant was decreased when combined with another lactobacillus strain. These results agreed with [13] and [14].

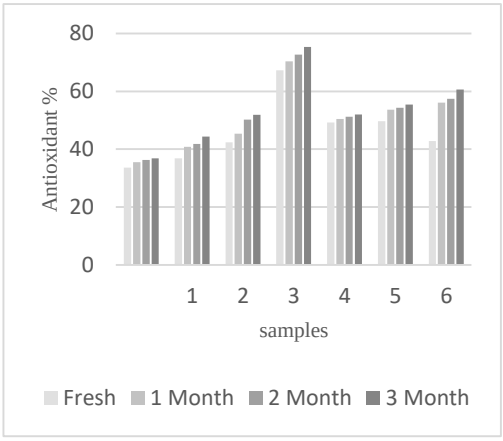

Fig.1. Antioxidant % of Domiati cheese as affected by different lactobacillus strains and storage period.

3.3 The antibacterial effect of Lactobacillus strains

The inhibitory effects of the purified CFS were related to bacteriocin. It is sure that almost all CFS of bacteriocin from different *Lactobacillus* strains had antibacterial effect against the tested pathogenic strains. Generally, all CFS concentrations had antibacterial effect on *E. coli, B. cereus, Staph. aureus, Sal. typhimurium and L. monocytogenes.* At a concentration of 2.5µg/ml CFS from *Lb. helveticus*

showed 90% inhibition of pathogenic bacteria at 30 days and 98% at 90 days, followed by *Lb. rhamnosus* and Lb. acidophilus with no significant differences between all samples. In all samples % inhibition increased during storage of Domiati cheese. Antibacterial activity of the two different concentrations (1.5 and 2.5 µg/ml) of Cell-free supernatant (CFS) on pathogenic bacteria are presented in fig. 2. And 3.

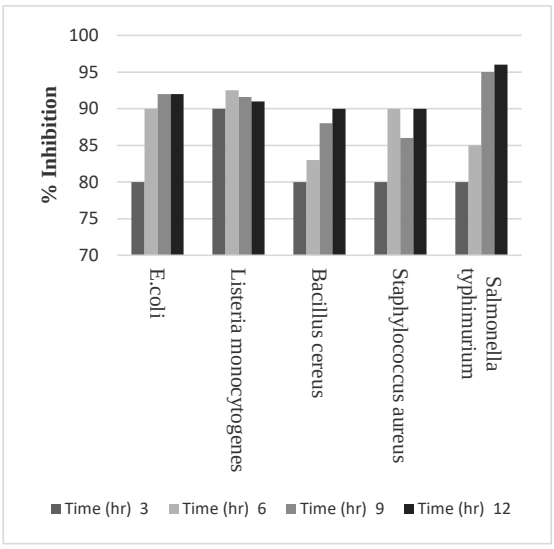

Fig.2. a. Inhibition of 1.5µg/ml CFS of *Lb. helveticus*

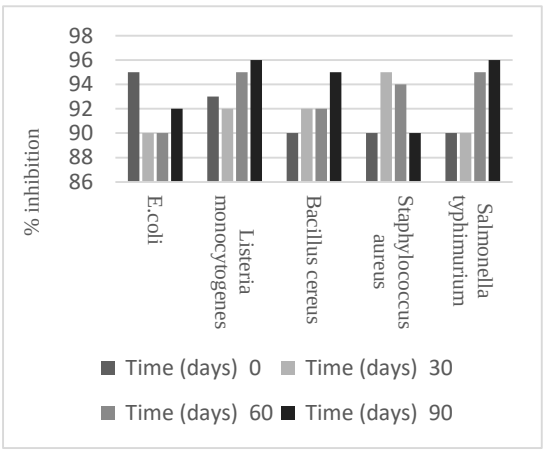

Fig. 2.b. % inhibition of 1.5µg/ml GFS of Lb.rhamnous

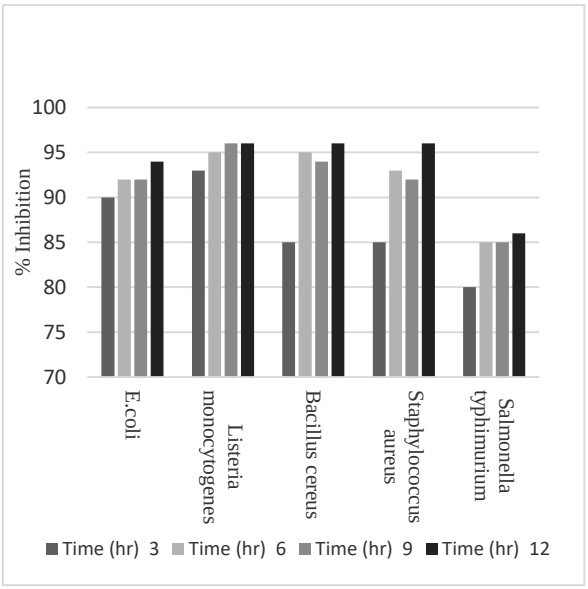

Fig. 2. c. % Inhibition of 1.5µg/ml CFS of Lb.acidophilus

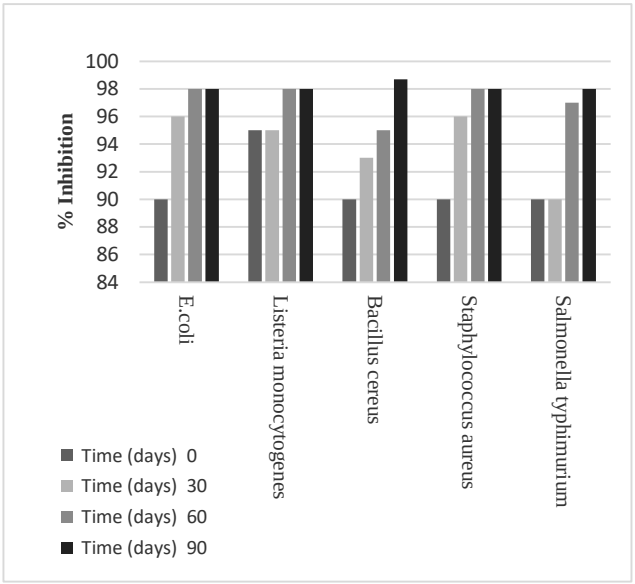

Fig.3. a. % Inhibition of 2.5µg/ml CFS of *Lb.helveticus*

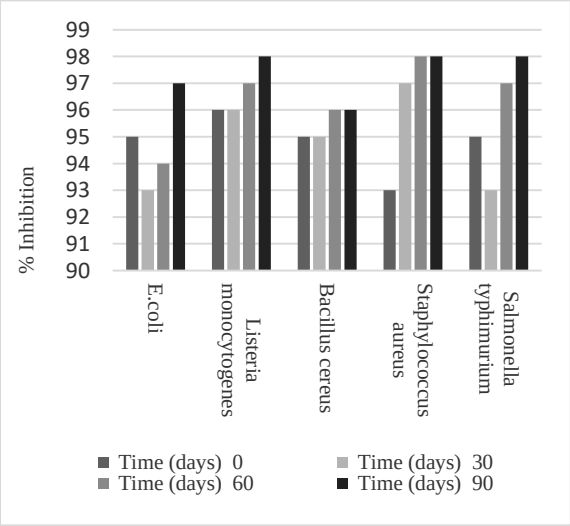

Fig. 3. b. % Inhibition of 2.5µg/ml CFS of *Lb rhamnous*

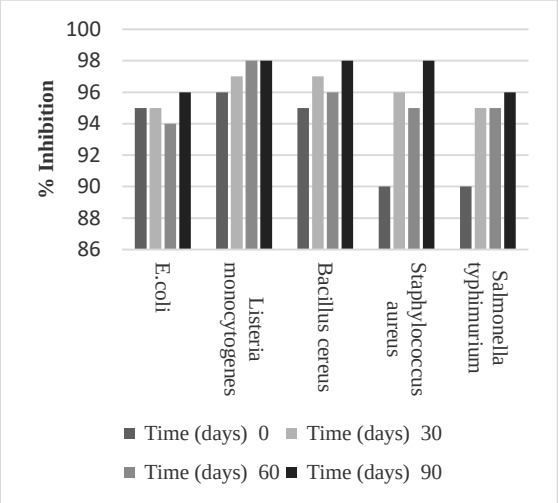

Fig.3. c. % Inhibition of 2.5µg/ml CFS of *Lb. acidophilus*

3.4 Molecular Weight of Bacteriocins.

The molecular mass of Bacteriocins from *Lactobacillus* spp. was evaluated by SDS-PAGE alongside molecular weight marker proteins. Purified phages particles were subjected to SDS-PAGE electrophoresis analysis and proteomic patterns were obtained after Coomassie staining and destining (Figure 4). The purified bacteriocin of *L. helveticus* gel showed a molecular weight of approximately 2 kDa on SDS-PAGE gel. A major protein band at 8 kDa was found in *L. acidophilus.*

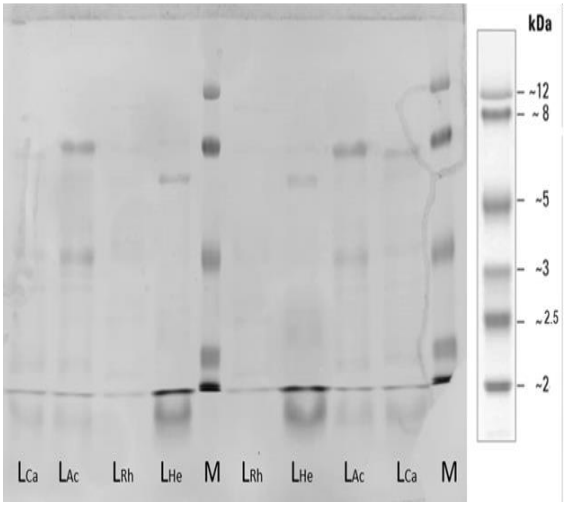

Fig.4: Coomassie Brilliant blue-stained 12% SDS-PAGE showing the Protein banding pattern of different lactobacillus isolates, lane M: protein molecular weight marker.

3.5. MIC of Lactobacillus strains

The minimum inhibitory concentration (MIC) of *Lactobacillus* strains against Gram - positive and negative pathogenic bacteria were determined and the results are shown in table 2. It ranged from 50 to 120 mg/ml., whereas [3] reported that MIC ranged from 50 to 128 mg/ml. [15] reported that the growth of gram-positive pathogens such as *B. cereus, L. monocytogenes* was significantly inhibited by bacteriocin of *lactobacillus acidophilus* NX2-6 (NX371) which was ranged from 20 to 160 mg/ml.

Table 2.
Minimum inhibitory concentrations MIC (mg/ml) of lactobacillus strains

Bacterial Strain	*E.coli*	*S. typhii*	*S.aureus*	*L.monocyto -genes*
	MIC (mg/ml)			
Lb. acidophilus	75	100	50	50
Lb. helveticus	50	50	75	100
Lb. rhamonus	50	50	50	50

3.6 Sensory evaluation

All fresh cheese samples had good body and texture with no significant differences. using of different types of *Lactobacillus* strains in cheese manufacture can contributes to improving flavour

and texture of Domiati cheese during storage. The results showed that, after 90 days of storage, the scores of all the treated cheese were higher than the control. During the ripening of cheese, proteolysis plays most important role in flavour development. The body and texture, and appearance followed the same trend as the flavour. Total scores show that *lb.rhamnosus* have the highest acceptability followed by *lb. helveticus*, in all samples the organoleptic properties increased during the early stage of storage. These results are in agreement with those obtained by [16].

4. Conclusions

Our results revealed that *Lb.helveticus* would be used as a protective culture to delay the growth of pathogenic bacteria, particularly *S. typhimurium* and *L. monocytogenes* in Domiati cheese. At a concentration of 2.5µg/ml CFS produced by *Lb. helveticus* showed an inhibition effect of 90% after 30 days and 98% at 90 days, followed by *Lb. rhamnosus* and *Lb. acidophilus*. *lb.rhamnosus* showed the highest acceptability for organoleptic properties followed by *lb. helveticus.* This finding ensures that *Lb.helveticus* can be used as a protective culture where it showed the highest value of antioxidant and antibacterial activity during storage of Domiati cheese.

5. Acknowledgments

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