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Utilization of Cyanobacteria Extracts in Improving the Microbial Quality of Soft White Cheese

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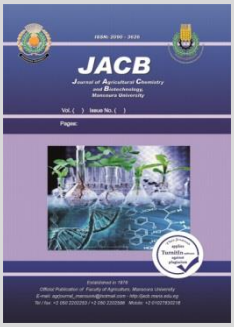

ABSTRACT

The current study was evaluated to determine the prevalence of *Escherichia coli* and *Staphylococcus aureus* in raw milk in addition to some dairy products which are sold in Dakahlia Governorate, Egypt. Hundred and fifty samples including raw milk, soft cheese (Talaga cheese), kariesh cheese; Ras cheese (Romy), ice cream and yoghurt. (25 samples from each product) were collected and then subjected to bacterial examination. In order to identify recovered isolates, an array of biochemical and polymerase chain reaction (PCR) was used. Our results detected that *E. coli* was noticed in 68%, 56%, 88%, 68%, 52% and 80% of raw milk, soft cheese (Talaga cheese), kariesh cheese; Ras cheese (Romy), ice cream and yoghurt, respectively. The prevalence of *S. aureus* was detected in 52%, 88%, 80%, 76%, 28% and 52%, respectively. The results showed that the aqueous extracts of the cyanobacteria such as *Sprirulina plantensis* and *Arthrospira fusiformis* were evaluated for their antibacterial activity by inhibiting the growth of *E. coli* and *S. aureus*. The soft cheese treated with cyanobacteria extract and wrapped with banana leaves showed a potentially reduction of bacterial contamination than that wrapped with plastic material. The results proved that the using these species of cyanobacteria could be used a good source for the production of promising antibacterial agents.

***Keywords*: *E. coli*, *S. aureus*,** Milk, Dairy products, Cyanobacteria , banana leaf.

INTRODUCTION

Milk is an extremely nutritious food. It is an aqueous colloidal suspension of proteins, fat and carbohydrates that contains numerous vitamins (Sangoyomi *et al.*, 2010). The nutritional richness of milk is a good source of high biological value proteins and important vitamins and essential minerals (Pereira, 2014). Soft white cheese is considered a common delicious cheeses manufactured and consumed in Egypt. However, the techniques of manufacturing and handling this cheese in Egyptian markets are still primitive and unhygienic (Sadek *et al*., 2009). Kariesh cheese is one of the most popular local types of fresh soft cheese in Egypt. There is a growing demand by Egyptian consumers due to its high protein content and low price (Osman *et al.*, 2010). Ras cheese (Romy) is an Egyptian hard cheese it is made in great amounts under artisan conditions from cows and buffalos milk (Dabiza and El-Deib, 2007 and Hattem *et al.*, 2012). Ice cream is a comparatively, easily digestible, well-balanced, healthy, delicious food and it has about four times as much carbohydrates such as in milk (Deosarkar *et al*., 2016). Yoghurt is an excellent basis of protein of high biological worth that contains all essential amino acids (Mckinley, 2005).

The main causes of the Contamination of milk and milk products are the human factor and lack of hygienic conditions. Milk is often contaminated with many kinds of microorganisms at milk-collecting places (Soomro *et al.*, 2002). The most common spoilage micro-organisms in milk and dairy products are *Pseudomonas*, coliforms, *Bacillus* spp., *Clostridium* spp., lactic-acid producing bacteria, yeasts and molds, enterococci, etc. (Torkar and Teger, 2006). *Escherichia coli* (*E.coli*) is the gram negative bacterium that present in human intestine. *E. coli* commonly contaminates the food and it is a good pointer of fecal contamination (Benkerroum *et al.*, 2004). *Staphylococcus aureus* (*S. aureus*) is the gram positive bacterium that generally responsible for post-operative wound contaminations, endocarditis, osteomyelitis, and food poisoning and toxic shock syndrome (Benayache *et al*., 2001). The polymerase chain reaction (PCR) is a tool to identify microorganisms. A scientific technique in molecular biology is to amplify one or a small number of copies of a piece of DNA across several orders of magnitude, to generate thousands to millions of copies of a particular DNA sequence (Joshi and Deshpande, 2011). Seaweeds, particularly cyanobacteria, are regarded a likely source of antimicrobial substances because of their diversity of secondary metabolites with antiviral, antibacterial and antifungal activities (Val *et al.*, 2001).

The aim of this study is the evaluation of cyanobacteria extract for controlling *E. coli* and *S. aureus* which contaminate the raw milk in addition to some dairy products sold in Dakahlia Governorate, Egypt.

MATERIALS AND METHODS

1.Collection and preparation of samples:

Hundred and fifty samples of milk and dairy products were collected from retail shops and supermarkets in Dakahlia Governorate, Egypt. These samples include raw milk, soft

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cheese (Talaga cheese), kariesh cheese; Ras cheese (Romy), ice cream and yoghurt (25 samples from each product).

2.Identification and characterization of *E. coli* and *S. aureus*:

Colonies of *E. coli* were recognized based on colonial morphology appeared as distinctive metallic green sheen (Howard, 1994), and biochemical reactions according to MacFaddin (2000). API 20 E, biochemical rapid test, Bio-Mérieux (Fig. 1). Colonies of *S. aureus* were recognized based on colonial morphology appeared as yellow colonies surrounded by yellow halo on mannitol salt agar media (Shittu *et al*., 2006), and biochemical reactions according to Arora (2003). Isolates were examined by Gram's stain (Cruickshank *et al.*, 1975) and identified by PCR for detection of *sau* and *eco* gene for detection *S. aureus* and *E. coli* (Riffon *et al.*, 2001) using the primer sequence outlined in Table (1).

3.Preparation of cyanobacteria extracts:

Soft cheese was manufactured according to Haddadin (2005), and aqueous extracts from two cyanobacteria species were added to cheese during manufacture. These cyanobacteria species were *Spirulina platensis* (Code 2S) and *Arthrospira fusiformis* (Code 2T) and they obtained from the Culture Collection of the Biotech International Research (Abdel-Hamid *et al.*, 2012). The soft cheese treated with cyanobacteria extracts were wrapped by banana leaf (Haddadin, 2005).

Table 1. Primer sets for PCR amplification of *sau* and *eco* genes specific for molecular identification of *S. aureus* and *E.coli*.

Primer name and direction	Nucleotide sequence 5`-3`	Amplicon Size	Reference
Sau 327	GGA CGA CAT TAG ACG AAT CA	1318 bp	Riffon *et al.*, 2001
Sau 1645	CGG GCA CCT ATT TTC TAT CT		
Eco 223	ATC AAC CGA GAT TCC CCC AGT	232 bp	Riffon *et al.*, 2001
Eco 455	TCA CTA TCG GTC AGT CAG GAG		

RESULTS AND DISCUSSION

Results:

In this study, *E. coli* was detected on EMB agar among all milk and dairy products with mean counts of $8.2\times10^6\pm 2.5\times10^6$, $4.1\times10^6\pm1.2\times10^6$, $1.8\times10^7\pm4.5\times10^6$, $1.4\times10^6\pm 3.9\times10^5$, $1.5\times10^5\pm0.6\times10^5$ and $8.1\times10^6\pm2.3\times10^6$ CFU/g or ml in kariesh cheese, Ras cheese, Talaga cheese, yoghurt, ice cream and raw milk samples, respectively (Table 2). *S. aureus* was detected respectively with mean counts of $1.1\times10^6\pm 2.3\times10^5$, $1.1\times10^6\pm 2.3\times10^5$, $1.0\times10^7\pm 2.2\times10^6$, $5.2\times10^5\pm 2.8\times10^5$, $0.3\times10^5\pm 0.2\times10^5$ and $6.7\times10^6\pm 2.5\times10^6$ colony-forming units (CFU)/g or ml among tested kariesh cheese, Ras cheese, Talaga cheese, yoghurt, ice cream and raw milk samples, respectively (Table 3).

Table 2. Total *E. coli* count in raw milk and some dairy products on EMB agar medium.

Milk & dairy products	Total number of samples	+ve sample No.	%	*E. coli* min	max	Mean ± SE	Std. Deviation
Kariesh cheese	25	22	88	1.0×10^4	4.5×10^7	$8.2\times10^6\pm2.5\times10^6$	1.2×10^7
Ras cheese	25	17	68	2.0×10^4	1.7×10^7	$4.1\times10^6\pm1.2\times10^6$	5.4×10^6
Talaga cheese	25	14	56	4.0×10^3	7.5×10^7	$1.8\times10^7\pm4.5\times10^6$	2.1×10^7
Yogurt	25	20	80	2.0×10^3	6.7×10^6	$1.4\times10^6\pm3.9\times10^5$	1.8×10^6
Ice cream	25	13	52	2.0×10^2	1.1×10^6	$1.5\times10^5\pm0.6\times10^5$	2.7×10^5
Raw milk	25	17	68	1.0×10^4	3.7×10^7	$8.1\times10^6\pm2.3\times10^6$	1.1×10^7

Table 3. Total S. *aureus* count in raw milk and some dairy products on mannitol salt agar.

Milk &dairy products	Total number of sample	+ve sample No.	%	*S. aureus* min	max	Mean ± SE	Std. Deviation
Kariesh cheese	25	20	80	3.0×10^4	3.2×10^6	$1.1\times10^6\pm2.3\times10^5$	1.2×10^7
Ras cheese	25	19	76	4.0×10^4	3.6×10^6	$1.1\times10^6\pm2.3\times10^5$	1.1×10^6
Talaga cheese	25	22	88	3.0×10^5	3.6×10^7	$1.0\times10^7\pm2.2\times10^6$	1.1×10^7
Yogurt	25	13	52	2.0×10^3	6.0×10^6	$5.2\times10^5\pm2.8\times10^5$	1.3×10^6
Ice cream	25	7	28	4.0×10^2	3.1×10^5	$0.3\times10^5\pm0.2\times10^5$	0.8×10^5
Raw milk	25	13	52	1.0×10^4	3.3×10^7	$6.7\times10^6\pm2.5\times10^6$	1.2×10^7

The development of API-20E system was originally to identify genus and species level. The results of coding strains with the API20E system will be referred to as "API codes" while the results of fermentation tests, hemolysis and motility as "biotypes". Different genera could be easily distinguished from one another and identified using the API 20E (Fig. 1) Analytical Profile Index. This identification is based on Biochemical characteristics using API20E set.

These results were confirmed by PCR detection of *sau* and *eco* genes specific for molecular identification of *S. aureus* and *E. coli* (Figs 2 & 3). The specific oligonucleotide primer sequences were designed towith the aim of amplifying and identifying tested bacteria. Pairs of primers were tested (*E. coli / S. aureus*). Before carrying out PCR, purified DNA was run on 1.5 % agarose gel and quantified by UV absorbance at 260 and 280 nm to confirm affirm its amount and existence in PCR. The specificity of the primer pairs was confirmed by positively amplifying the DNA from bacteria. No observation of amplification in negative controls of PCR mix. These primer sets were designed from DNA regions coding for16SrRNA.

The present study showed that the extracts from *S. platensis* (Code 2S) and *A. fusiformis* (Code 2T) exhibited an antibacterial activity (Figs 4 & 5) and proved that the extracts was active against gram (positive and negative) bacteria. Two cyanobacteria extracts at 10, 15 and 20 ml/L concentrations showed different ranges of the mean of inhibition of bacteria. When the concentration of extracts was increased to 20 ml/L, the increase of inhibitory activity was detected. The highest inhibitory activity was observed with *S. platensis* towards *S. aureus* at a concentration of 15-20 ml/L, and also inhibited by *A. fusiformis* at a concentration of 20 ml/L. High concentration of two extracts at 20 ml/L inhibited *E. coli* growth. The low concentration of both extracts showed a less inhibitory activity against the tested bacterial strains. Antibacterial activity of extracts against *E. coli* is shown in Fig. (4). Comparatively, the extracts were less active towards Gram negative bacteria when

compared with Gram positive ones (Fig. 5). When soft cheese wrapped by banana leaves, it exhibited a lower most probable number of *E. coli* and *S. aureus* than that wrapped by plastic material. These results appeared after two weeks from storage (Figs 4 & 5).

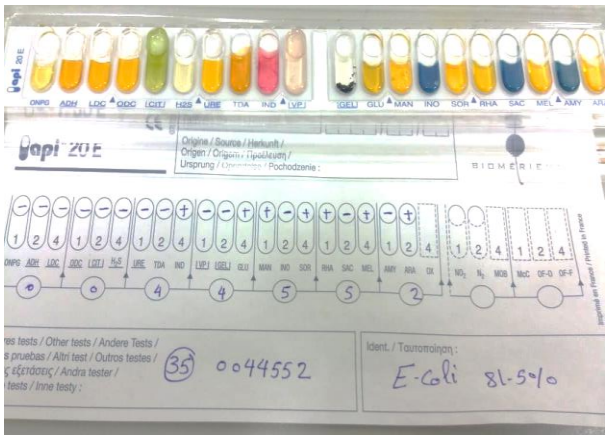

Fig. 1. Biochemical identification of isolates by using API 20E.

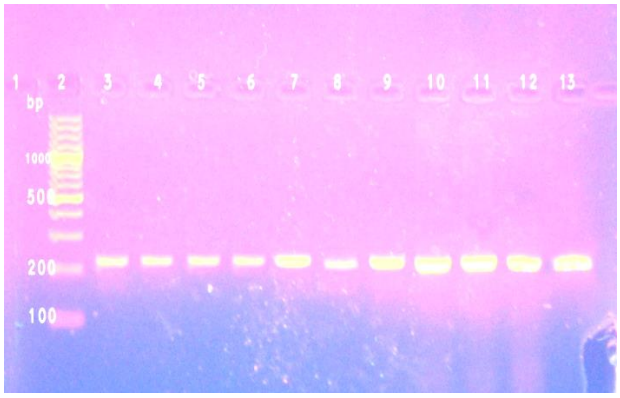

Fig. 2. Agarose gel electrophoresis of PCR amplicons of the marker genes identified in (*E.coli)* isolates from milk Dairy products samples. Amplified bands of the expected sizes of 232bp for *eco* gene. Lane1: control negative, Lane 2: 100-bp DNA ladder Lane 3: control positive (field isolate) and Lanes: 4-13, samples.

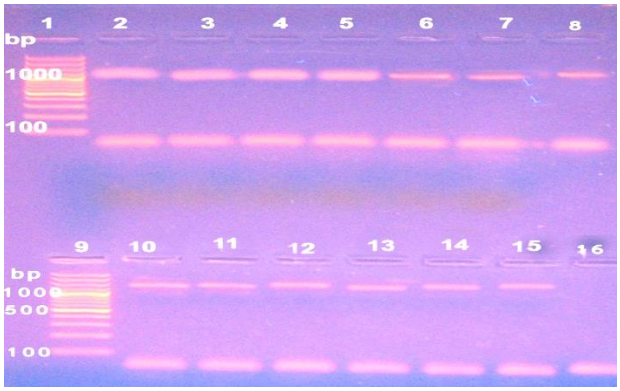

Fig. 3. Agarose gel electrophoresis of PCR amplicons of the marker genes identified in (*S. aureus)* isolates from milk Dairy products samples. Amplified bands of the expected sizes of 1318 bp for *sau* gene. Amplification products were analyzed by electrophoresis on a 1.5% agarose gel. Lanes: 1, 9, 100-bp DNA ladder, Lanes: 2-8, samples, Lanes: 10-14 samples,
Lane: 15: control positive (field isolate) and Lane: 16: control negative.

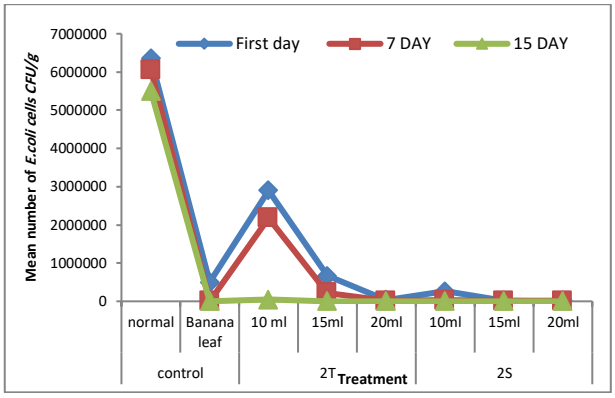

Fig. 4. Effect of some treatments against *E. coli* count as an experimental study on soft cheeses.

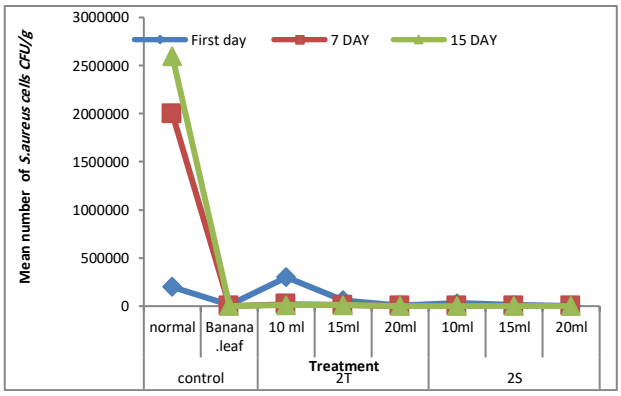

Fig. 5. Effect of some treatments against *S. aureus* count as an experimental study on soft cheeses.

Discussion:

Microbial contamination can occur from different sources as inefficient sterilization, contamination from environment and poor packaging (Fenlon *et al.*, 1995). In the present study, 88% of samples were satisfactory for *E. coli* in kariesh cheese which lies within the range of $10^4 \leq 10^6$. The different rates of isolation between studies is likely because of difference in salt concentration, acidity of kariesh cheese and the keeping temperature (EL-Sayed and EL-Kaseh, 2009). Kariesh cheese which was made from raw milk was often of poor bacteriological quality as it the conditions under which it was produced was unsatisfactory Moreover, this cheese is sold while uncovered, which increases contamination risk. Consequently, it can be regarded as a suitable medium for the different types of spoilage and pathogenic microorganisms to grow (Dawood *et al.*, 2009). In Ras (Romy) cheese, the frequency distribution of *E. coli* was 68% which lies within $10^4 \leq 10^6$. Contamination of ready to eat food is attributed to post-processing contamination due to many reasons such as unhygienic practices during food handling, lack of personal hygiene, unhygienic kitchen, utensils, equipment and packaging (Darwish *et al.*, 2015). The existence of *E. coli* in fresh white cheese indicates low quality milk, poor production practices or after-processing contamination (Tekinsen and Ozdemir, 2006). The percentage of isolated *E. coli* in Talaga cheese was 65% within the range of $10^3 \leq 10^6$. Moisture content of Soft cheese is higher in comparison to hard cheese in addition to its lower shelf life as a result of microbial spoilage. It was found that the majority of soft, unripened cheeses are microbiologically unstable as a result of metabolic activity of bacteria, yeast or mold contaminants

(Haddad and Yamani, 2017). The frequency distribution of *E. coli* in yoghurt was 80%, lying within the range of $10^3 \leq 10^{5.}$ Prevalence of *E. coli* in yoghurt indicated post pasteurization contamination of the cheese whether before or throughout the process of packaging (Omola *et al*., 2014). The results of ice cream samples indicated that 52% of samples were contaminated with *E. coli*, lying within $10^2 \leq 10^5$. Water and raw milk were among the Primary sources of microbial contamination to ice cream whereas flavoring agents, utensils and handling were among the secondary sources (El Khair *et al.,* 2014). A large number of studies agree that ice cream is considered an excellent medium for the growth of many microorganisms (Ahmad *et al.,* 2009). Raw milk acts as an excellent medium for the growth of many microorganisms and the highest frequency distribution of *E. coli* in raw milk was 68%, lying within $10^4 \leq 10^6$. Obviously, existence of *E. coli* in milk may be caused by machines, ways of manual milking in addition to inferior quality of water (Chye *et al*., 2004). Moreover, these results indicated a general absence of hygiene in handling and inadequate storage (Shojaei and Yadollahi, 2008). *E. coli* is the most frequently observed microorganism among the microorganisms causing food intoxications (Hayaloglu and Kirbag, 2007). The frequency distribution of *S. aureus* in kariesh cheese was 80%, lying within the range of $10^4 \leq 10^6$. Presence of *S. aureus* in kariesh cheese indicates that cit contaminated by skin, mouth of dairy workers or handling in additioin to inadequately cleaned utensils or equipment which are likely to be other contamination sources (New Some, 1988). In the present study, 76% of samples were satisfactory for *S. aureus* in Ras (Romy) cheese which lies within the range of $10^4 \leq 10^6$. There was an increase in contamination with *S. aureus*, in traditionally made cow milk cheese, especially at the stage from milk to curd (Jakobsen *et al.,* 2011). In the present study, 88% of samples were satisfactory for *S. aureus* in Talaga cheese which lies within the range of $10^5 \leq 10^7$. Contamination can occur from different source include the poor quality of raw milk and processing in uncontrolled environments (Sameh, 2016). The presence of *S. aureus* in cheese usually indicates contamination of milk from diseased udder or external surface of the dairy animals, or from contaminated, unclean hands of the dairy workers or from their sneezing and coughing (Salem *et al.,* 2016). The maximum incidence distribution of *S. aureus* in yoghurt was 52% which lies within the range of $10^3 \leq 10^6$. Occurrence of *S. aureus* in yoghurt is a common indicator of contamination caused by food handlers' injured hand or arm infected with S. *aureus*, or through coughing and sneezing, which is common during respiratory infection (Hussain, 2010). Frequency distribution of *S. aureus* in ice cream was 28%, lying within $10^2 \leq 10^4$. Microbial contamination can occur from different sources as inefficient sterilization, contamination from environment and poor packaging (Fenlon *et al*., 1995). The highest frequency distribution of *S. aureus* in raw milk was 52%, lying within of $10^4 \leq 10^7$. Existence of *S. aureus* or its enterotoxins in milk indicates poor sanitation as it is damaged by heat treatment and almost all sanitizing agents. *S. aureus* can cause strict food poisoning (FDA, 1998). *S. aureus* is considered one of the most influential opportunistic pathogen among staphylococci belonging to family Micrococaceae resulting in serious infections when appropriate conditions exist (Prescott *et al*., 2002).

The results of this study showed that the cyanobacteria extracts possess an antimicrobial activity by reducing the bacterial number. However; among the bacteria tested, S. *aureus* showed an inhibition in its number at all the studied concentrations. When the concentration level increases, the inhibitory effect also increased which is similar to the findings of Indira *et al.* (2013). Similar results were observed through a study carried out by Chattopadhyay *et al*. (2002). Moreover, in this study the extract of *Spirulina platensis* showed more effective against Gram-positive bacterium (*S. aureus*) when compared to Gram-negative bacterium (*E. coli*). These results are similar to a previous study performed by Afifah *et al.* (2010) who argued that the cyanobacteria extract was more influential against Gram-positive bacteria and less against Gram-negative bacteria. Basically, the differences in the antimicrobial activities might be due to differences in cell walls of the bacteria. The cell wall of Gram-positive bacteria has only one outer peptidoglycan layer. This structure enables other substances to easily permeate through the cell wall. In contrast to Gram-positive bacteria, cell wall of Gram negative bacteria consists of lipopolysaccharides in their outer membrane. These structures make the cell less permeable to other substances (Afifah *et al*., 2010). As reported by Shafay *et al*. (2015), bacterial cell wall of Gram-positive bacteria consists of 90- 95% peptidoglycan and only 5-10% of lipid. This small percentage of lipids results in less rigidity in bacterial cell wall. However, 90-95% of Gram-negative bacterial cell wall consisted of lipid and 5-10% of peptidoglycan. This may illustrate the reason why Gram-negative bacteria are more resistant compared with Gram-positive bacteria. There are numerous reported on *Spirulina platensis* extract which inhibited both Gram negative and Gram positive bacteria (Ericcsson and Sherris, 1971). Due to the increase in antibiotic resistance in bacteria, a search for antibacterial drug from cyanobacteria has increased worldwide (Rania *et al.,* 2008). The process of wrapping or packaging food using plastic or styrofoam is rising every year and this is likely to threaten health unintentionally or unwittingly. The material that forms plastic or styrofoam contains ingredients such as polystyrene, polyvinyl chloride, and acrylonitrile which could act as a carcinogenic agents (Hidayat, 2011). Banana leaves are available and can be easily obtained without affording production costs, in addition to their elastic nature which makes them easy to use. Polyphenols activity on banana leaves can inhibit bacterial decomposition. Moreover, microbial contamination would be affected by the content of water on dangke (Zakariah *et al.,* 2019). Based on traditional practice of utilising banana leaves as food wrapping, it is the most dependable as food and cuisine wrapping; these should be the criteria to look for in other species or varieties of banana as potential wrapping (Harijati *et al.,* 2013).

CONCLUSION

Overall, these findings suggested that bacteriological quality of raw milk and dairy products were highly contaminated with *E. coli.* and *S. aureus*. Therefore, the use of natural products such as cyanobacteria extracts during cheese manufacture and packaging may reduce the growth of bacteria and prolonged the shelf-life of cheese.

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الاستفادة من مستخلصات البكتريا الخضراء المزرقة لتحسين الجودة الميكروبية للجبن الابيض الطري

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يهدف البحث إلى استخدام مواد طبيعية مثل مستخلصات السيانوبكتريا أثناء تصنيع وتغليف الجبن وذللك لتقليل والحد من التلوث البكتيري. تمت هذه الدراسة بعزل وتعريف كل من: بكتريا الايشريشا القولونية و المكور العنقودي الذهبي من اللبن الخام ومنتجاته حيث تمت الدراسة علي 150 عينة (الجبن القريش، الجبن الرومي، الجبن الثلاجة، الزبادي، الآيس كريم واللبن الخام) وتم تجميع 25 عينه من كل منتج من الأسواق والمحلات التجارية في محافظة الدقهلية، مصر. وقد تم عزل البكتريا باستخدام بيئات خاصة لكل ميكروب ثم عمل الاختبارات الكيميائية وتفاعل انزيم البلمرة التسلسلي للتأكد من البكتريا المعزولة . وقد كانت نسبة تواجد بكتريا الايشريشا القولونية هي (88%،68%،56%،80%،52%،68%) في كل من (الجبن القريش، الجبن الرومي، الجبن الثلاجة، الزبادي، الآيس كريم واللبن الخام بالترتيب). اما بالنسبة للميكروب المكور العنقودي كانت النسبة كالاتي: (80%،76%،88%،52%،28%،52%) في كل من (الجبن القريش، الجبن الرومي، الجبن الثلاجة، الزبادي، الآيس كريم و اللبن الخام بالترتيب) .وجد ان نسبة تواجد كلا من ميكروب الايشريشا القولونية و ميكروب المكور العنقودي عالية في الجبن الطري(القريش والثلاجة) علي الرغم من أنه من اكثر أنواع الجبن استهلاكا في مصر وذلك لكثرة فوائده لذا تم القيام بدراسة تأثير استخدام مستخلصات بعض أنواع السيانوبكتريا واضافتها الي الجبن كما تم أيضا استخدام أوراق شجر الموز في تغليف الجبن وتم عزل كلا من البكتريا السابق ذكرها ومقارنتها بالجبنة الخالية من الاضافات لوحظ ان الجبنة المصنعة والمضاف اليها السيانوبكتريا والمغلفة بورق الموز حققت انخفاضا معنويا جدا في اعداد البكتريا موضع الدراسة .