PRODUCTION AND EXTRACTION OF REUTERIN FROM LOCAL ISOLATE *Lactobacillus reuteri* AND USING IT IN SOFT CHEESE PRESERVATION

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(Received 8 January 2020 ,Accepted 19 January 2020)

Keywords: Lactobacillus, Reuterin, pathogens, cheese

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ABSTRACT

A local bacterial isolate from fecal samples of infants 1-3 months, previously isolated and identified as Lactobacillus reuteri, was used for reuterin production and characterization then applying it in local soft cheese preservation. Reuterin was produced under anaerobic conditions by growing these bacteria in the MRS broth medium at 37°C. After 18 hours of incubation , cell biomass was harvested and washed, then suspended in 278 mMol glycerol-water solution (pH=7.4) at 37°C for 3 hours . After cells removing from suspension by centrifugation and filtration through millipore filter paper (0.45), the reuterin was tested against four species of bacteria. Addition of produced reuterin as preservative substance was applied on locally industrialized soft cheese. Five concentrations of reuterin with control , (0.2 , 0.4 , 0.6 , 0.8 , 1) % , were used in four different solutions to detect the inhibition efficiency against different test pathogens ; total plate bacteria , Coliforms and Staphylococcus aureus . Viability of all the tested pathogens decreased with the increasing of contact reuterin concentration. The concentration 1% showed a significant difference ($p<0.05$) in opposite of the rest of concentrations and control sample. The number of viable cells hugely declined as the contact with reuterin continued, resulting in a reduction of 95.8 % inhibition of the total count cells after 7 days of treatment. The reduction of Coliform bacteria count, at the same period, was 94.2% Inhibition ,

while , it was 98.1% inhibition for Staphylococcus aureus count. The sensory assessment of 1% reuterin has the highest score as compared with the other treatments.

INTRODUCTION

Reuterin is described as a neutral water soluble and non - protein substance with low molecular weight (< 200 g mol-1) and it is derivative from glycerol. Reuterin is produced from L. reuter as an intermediate compound during the anaerobic growth of L. reuteri by the action of glycerol dehydratase which catalyzes the removal of water molecule from the glycerol and converse it into reuterin. Reuterin can form additional compounds in aqueous solution [(1),] where it can dimerize forming HPA dimer, or can be hydrated to form HPA hydrate and can also be dehydrated into the toxic compound acrolein [(2).]

This compound has been detected to possess a biological activity and probiotic health benefits ascribed to L. reuteri, where it has a broad antimicrobial effect towards the gram positive bacteria like Staphylococcus , Clostridium , Listeria, the gram negative bacteria like Salmonella , E.coli , Shigella, yeasts like Candida, fungi , protozoa like Trypanosoma , and eukaryotes [(3,4,5).] There is an evidence that reuterin induces oxidative stress in cells, most likely by modifying thiol groups in proteins and small molecules [(6).] In addition, it is expected that the inhibitory effect of it may be related to its action on DNA synthesis. Reuterin is water soluble, active in a wide range of pH about 2 to 8, resists the proteolytic and lipolytic enzymes [(7),] therefore it is suitable for biopreservation of foods. It has previously been reported that reuterin has the ability to preserve meat, milk and cottage cheese [(8).] The main objective of our research is to detect the bactericidal effect of reuterin produced by L. reuteri against food-borne pathogens including, total plate count, Coliform and Staphylococcus aureus in laboratory industrialized soft cheese.

MATERIALS AND METHODS

Microorganisms: *L. reuteri*, our previous isolate was identified as reuterin-producing strain. Other strains used such as Escherichia coli, Staphylococcus aureus, Bacillus subtilis and Enterococcus sp. They obtained from Biotechnology Lab. Agriculture College, University of Basrah.

Chemical culture media: De Man, Rogosa and Sharpe agar, De Man, Rogosa and Sharpe broth, MacConkey Agar, Mannitol salt agar, Nutrient agar, Mueller Hinton agar.

Screening of antibacterial spectrum of *L.reuteri* : *L. reuteri* was screened for their antibacterial Inhibitory spectrum. Using the well diffusion agar method, the activity of the cell free culture supernatants were determined against a broad range of Gram-positive and Gram-negative strains. 0.1 (107 cfu/ml) milliliter of 24 hour old cultures of each indicator bacterial cultures was spread on Mueller Hinton agar plates independently then 6 millimeter wells were spread onto agar plates. The wells were then filled with 0.05 milliliter of the cell free supernatants from four genetically identified isolates and the plates were kept undisturbed for 2 h and subsequently incubated at 37oC. After 24 hours, the diameters (mm) of the growth inhibition zones were measured [9].

Reuterin Production: Reuterin was produced from L. reuteri cultures using the methods described previously by [10,11] with some modifications. Briefly. The overnight activated cells of L. reuteri were grown in 50 ml tubes of MRS broth (total size was 1litre). After 18 hours incubation period, cells were harvested by centrifugation (7000 rpm for 10 min) and washed twice with 50 mMol sodium phosphate buffer (pH 7.4). The washed cells were then suspended in 100 ml of 278 mMol water-glycerol solution. An amount of (1% ml) of other bacteria (E. coli) has been inoculated in the suspension as stimulation factor for Reuterin production. The water-glycerol suspension was then incubated under anaerobic conditions at 37°C for 3 hour. Reuterin was collected by centrifugation of L. reuteri suspension in 10 ml screw-capped tubes at (8000 rpm for 15 min) and filtered through 0.45 mm Millipore filter paper. The resulting cell-free supernatant was then stored in refrigerator at -4°C.

Bioactivity comparison of pure and crude reuterin against *E coli* :The bioactivity of the pure and crude reuterin were assessed by using the paper disc diffusion method [12]. As passed previously, both of the pure and crude reuterin were produced in a test tubes of glycerol-water solution and MRS broth respectively with taking in care the neutralize of pH to 7.4 for prevention of organic acids production. Tubes then subjected to centrifugation at 7000 rpm and Millipore filtering before a sterilized filter paper discs were immerged and saturated with the pure and crude supernatants. After

that, the discs were air-dried and put directly on the surface of sterilized Mueller Hinton agar plates were freshly inoculated with E. coli. After incubation of plates at 37°C for 24 hour, the zone of growth inhibition was measured in millimeter.

Application of extracted reuterin in food preservation

Preparation of reuterin solutions The Effect of both pure and crude Reuterin were assayed against Food Borne Pathogens using industrial laboratory tender cheese as a test food substrate. Inhibitory activity against the total plate count, total E coli and total staphylococcus was investigated after different time intervals (1-4 weeks). Five concentrations of Reuterin with control (free of Reuterin) were prepared in four groups of solutions (pH 7.4) and stored at -4°C. prior to be use in this experiment (table 1). The first solution was of pure reuterin in 1% NaCl. The second and the third solutions were respectively prepared from pure and crude reuterin in distilled water. The fourth distilled water- pure reuterin solution was voluntary contaminated with Staphylococcus aureus to ensure and compare its growth**.**

Table 1: Reuterin solutions used in biopreservation of food

Solution	Reuterin Concentrations (%)	content
Solution 1	0.0 , 0.2 , 0.4, 0.6 , 0.8 , 1	**1% NaCl buffer + pure Reuterin**
Solution 2	0.0 , 0.2 , 0.4, 0.6 , 0.8 , 1	**Distilled water + pure Reuterin**
Solution 3	0.0 , 0.2 , 0.4 , 0.6 , 0.8 , 1	**Distilled water + crude Reuterin**
Solution 4	0.0, 0.2 , .0.4, 0.6 , 0.8 , 1	**Distilled water + pure Reuterin + *Staphylococcus aureus***

Industrialization of tender cheese in laboratory

The method reported in [13] was used for industrialization of tender cheese in the laboratory with some modifications. Five liters of milk were subjected to pasteurization at 72°C for 1 minute then leaving it to cool at 40°C. After that, Rennin enzyme was added to the milk with shaking for a period and then leaving it for 20-30 minutes. The cheese lump formed was then distributed in water-filter cups and leave it overnight to remove the whey from it. 25ml of each Reuterin-containing solution was

independently added to 25g of dry cheese has been weighted in plastic containers (figure ١) and kept in 4°C for counting of bacteria after the first storage week till four weeks periodically.

Fig. 1: Plastic containers containing 25g: 25ml of prepared tender cheese and reuterin solutions.

Microbial assay of the test industrialization cheese Parts of cheese taken from the outer surrounding of each test sample (about 1g) was added to 9ml sterilized peptone water containing 0.2 ml of sodium citrate. The mixture was then shacked for 5 minutes to disintegration of the sample before preparation of dilutions. 0.1 ml of each dilution was cultured by pour plate method on both Nutrient agar for the total bacteria count, MacConkey agar for total Coliform count and Mannitol agar for the *Staphylococcus aureus* count. Each plates were then incubated aerobically at 37°C for 24 hours.

Sensory evaluation of storage cheese The sensory evaluation list, reported by (14), was used for Assessment of 5 reuterin-treatment cheese samples, NaCl-treatment cheese sample and control cheese sample (figure ٢). The sensory tests were performed by sharing of nine academics; some were specialist in human health and the others in food sciences.

Fig. 2 : The treatment cheese samples were subjected to sensory evaluation.

Statistical analysis The obtained data has been treated with statistical analysis by using chi square and T-test to count the significant values at ($P<0.05$) level using the statistical analysis program SPSS version 16.

RESULTS AND DISCUSSION

Reuterin production Due to high solubility of reuterin in water, as referred to in most references, it was preferred to produce after separation the cells of *L. reuteri* from broth culture medium and suspended in a mixture of distilled water-glycerol solution. This provides some advantages for the process e.g. time shortening, low cost, easy purification. For this reason, water-glycerol solution was considered as optimum medium for reuterin production. Also, it was indicated that adding other microorganisms into aqueous glycerol solutionto be in direct contact with *L. reuteri* in the stationary phase where incubated for 3 hours, is stimulated to a sufficient in aqueous glycerol solution that led to a corresponding increase in reuterin production levels which were detected by inhibition of growth zone test for the cell-free reuterin supernatants as comparing with the supernatants from *E. coli* free-aqueous glycerol solution and this agree with what reported in [15,16,17] .

No effect was appeared in the size of *E. coli* inoculum which has been added, on reuterin production where no difference among them. The best pH value for reuterin production was (7.4) which eliminate and prevent the production of organic acids and hydrogen peroxide in the glycerol aqueous solution , therefore reuterin will synthesized as pure yield and no other compounds interact with its bioproduction giving perfect result of it. Furthermore , the best temperature and inoculum size=for

reuterin production were 37°C and 2% ml that caused an increase in cell biomass that harvested and suspended in the production solution leading to enhancing of reuterin production. Harvesting and transferring of *L. reuteri* cells after 18 hours growing period were exactly perfect for production because cells were in the stationary period where the secondary metabolisms be active for 3 hours. Glycerol concentration at 3% was also the best as a primary source for production, while no role was observed to vancomycin in the production solution in contrast with its presence in isolation process for elimination of bacterial strains that are not wanted; hence its addition is not necessary in the reuterin production process.

Antibacterial spectrum of produced reuterin: Table (2) shows the inhibition effect of the culture supernatant, of *L. reuteri*, against a wide range of Gram positive and Gram negative indicator bacterial isolates. The test was included four food spoilage and food borne pathogen as indicator bacteria. The spectrum showed a weak inhibition zones (< 10mm) to strong inhibition zones (>20mm) while the range in between was divided to intermediate inhibition zones (from 10 mm up to 15mm) and less strong inhibition zones (from 16 mm up to 20mm). On the other hand, no sensitivity shown when we subjected *L. reuteri* to the culture supernatants.

The results were in agreement with many other studies, which indicated the antibacterial ability of reuterin. [18] was stated *L. reuteri* as all other LAB has been reported to produce various organic acids during fermentation, such as lactic acid and acetic acid, which lead to lowering of pH in GIT. The organic acids besides the production of reuterin gave *L. reuteri* a strong antagonistic effect, where they served as potent antibacterial agents against pathogenic bacteria [3]. The antagonistic effect of *L. reuteri* as well as their ability to survive at lower pH were considered beneficial in maintaining general health of GIT and female genital tract of the host.

Table 2 : Antibacterial activity spectrum of *L. reuteri* culture supernatants

No.	Test isolates	Inhibition zone (mm)
1	*E.coli*	++++
2	*S. aureus*	++++
3	*B. subtilis*	+++
4	*Enterococcus sp.*	++

* - = no inhibition ; + < 10mm ; ++ = 10-15mm ; +++ =16-20mm ; ++++ >20mm

Bioactivity comparison of pure and crude reuterin against bacteria Figure (3) shows the bioactivity of pure and crude reuterin against the test E. *coli* bacterium. The inhibition zone of pure reuterin supernatant was much larger (34 mm) than crude reuterin supernatant (9mm). The high effect of pure reuterin may be due to aldehyde group of the compound. Aldehyde group of the reuterin is highly reactive, thus reuterin compound can be converted to various compounds in the aqueous solutions. The aldehyde group of the reuterin is highly reactive with the thiol groups and the primary amines, therefore , reuterin compound could inactivate or inhibit the proteins and the small molecules that containing such groups , so this explain the broad spectrum of reuterin effect against most bacteria, fungi and also viruses[1]. Another hypothesis was proposed that reuterin could be demonized to form HPA dimmer, which is similar in structure to the ribose sugar and could, specifically, block the enzyme of ribonucleotide reeducates by its action as a competitive inhibitor for it.

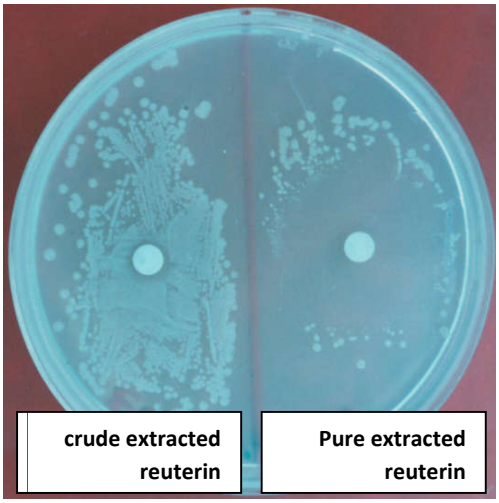

Fig. 3 : The bio-activity of pure and crude reuterin against *E.coli*

Application of reuterin in the soft white cheese:The results that have been obtained, from adding different concentrations of reuterin at different time intervals, revealed an effect on the number logarithm of total aerobic bacteria found in soft white cheese. We showed that the number logarithms were reduced after reuterin adding till 14 days of storage period, then the number logarithms began to rise in all the concentrations used but did not reach the logarithm of the number found at the beginning of the experiment. The reducing in bacterial number is a result of increasing in reuterin concentrations. The logarithms of total aerobic bacteria count , on nutrient agar and

after one week of storage , were (5.4, 5.3, 5.1, 4.9, 4.5, 3.9) Log cfu/g for the solution of pure reuterin concentrations (solution 2) which were (0.2, 0.4, 0.6, 0.8, 1) % respectively, while the logarithm of the numbers was higher for the same Concentrations of crude reuterin solution (solution 3) (5.5, 5.3, 5.2, 4.9, 4.8, 4.4) as compared with the saline solution (solution 1) where the logarithm of the numbers (5.3, 5.2, 5.1, 4.9, 4.5, 4). From such results, we observed that there was a relative superiority of 1% concentration of pure reuterin solution on the brine. This superiority continued until the end of the fourth week, while there was a convergence in the effect between them for the other concentrations. The crude solution of reuterin showed an effect of increasing the concentration up to 1%, but it was less than the two previous solutions in the effect of bacterial numbers. The solution , deliberately , contaminated with *Staphylococcus aureus* (solution 4) showed a similar effect in reducing the bacterial logarithm numbers with the increase of reuterin concentrations, where 1% concentration was also more effect here and there was on no significant differences in opposite of pure reuterin solution till the fourth week of storage .

The results of the statistical analysis showed a significant difference ($p<0.05$) between the concentrations used in the experiment and the storage periods and the type of solutions used when calculating the logarithm of total aerobic bacteria. There was a significant difference between the concentration 1% against the rest of concentrations and control sample in terms of effect on bacterial numbers throughout the storage period. There were no differences between the solutions 1, 2 and 3 but there was a difference between them and the solution 4. In addition, there were no differences between concentrations at storage for 4 weeks (figure 4).

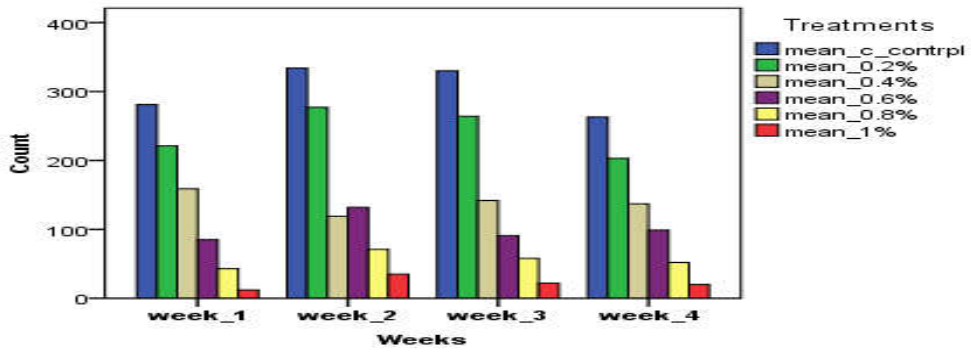

Fig. 4 : The mobility of total bacterial count through four weeks of storage according to the effect of reuterin concentrations .

On the other hand, the logarithm number of Coliform bacteria was decreased on MacConkey agar as a result of increasing of reuterin concentration in the four solution for two weeks of storage then rises again in the third and fourth weeks. Likely 1% concentration was the more effect in numbers of this bacteria , where the logarithm number after the end of fourth week storage, reached to 3.9 Log cfu /g from 3.6 in the first two weeks .The effect in the solution 1and 2 was better than solution 3 while no growth appeared in all concentrations of solution 4. In calculating the logarithm of Coliform bacteria, the concentration 1% showed a significant difference from the rest of the concentrations throughout the storage period. No difference appeared between the solutions 1 and 2 till the end of second week while the difference was found between them from third to fourth week. They also showed a difference from solution 3, which was less effective (figure 5).

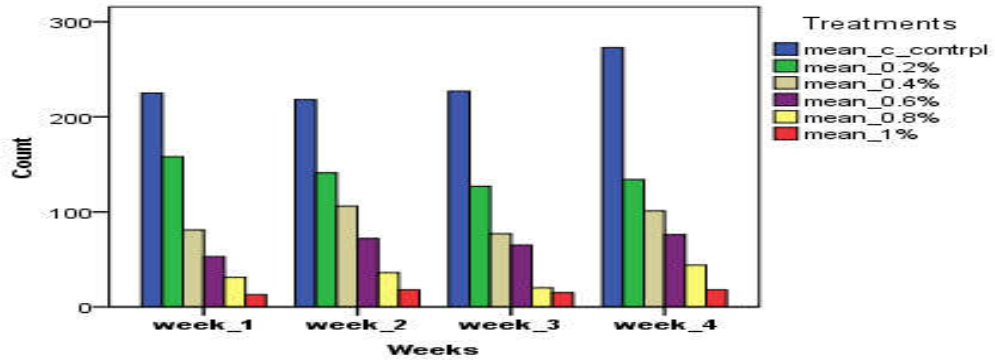

Fig. 5 : The mobility of Coliform bacterial count through four weeks of storage according to the effect of reuterin concentrations .

The growth of *Staphylococcus aureus* on Mannitol salt agar disappeared in all concentrations used in the solutions 1, 2 and 3 while growth appeared on the culture medium for solution 4 that contaminated with these bacteria. High decreasing in bacterial growth was observed in all concentrations of this solution, especially 1%, after the first week of storage. The logarithm number of bacterial count was 3.6 for 1% concentration then logarithm number rose again from the second storage week to reach 4.2 in fourth week. In the calculating of *Staphylococcus aureus* no significant difference was observed between 1% and 0.8% concentrations until the end of the second week, but after the third to fourth week, the difference was observed when the

concentration 1% overcame all other concentrations. The rest other concentrations (0.2, 0.4, 0.6) % did not appear significant differences during the storage period (figure 6).

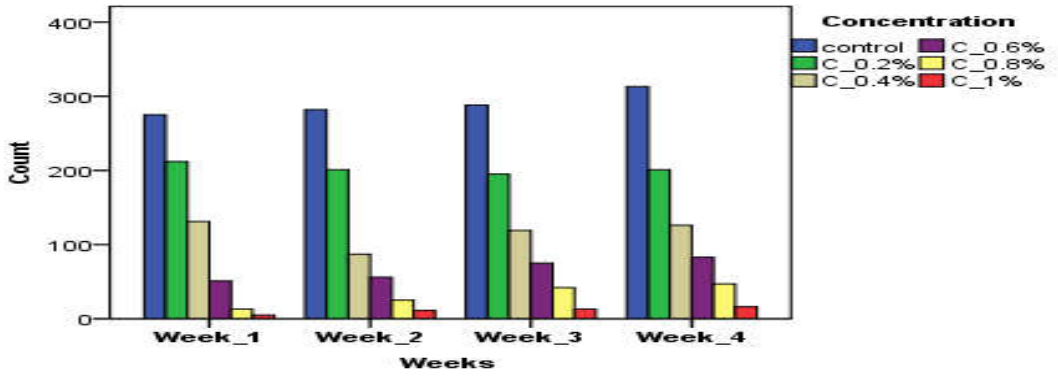

Fig. 6 : The mobility of *Staphylococcus aureus* count through four weeks of storage according to the effect of reuterin concentrations .

Sensory evaluation of laboratory cheese Table (3) shows the average of degrees of sensory evaluation of the processed cheese in which different concentrations of reuterin were added to it as compared with the control sample (free of reuterin and any other preservatives) and sample with 2% NaCl, according to the characteristics of color and appearance, taste and flavor, texture, the general acceptance of the model and for each character, the degree of evaluation in front of her. The sensory evaluation of the cheese was carried out after three months of storage at 4 °C. The results and their statistics (table 4) showed that the sensory characteristics of the model containing 1% reuterin concentration were not changed. A score of 74% was obtained compared to the NaCl containing model which collected a 50% evaluation score and has crisp texture with salinity taste. The control model , which collected a 36% evaluation score, also showed crisp textures with a bitter bait. The other models of reuterin concentrations showed different results. 0.6% and 0.8% concentrations were brittle or tourist textures and received 40% and 43% scores respectively, while the concentrations of 0.2% and 0.4% showed color change into yellow with a passing taste and received 38% and 39% scores.

Table 3: Sensory evaluation of the test cheese treated with reuterin.

character	Degree	Co. 0٪	Sample 0.2٪	Sample 0.4٪	Sample 0.6٪	Sample 0.8٪	Sample 1٪	Sample 2٪NaCl
Color and External Appearance	10	4	5	5	5	5	8	5
Taste and Flavor	40	13	14	14	13	15	29	20
Texture	40	16	16	17	18	19	30	19
general acceptance	10	3	3	3	4	4	7	6
Total	100	36	38	39	40	43	74	50

Table 4: Compare Treatment samples with control sample by T- test.

Treatment Samples	Compared sample	T – test value	P- value	df	result
Sample 1% reuterin	Control	4.477	0.002	8	significant
Sample 0.2% reuterin		0.541	0.603		insignificant
Sample 0.4% reuterin		0.958	0.366		insignificant
Sample 0.6% reuterin		1.339	0.217		insignificant
Sample 0.8% reuterin		0.894	0.397		insignificant
Sample 2% Nacl		5.656	0.000		significant

ACKNOWLEDGMENT

The authors want to thank the department of food science and biotechnology, college of agriculture, University of Basrah for financial support of this science work.

انتاج واستخلاص الرتيرين من العزلة المحلية *Lactobacillus reuteri* واستعماله في حفظ الجبن الطري

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الخلاصة

استخدمت العزلة البكتيرية المحلية *Lactobacillus reuteri* ، المعزولة والمشخصة سابقا من عينات الغائط لأطفال رضع بعمر 1- 3 اشهر ، لإنتاج وتوصيف مركب الرتيرين واستخدامه في حفظ الجبن المنتج محليا . تم انتاج الرتيرين لاهوائيا بتنمية هذه السلالة ، بعد تنشيطها ، في وسط MRS السائل بدرجة حرارة م 37° لمدة 18 ساعة تم بعدها حصاد الكتلة الحيوية للخلايا من الوسط السائل وغسلها ثم اعادة تعليقها في تركيز 278 ملي مول محلول كليسيرول - ماء مقطر بدرجة حامضية pH ٧.٤ ثم حضنها لاهوائيا في درجة حرارة م 37° لمدة 3 ساعات . بعدها تم ازالة الخلايا من المعلق البكتيري بواسطة الطرد المركزي وتعقيمه بالترشيح من خلال ورق ترشيح Millipore filter paper (0.45) ثم اختبر الرتيرين الناتج تجاه اربع انواع من البكتيريا . تم تطبيق استخدام الرتيرين المنتج كمادة حافظة للجبن الطري المنتج محليا حيث استخدمت خمسة تراكيز من الرتيرين (1 % , 0.8 , 0.6 , 0.4 , 0.2) مع عينة سيطرة في اربعة محاليل مختلفة لكشف فعالية التثبيط ضد انواع مختلفة من الممرضات ومسببات تلف الاغذية من خلال حساب البكتيريا الكلية وبكتريا القولون والمكورات العنقودية . وجد هناك تناقص في الاعداد الحية لكل البكتيريا المختبرة تناسب طريا مع زيادة التركيز للرتيرين واظهر التركيز 1% فرقا معنويا ($p<0.05$) مقابل بقية التركيز وعينة السيطرة وتناقص اعداد الخلايا الحية مع استمرار تعرضها للرتيرين مما ادى الى نسبة تثبيط 95.8 % لعدد البكتيريا الكلي بعد سبعة ايام من المعاملة ونسبة 94.2% لبكتيريا القوقون بينما كانت النسبة 98.1% للعنقوديات *Staphylococcus aureus* . واعطت اضافة نسبة 1% من الرتيرين اعلى درجة في التقييم الحسي مقارنة بالتراكيز الاخرى المضافة للجبن المصنع.

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