

Application of Reuterin Produced by *Lactobacillus reuteri* DSM 20016 to Inhibit Some Food-born Pathogens in UF-Feta-Cheese

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Abstract

A broad-spectrum reuterin produced during anaerobic fermentation of glycerol by *Lactobacillus reuteri* strain 20016 was found to be inhibitory and bacteriocidal for *Listeria monocytogenes*, *Escherichia coli*, *Salmonella typhimorium*, *Pseudomonas aerogenes*, *Klebsiella penomoniae* and *Staphilococcus aureus*. Reuterin was produced by a two-step fermentation process. A batch fermentation in a 1.5 liter flask fermentor was applied to produce a biomass of Lb. Reuteri using a modified MRS broth at pH 5.5. Further, harvested cells were used to ferment glycerol (250 mMol) under anaerobic conditions (flushed with nitrogen). The MIC values of reuterin for *Listeria monocytogenes*, *Staphilococcus aureus*, *Escherichia coli*, *Salmonella typhimorium*, *Pseudomonas aeruginosa* and *Klebsiella penomoniae* were 10, 4, 2, 2, 1 and 1 AU/ml, so the strain *L. monocytogenes* was more resistant to reuterin than the others. In potassium phosphate buffer (pH=7.2) using 80 AU/ml reuterin the count of *Listeria monocytogenes* and *Staphilococcus aureus* decreased from 10^7 to 5.5×10^6 and 2.5×10^6 respectively and the counts of *Escherichia coli*, *Salmonella typhimorium*, *Pseudomonas aerogenes* and *Klebsiella penomoniae* decreased to nondetectable level in 5 hours. Addition of reuterin (40 units per gr) to the UF-Feta-Cheese reduced the viability of all organisms. The inactivation rate was more pronounced with *Pseudomonas aeruginosa* and less with *Listeria monocytogenes*.

1-Introduction

Reuterin is a small antimicrobial compound that is produced as an intermediate metabolite during anaerobic fermentation of glycerol (8). It consists of an equilibrium mixture of monomeric, hydrated monomeric and cyclic dimeric forms of β -hydroxypropionaldehyde (7). Reuterin is produced by some strains of *Lactobacillus reuteri*, with antimicrobial activity towards a range of food-borne pathogens and spoilage organisms, including both Gram-positive and Gram-negative bacteria, yeasts, moulds and protozoa (1,7) so it is expected that its inhibitory effect be related to its action on DNA synthesis (7). It is water soluble, effective in a wide range of pH values (2 to 8), and resistant to proteolytic and lipolytic enzymes (1), and it is therefore suitable for biopreservation. The possible use of reuterin for biopreservation has been investigated in meat (4), milk and cottage cheese (3) and herring filets (5).

It has previously been reported that *L. reuteri* strain 12002 has the ability to produce reuterin which shows a potential inhibitory effect against a wide range of gram-positive bacteria, e.g., *Bacillus cereus*, *Staphilococcus aureus* and

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Listeria monocytogenes and gram-negative bacteria e.g., *Escherichia coli*, *Yersinia enterocolitica* and *Pseudomonas fluorescens* in synthetic media (4).

The main objective of the present work is to investigate the bactericidal effect of reuterin produced by *L. reuteri* 20016 against food-borne pathogens including *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *E.coli* and *Salmonella typhimorium* in phosphate buffer and UF-Feta-Cheese media.

Materials and Methods

Microorganisms: *L. reuteri* DSM 20016 was purchased as the producing strain of reuterin from DSMZ. Other strains used included *E.coli* K-12 as the indicator organism (Pasteur Institute, Tehran, Iran), *Listeria monocytogenes* PTCC 1294 (Persian Type Culture Collection, Iran), *Staphylococcus aureus* PTCC 1337, *Pseudomonas aeruginosa* PTCC 1074, *Klebsiella pneumoniae* PTCC 1053, *E.coli* PTCC 1338 and *Salmonella typhimorium* ATCC 14028.

Reuterin Production: Reuterin was produced by a two-step fermentation process. *L.reuteri* DSM 20016 was first propagated overnight at 37 °C in a 1.5 lit. flask fermentor, in modified MRS broth (m-MRS). The stirring rate was 200 rpm. m-MRS was prepared using sodium acetate and tween 80, respectively at 1.5 and 1.2 g/l concentrations. Glucose (D+ glucose-monohydrate, Merck, Germany) was autoclaved separately and added afterwards to a concentration of 60 mMol/l. The pH was adjusted to pH 5.5 with 3M H₃PO₄ and 1.5M NaOH (Merck-Germany) using pH-meter (Jenway 3510- UK). Cells were harvested after 20h by centrifugation (7000× g, 10 min, 4°C, BECKMAN- Avanti J-25- USA) and washed twice with phosphate buffer (pH=7.2, 50 mMol). Washed cells were incubated in water-glycerol solution (250 mM, Merck, Germany) under anaerobic (flushed with nitrogen) conditions (6). The resulting supernatant was sterilized by filtration (pore size 0.22 µm, Schleicher & Schuell, FP 3010 CA-S) then stored in refrigerator.

Quantification of reuterin: Reuterin activity was quantified by the MIC method using microtiter plates as described by Chung et al.(1989). In general, the culture of *E.coli* K-12 (Indicator organism) grown overnight was harvested, washed twice with phosphate buffer (pH 7.2, 50 mM), suspended in the same buffer, and diluted to an A₄₂₀ of 0.2 measured with a shimadzu spectrophotometer. This suspension was diluted 1/10, which corresponds to about 6log₁₀ CFU/ml. The diluted suspension (0.1 ml) was used to inoculate 0.2 ml of serial dilutions of reuterin diluted in Muller-Hinton medium (Himedia, India Laboratories). Glucose was autoclaved separately and added to a concentration of 20 mMol/l. The microplate was incubated at 37 °C and growth was examined after 48 h. Reuterin concentration was defined as the reciprocal of the highest dilution that did not permit visible growth of the indicator strain and expressed as Arbitrary Units (AU) with 1 AU of reuterin being defined as the reciprocal of the highest dilution that did not permit visible growth of the indicator strain (3).

Determination of reuterin concentration to inhibit *Listeria*, *Salmonella*, *E.coli*, *Klebsiella*, *Pseudomonas* and *Staphilococcus*.

Listeria monocytogenes and *E.coli* PTCC 1338 was grown in Brain Heart Infusion broth (BHI) at 37°C for 20h. *Salmonella typhimorium*, *Klebsiella pneumoniae*, *Staphilococcus aureus* and *Pseudomonas aeruginosa* were grown at 37°C for 20 h in Nutrient broth (Merck, Germany). Inocula of the cultures were then prepared (ca. $6 \log_{10}$ CFU/ml) as mentioned above and used (0.1 ml) to inoculate aliquots of the diluted reuterin (0.2 ml) in 96 wells microplates. Quantified reuterin was serially diluted in Muller-Hinton medium supplemented with 20 mMol sterilized glucose to obtain dilutions with 0, 2, 4, 6, 8, 10 and 12 AU/ml. The microplates were incubated at 37°C and visible growth was controlled after 48 h.

The inhibitory effect of reuterin against *Listeria*, *Salmonella*, *E.coli*, *Klebsiella*, *Pseudomonas* and *Staphilococcus* in phosphate buffer medium.

The mentioned strains were grown, harvested and washed as described above and then suspended in phosphate buffer at room temperature. The bacterial suspension (approx. 10^6 CFU/ml) was used to inoculate 1ml of reuterin solution (80 AU/ml). After the defined exposure times at room temperature, 8 ml of a solution containing 0.5% (wt/vol) peptone (merck), 0.8% (wt/vol) NaCl and 0.1 ml of 15% (wt/vol) K₃PO₄, which gives a pH of 9 was added to neutralize the antimicrobial effect of reuterin (4). The exposure times were 1, 2, 3, 4 and 5 hours. Viable cells were determined by the pour plate method in Eosin Methylene Blue Agar for *E.Coli*, Salmonella Shigella Agar for *salmonella typhimorium*, Bird Parker Agar for *staphilococcus aureus*, MacConkey Agar for *pseudomonas aeruginosa* and *Klebsiella pneumoniae*, and in Trypton Soy Yeast Extract Agar for *Listeria monocytogenes*.

The inhibitory effect of reuterin against *Listeria*, *Salmonella*, *E.coli*, *Klebsiella*, *Pseudomonas* and *Staphilococcus* in UF-Feta-Cheese medium.

One gram of cheeses was aseptically weighted into a sterile tube and inoculated with 1ml of bacterial suspensions to give ca $5 \cdot 10^6$ CFU/g. Reuterin stock solution was added to achieve final concentration of 40 units per g in contaminated cheese samples. Then the samples were placed in 7°C and survival of organisms was monitored in 1, 3, 6, 9, 12 and 15 days.

Statistical analysis

Data were analyzed by SPSS software (version 9.0) for the analysis of variance and determination of significance ($p < 0.05$) between the treatments using Duncan's multiple test.

Results and Discussion

The reuterin concentration required to inhibit the growth of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *E.Coli*, *Salmonella typhimorium*, *Staphilococcus aureus* and *Listeria monocytogenes* was respectively 1, 1, 1, 2, 2, 4 and 10 AU/ml(table 1). So the strain *L. monocytogenes* was more resistant to reuterin than the others. Chung et al., (1989) reported the MIC of reuterin for *Pseudomonas aeruginosa*, *E.Coli*, *Salmonella typhimorium*, *Staphilococcus aureus* and *Listeria monocytogenes* respectively as 2, 4, 4, 2 and 4-8 AU/ml which agrees to our results in some aspects(2). There was no report about *Klebsiella pneumoniae*. Another research shows the MIC for 6 strains of *E.Coli* as 4 AU/ml.

Table 1- Minimum Inhibitory Concentration of reuterin against different pathogens.

Organism	Minimum Inhibitory Concentration(AU/ml)
<i>E.coli K-12</i>	1
<i>E.coli PTCC 1338</i>	2
<i>Salmonella typhimorium ATCC 14028</i>	2
<i>Staphilococcus Aureus PTCC 1337</i>	4
<i>Klebsiella pneumoniae PTCC 1053</i>	1
<i>Pseudomonas aeruginosa PTCC 1074</i>	1
<i>Listeria monocytogenes PTCC 1294</i>	10

Survival curves of the mentioned strains in phosphate buffer (pH 7.2, 50 mM) with 80 AU/ml of reuterin added are shown in Figure 1. The cell count was enumerated during a 5 hrs period at room temperature. As there was no differences between the growth curves of untreated samples in phosphate buffer, all the curves are shown in one. No significant reduction was observed in the counts of control samples, whereas during the same 5hrs period, reuterin resulted in reduced viability of all organisms ($p < 0.05$). The reduction in the counts of *Pseudomonas aeruginosa*, *E.Coli*, *Salmonella typhimorium* and *Klebsiella pneumoniae* was more pronounced compare to the counts of *Staphilococcus aureus* and *Listeria monocytogenes*. The first group reduced to nondetectable limit within 5 hrs and the count of *Listeria monocytogenes* and *Staphilococcus aureus* decreased from 10^7 to $5.5 * 10^6$ and $2.5 * 10^6$ respectively. Statistical analysis showed that the antimicrobial agent has had significant differences on the microbial counts. The difference exists not only between the treated samples and control but also between the treated samples together, except of *E.Coli* vs. *Salmonella* and *E.Coli* vs. *Pseudomonase*.

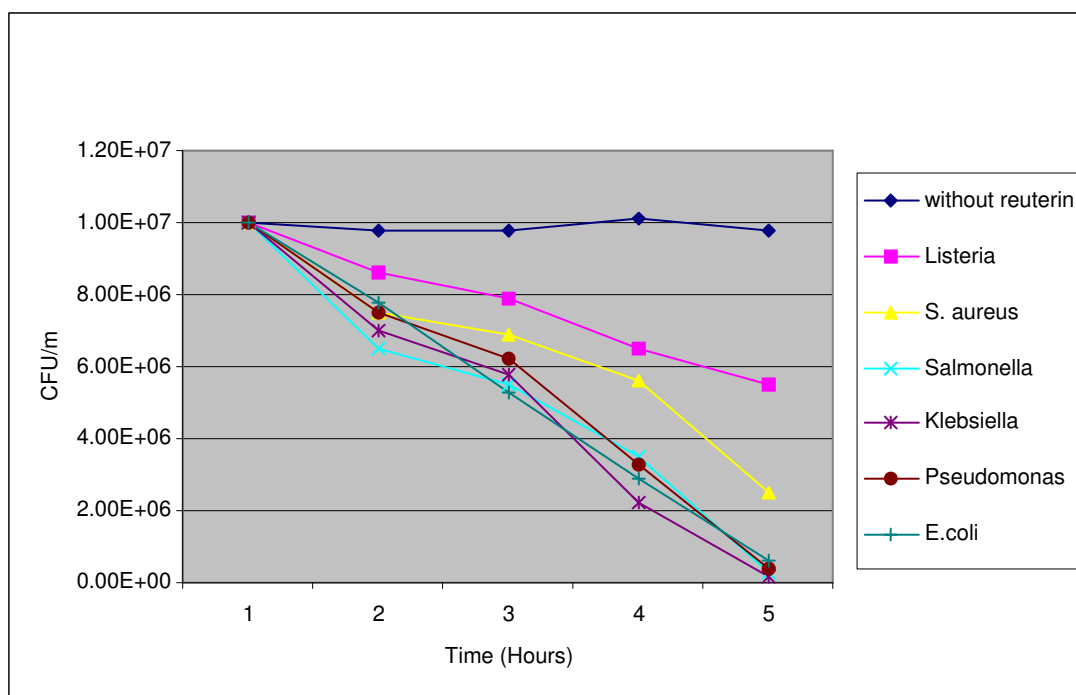


Fig.1- Survival curves of different pathogens exposed to reuterin, 80 AU/ml, in phosphate buffer at room temperature.

The effect of reuterin at 40 units per ml on the growth of different pathogens in UF-Feta-Cheese medium at 7 degrees centigrade during a period of 15 days is shown in Figures 2 to 7. As the behaviour of pathogens on cheese medium differ from one to another, we presented the curves separately. The counts of pathogens including *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonase aeruginosa*, *Klebsiella peunomoniae*, *E.coli* and *Salmonella typhimorium* in reuterin treated samples decreased from 5×10^6 to 4.5×10^6 , 3.8×10^6 , 1.8×10^6 , 1.7×10^6 , 3.2×10^6 and 1.5×10^6 respectively. Also the counts in untreated samples was respectively 5.4×10^6 , 5.8×10^6 , 4.5×10^6 , 4.7×10^6 , 5.5×10^6 and 3.5×10^6 .

EI-ziney & Debevere (1998) showed that at a reuterin concentration of 50 AU/g of creamed cottage cheese (pH=5.4), the population of *L. monocytogenes* decreased by 1.5 log cycles after three weeks, whereas *E.Coli* decreased by 3.5 cycles during the same time(3). In the absence of reuterin the inoculated number of viable *Listeria* increased by 0.4 log, whereas *E.Coli* decreased by 0.5 log within 21 days at 7°C. In our study *Listeria monocytogenes* and *E.coli* decreased respectively by 0.1 and 0.4 log cycles. The difference might be due to lower activity of our reuterin solution and also the shorter period of survey time.

Results of another research showed that the count of *Listeria monocytogenes* increased by 0.4 log. Cycle after 21 days at 7 degrees centigrade in cottage cheese medium. The count of *E.coli* decreased by 0.5 log. Cycles (4).

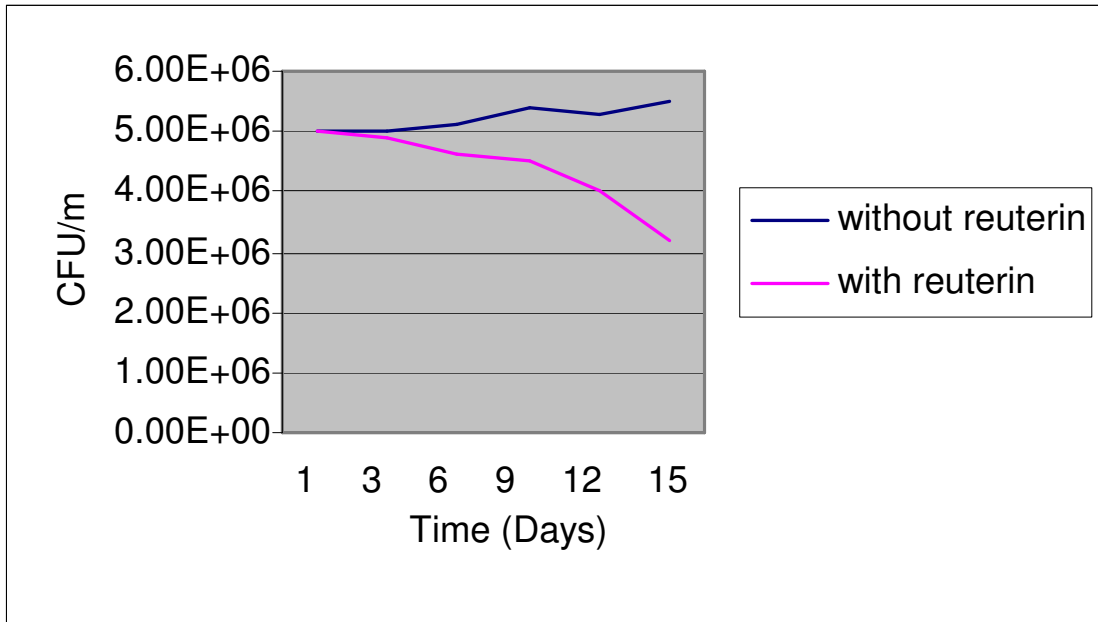


Fig 2- Effect of reuterin at 40 units reuterin per ml on the growth of *E.Coli* PTCC 1338 in UF-Feta-Cheese at 7°C.

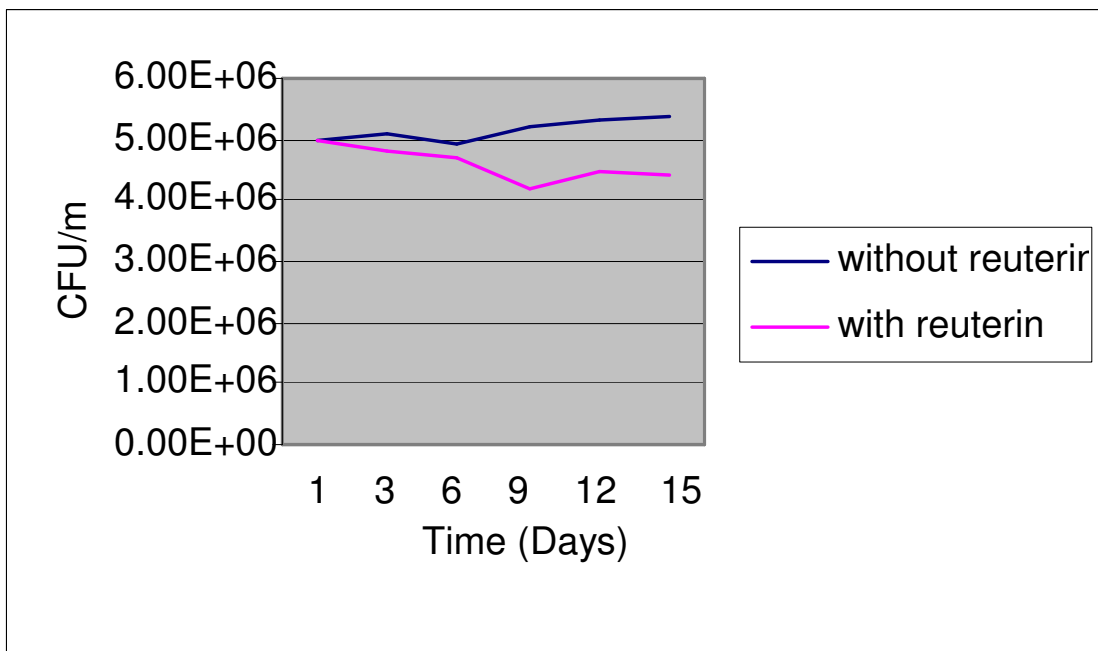


Fig 3- Effect of reuterin at 40 units reuterin per ml on the growth of *L. monocytogenes* PTCC 1294 in UF-Feta-Cheese at 7°C.

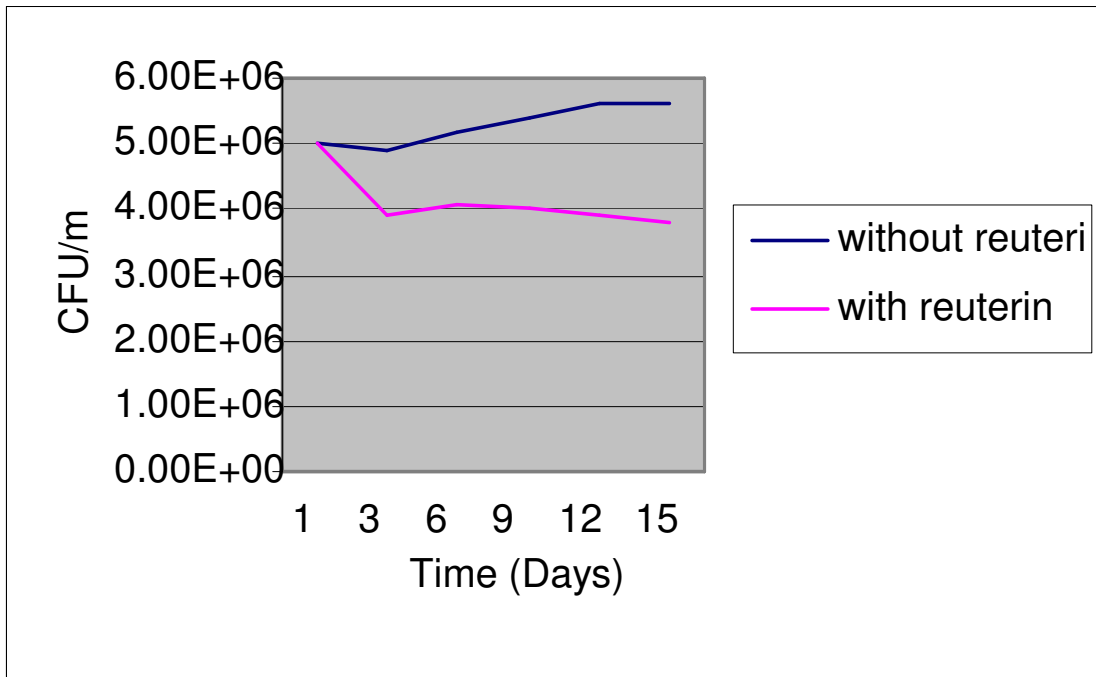


Fig 4- Effect of reuterin at 40 units reuterin per ml on the growth of *S. aureus* PTCC 1337 in UF-Feta-Cheese at 7°C.

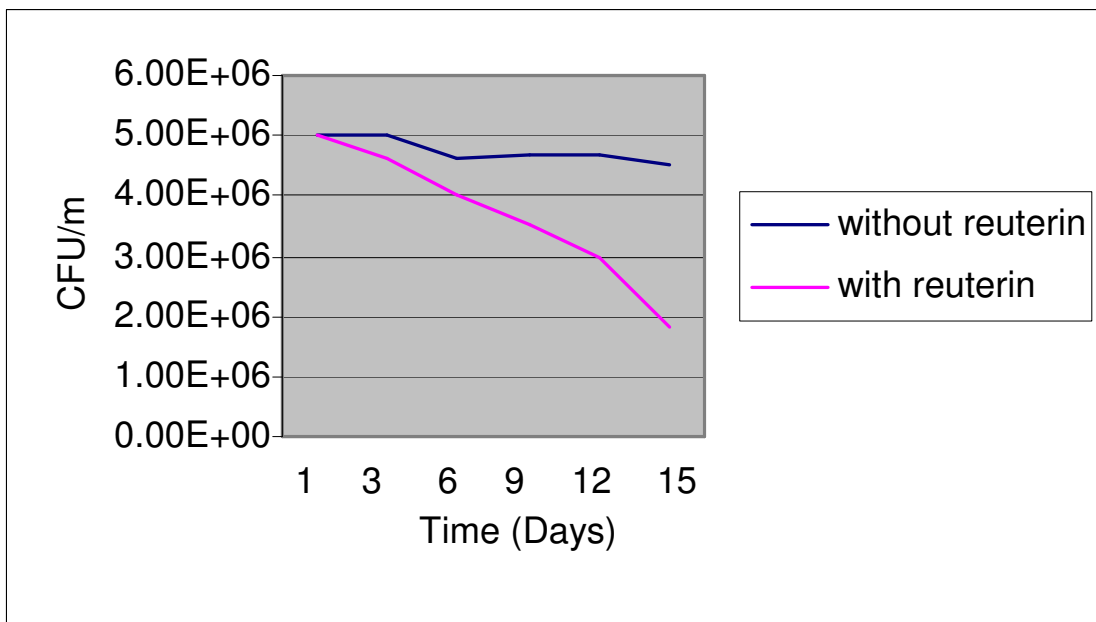


Fig 5- Effect of reuterin at 40 units reuterin per ml on the growth of *Pseudomonas aeruginosa* PTCC 1074 in UF-Feta-Cheese at 7°C.

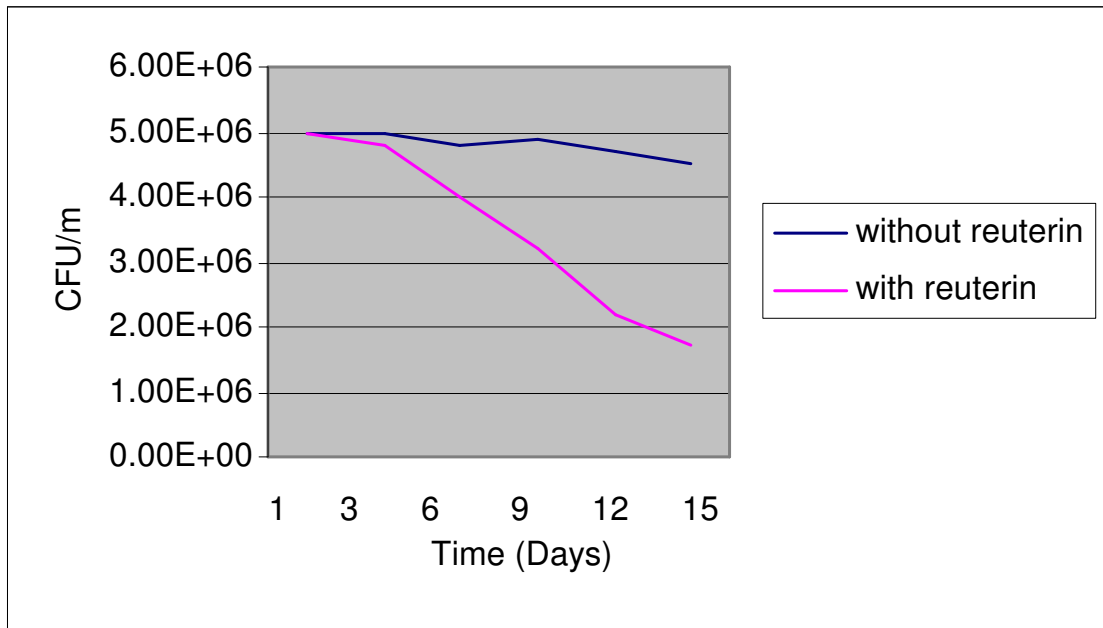


Fig 6- Effect of reuterin at 40 units reuterin per ml on the growth of *Klebsiella pneumoniae* PTCC 1053 in UF-Feta-Cheese at 7°C.

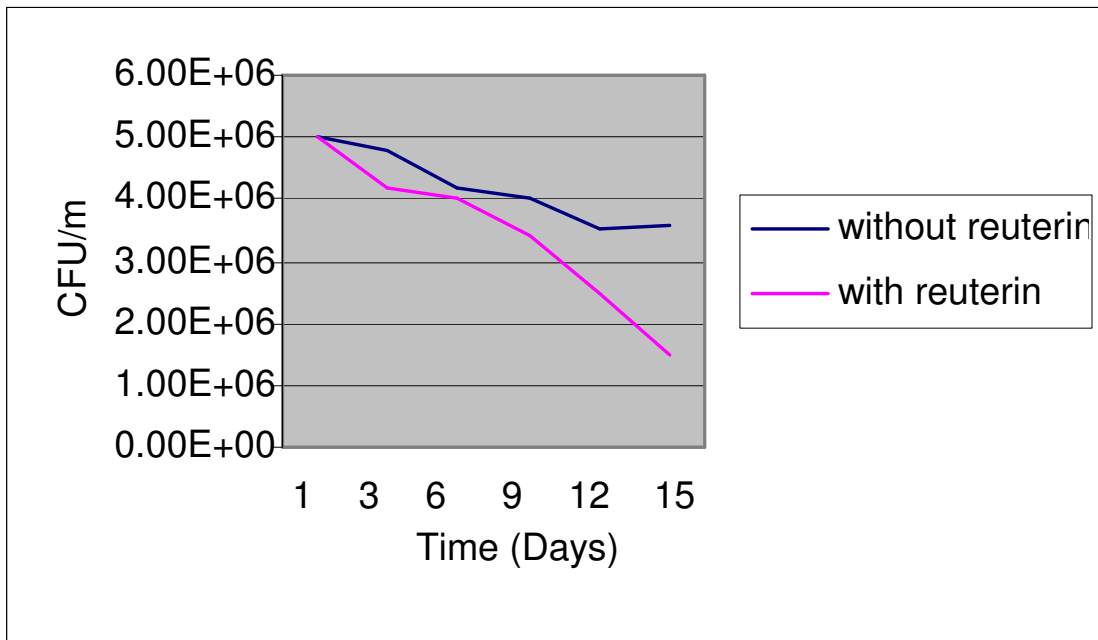


Fig 7- Effect of reuterin at 40 units reuterin per ml on the growth of *Salmonella typhimorium* ATCC 14028 in UF-Feta-Cheese at 7°C.

Conclusion:

It is concluded that reuterin is a suitable biopreservative which protects cheese effectively against some important pathogens especially against *Staphilococcus aureus*, *listeria monocytogenes* and *E.coli*. Also we can use *Lactobacillus reuteri* – glycerol system directly in cheese to produce reuterin.

Knowledgment

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