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Encapsulation of Platelet in Kefiran Polymer and Detection of Bioavailability of Immobilized Platelet in Probiotic Kefiran as a New Drug for Surface Bleeding

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ABSTRACT

***Background*:** Kefir contains lactic acid bacteria (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Acetobacter* and *Streptococcus*) and yeasts (*Kluyveromyces*, *Torula*, *Candida*, *and Saccharomyces*). Kefiran is the polysaccharide produced by lactic acid bacteria in kefir.

***Methods*:** Kefiran was prepared from milk containing 0.5% fat and 10 grams kefir grains and was separated from kefir by ethanol (0.02 gram) following entrapping the platelets to this polymer. Ligand of the platelet-polysaccharide was studied by FTIR.

***Results*:** FTIR results showed that the bands of C-O and C-O-C connections were formed and the polysaccharides had been attached to the receptors of the platelet glycoproteins (GP Ib, GPIIb / IIIa). Stability and encapsulation of the platelet and kefiran were assessed by Coulter Counter. Encapsulation of the platelets by polysaccharide at the beginning caused to reduce the number of platelets following by releasing of 50% of the platelets after 3 hours.

***Conclusion*:** The platelets were encapsulated in kefiran polymer and detected for bioavailability as new drug for surface bleeding. Also, kefiran has antimicrobial and antifungal properties. On the other hand, the existence of nisin in kefiran could be useful as an antibacterial lantibiotic.

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Introduction

Living organisms can synthesize a variable series of polymers (1). Kefir is a microbial symbiont mixture containing lactic acid bacteria (*Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Acetobacter* and *Streptococcus*) and yeasts (*Kluyveromyces*, *Torula*, *Candida*, and *Saccharomyces*) (2, 3). Lactic acid bacteria that excrete polysaccharide with high molecular weight have been extensively studied in the recent decade. Lactobacillus kefiranofaciens which is isolated from kefir grains produce an exopolysaccharide called kefiran (3). Kefiran reagents have various physical characteristics that make them suitable as viscosifying, stabilizing, gelling or emulsifying factors. Kefir polysaccharide known as kefiran is a branched glucogalactan soluble in water that has antibacterial, antifungal and antitumor properties (4, 5). Therefore, kefir could be used as a remedy for the patients affected with one or several strains of resistant microorganism. Moreover, the application of microorganisms for producing polymers by means of fermentation has several advantages such as high yields, reducing production time and complex separation methods (5, 6). On the other hand, nisin, a natural antimicrobial peptide, was isolated in 1947 from Lacto-coccus lactis by Mattick and Hirsch that is the oldest known and most widely studied lantibiotic. Nisin is used as a safe food additive in the world, particularly in dairy products, canned foods and plant protein foods (7). Platelets adhere to the site of vascular injury, generate biological mediators, discharge their granule contents, form multicellular aggregates and serve as a nidus for plasma coagulation reactions. In order to carry out these tasks, the platelet undergoes dramatic structural rearrangements utilizes multiple membrane receptors which bind small molecule mediators, adhesive glycoproteins and constituents of the vascular sub endothelium, and activates a network of complex signaling pathways (8, 9).

Multiple components from the bacteria (*Streptococcus mitis* or *S. sanguis*) could mediate direct binding of the bacteria to platelets. Direct binding of bacteria to platelets is essential for platelet aggregation. Cell wall polysaccharide composed of rhamnosus-glucose which is derived from Streptococcus mutans can stimulate platelet aggregation. Glycoprotein Ib and Glycoprotein IIb/IIIa receptors of platelet have essential role in connection with the polysaccharide (10). Also, poly-N acetylglucosamine nano fibers activate platelets (11). The ability of the bacterial componenets (rhamnosus-glucose) and poly-N acetylglucosamine nano fibers to induce in vitro platelet aggregation suggested that some common properties were involved in polysaccharide-platelet aggregation. Using FTIR, light microscopy and coulter counter, we demonstrated that platelet can be encapsulated with kefiran and released. Also the antibacterial characteristic of kefiran and investigation of nisin existence in kefir were demonstrated. The main object of this study was to investigate the interaction of polysaccharide of kefir with platelet for encapsulation of platelets in kefiran polymer and detection of bioavailability of immobilized platelet in probiotic kefiran as a probable new drug for surface bleeding.

Material and method

Extraction of kefiran polysaccharide from kefir grains

A 10 ml pasteurized milk containing 0.5% fat was heated and cooled down at room temperature and then 10 g kefir grains was added and kept for 48 hours at room temperature. After incubation, kefir grains were separated from the fermented product by a plastic sieve, and then the solvent collected and divided into two parts (12). Then using Gram staining to determine the form of microscopic organisms, catalase and oxidase tests based on differential tests such as TSI, Simmons Citrate Agar, urease, lysine, SIM and MR/VP and decarboxylase agar using standard diagnostic methods and biochemical were identified (17) (Figure 1).

Samples were kept in sterilized distilled water at 50 ºC and 100 ºC respectively for an hour in order to inactivating hydrolyzing enzymes and solving the polysaccharide. Then samples were centrifuged at 1180 g in 20 ºC for 15 minutes to remove all the cells from the sample. The supernatant contains polysaccharide, precipitated by adding two ethanol volumes in 96 % cold ethanol and kept 24 hours at -20 ºC. Then samples were centrifuged at 1180 g for 15 minutes at 4 ºC. The pellet was solved in hot distilled water. This step was repeated twice, and finally the obtained sample was lyophilized after solving the sediment in hot distilled water (13, 14).

Detection of peptide in kefiran

For evaluation of the protein in kefiran, Bradford assay was used to estimate the amount of protein. Standard curve was drawn by bovine serum albumin (BSA) (15).

Detection of polysaccharide in kefiran

The other one assay was used to estimate the amount of polysaccharide in kefiran. Standard curve was drawn by glucose.

Fourier transform-infrared (FTIR) spectroscopy

Purified polysaccharides, platelets and encapsulated platelet-polysaccharide were studied by using FTIR technique. Major structural groups of purified exopolysaccharide were identified by FTIR (4, 16, 17). The Fourier transform-infrared spectra were recorded on a Bruker Vector 22 instrument (Germany) in the region of 4000–400 cm^{-1}, at a resolution of 4 cm^{-1} and processed by Bruker OPUS software.

Platelet counting in encapsulated platelet-kefiran

Pure platelets were provided from Blood organization in Isfahan, Iran. At first, two methods were used to prepare platelet entrapped kefiran solutions (i) 0.5 ml platelets were added to 0.02 gr kefiran. (ii) 0.5 ml platelets were added to 4.5 ml normal saline and 0.02 gr kefiran. Then the numbers of platelets were counted by Coulter Counter (Sesmex, K 1000, USA).

Light microscopy

Evaluation of binding of polysaccharides to the platelets was carried out by light microscopy. Encapsulated Kefiran- platelets and polysaccharide of kefiran were dried on slide glass and then observed under light microscope.

Plasmid extraction and analysis of plasmid extraction by agarose gel electrophoresis and spectrophotometry

Kefir grains were grown in M17 broth and Eliker broth separately (Merck, Darmstadt, Germany). The equivalent volume of standardized microorganism suspensions including 108 colony forming units per ml (cfu/ml) (according to Mc Farland turbidity standards) of both media was used for plasmid extraction. Samples were centrifuged at 1180 g for 15 minutes. The supernatant was removed and 600 µl distilled water was added to the pellets individually followed by adding lysosyme to the samples. Then they were incubated in 37 ºC for 2 hours. Finally, plasmid extraction was done by Fermenase kit. In this study, the quality and quantity of plasmid extraction applied by Fermentase kit were assessed by using spectrophotometry and agarose gel.

Antimicrobial assay

Microbial strains

Staphylococcus aureus (*S. aureus*) (ATCC 25923), *Pseudomonas aeruginosa* (*P. aeruginosa*) (ATCC 27853), *Escherichia coli* (*E. coli*) (ATCC 25922), *Rizoctonia* and *Pseudomonas* sp. were isolated in Microbiology Research Laboratory of the University of Isfahan.

Antibacterial activity assay

Detection of the antimicrobial assay by disk diffusion

Blank disks impregnated with 50 µl of kefir, purified kefiran extraction and purified peptide were applied in the form of disk diffusion to test anti-microbial activities of these extracts. According to the procedure of the Clinical and Laboratory Standards Institute, cell suspensions of 3×10^8 CFU/ml (using McFarland turbidity standard solutions) from each organism were grown in Muller Hinton Agar medium. The microorganisms were *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Escherichia coli* (*E. coli*), *Rizoctonia* and *Pseudomonas* sp. isolated in Microbiology research Laboratory of the University of Isfahan. Plates were kept at 37 ºC for 24 hours and zone of inhibition was studied (19).

Detection of nisin gene in kefiran by PCR

pMRC01 plasmid of Lactococcus lactis was used for nisin primers. The Samples were examined by plasmid–based PCR using NisAF 5`-AAGAATCTCTCATGAGT-3` and NisAR5`-CCATGTCTGAACTAACA-3` primers (20).

The final reaction mix contained 5 µl of the extracted DNA sample, 10pM of each primers, 0.2 mM deoxynucleotide triphosphate (Cinnagen, Iran), 6 mM $MgCl_2$ (Cinnagen, Iran), and 1.5 U of Taq polymerase (Cinnagen, Iran), for a total volume of 25 µl. We used the above mentioned primer set in all the reactions in a touch up PCR program: 94 °C for 2 min, followed by 15 cycles of 94 °C-1 min, 42.5 °C-1 min, and 72 °C-30 sec, followed by 20 cycles of 94 °C-1min, 50 °C-1 min, and 72 °C-30 sec, and finally a final extension at 72°C for 5 min (21, 22).

Result

The measurement of polysaccharide and protein in kefiran

Protein concentration for treatment in 50 ºC and 100 ºC was determined as 41.57 and 55.68 µg/ml respectively, by comparing standard curve drawing by bovine serum albumin. Polysaccharide concentration for treatment in 50 ºC and 100ºC was determined as 796 µg/ml and 364 µg/ml by comparing standard curve drawing by glucose.

FTIR of polysaccharide, platelet and mixture of platelet with polysaccharide

FTIR of polysaccharide showed different peaks including 3224, 3036, 2846, 2053, 1414, 1092, 615. 3224 related to O-H group, C-H,N-H, 3036 is related to O-H, =CH, 2846 is related to C-H, aldehyde, O-H, 2053 is associated to triple, 1414 is related O-H, C0C, 1092 is related to C-C, COC, CO stretch, 615 related to C-CL. 1092 and 1414 contains bands of carbohydrates. 3224, 3036, 2846, 2053 related to chemical structure of kefiran.

FTIR of platelet had peaks 3315, 3067, 2958, 2928, 2871, 1658, 1542, 1449, 1395, 1307, 1241, 1153, 1111, 1078, 929, 837, 739, 700 and 621 (Figure.1). 3315 related to C-H, 3067 related to =CH, 2958 related to C-H, 2928 associated to C-H, 2871 related to C-H, 1658 associated with R2-C=N-R,C=C, 1542 related to N-H band, 1449 related to C-H bend, 1395 associated with CH(CH3)2 OR (CH3)3 bend, 1307 related to C-O stretch, 1241 associated with C-O stretch, 1153 related to C-C stretch, 1111 related to C-C stretch, 1078 associated with C-O stretch, 929 related to C-O stretch, 837 associated with C-H bend, 739 related to C-H bend, 700 associated with C-H bend (23). 1542 and 1658 contain bands of amide I and amideII. 3315, 3067, 2958, 2928, 2871, 1449, 1395, 1307, 1241, 1153, 1111, 1078 related to chemical structure of platelet. 600-900 contain fingerprint region.

Table 1. Platelet counting in 0.5 ml platelet+0.02 gram polysaccharide.

T(minute)	Blank (platelet)	Template (0.5 ml platelet+ polysaccharide)
0		290×10^3
30 (0.5 hours)		290×10^3
60 (1 hour)		290×10^3
90 (1.5 hours)		278×10^3
510 (8.5 hours)		248×10^3
840 (14 hours)		258×10^3

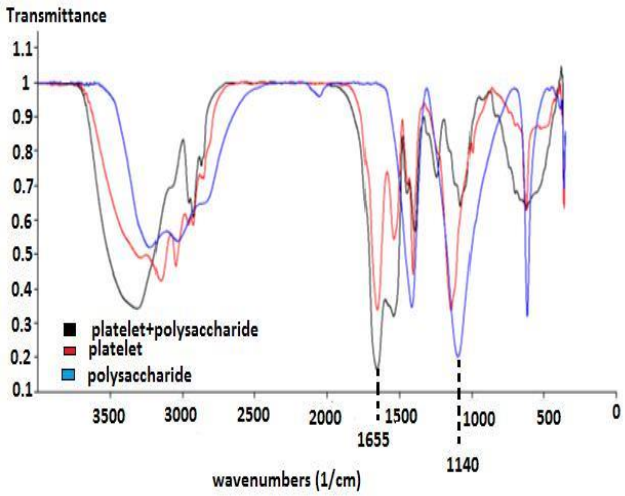

Figure 1. Infrared spectra of platelet and encapsulated of platelet-polysaccharide in 500-4000 cm^{-1}. In two FTIR, Amide I (1655 cm-1) and Amide II didn`t change. 1140 in polysaccharide region has been changed. Finger print region changes is less than polysaccharide region (28). All changes almost are in polysaccharide region, (900-1200 cm-1) in encapsulated of platelet-polysaccharide.

Encapsulated platelet-kefiran had peaks of 3291.89, 3154.01, 3049.87, 2961.16, 2923.56, 2855.1, 2815.56, 1655.50, 1539.88, 1443.46, 1230.36, 1140.69, 996.053, 743.424, 698.105, 621.931 cm-1(Figure.1). 3291 is related to O-H group, 3154, 3049, 2961, 2923, 2855, 2815 associated with C-H stretch, 1655 related to C=C stretch, 1539 related to N-H bend, 1443 related to O-H bend, 1406 associated to C-H in plane bend, O-H bend, 1230 related to C-O stretch, C-C,C-C-O-C , 1140 related to C-C, C-O stretch, C-N, 996 related to C-H bend monosubstituted, 743 associated with C-CL, C-H bend,(ortho), C-H bend mono, 698 related to C-H bend (disubstituted), 621 related to acetylenic C-H bend (23). Amide I (1655 cm-1) and Amide II haven`t been changed. 1140 in polysaccharide region has been changed. Finger print region (900-600 cm-1) (24) changes is less than polysaccharide region (Figure. 1). So, all the changes almost are in polysaccharide region.

Table 2. Platelet counting in 0.5 ml platelet+ 4.5 ml physiology serum+0.02 gram polysaccharide

T(minute)	Blank	Template (0.5 ml platelet+ 4.5ml physiology serum+ polysaccharide)
0	34×10^3	
30 (0.5 hour)		28×10^3
60 (1 hour)		26×10^3
90 (1.5 hours)		23×10^3
120 (2 hours)		23×10^3
870 (14/5 hours)		20×10^3
930 (15/5 hours)		26×10^3
960(16 hours)		30×10^3

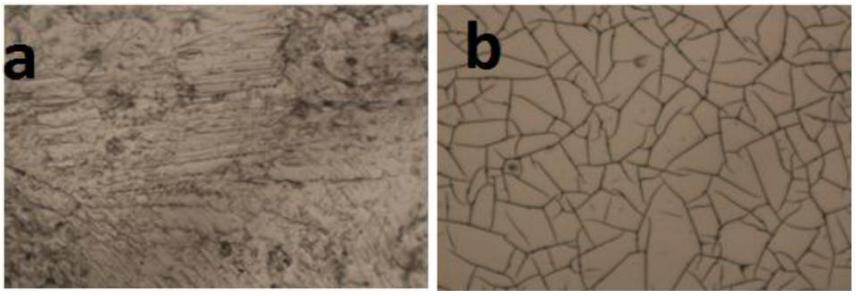

Figure 2. Structure of kefiran and encapsulated of platelet-kefiran were shown by light microscope. The Crystals made from kefiran and polysaccharide organized and neatly regulated shown by light microscope (Figure 2a). The connection of platelets and kefiran by optical microscope showed (Figure 2b).

Platelet counting in encapsulated platelet-polysaccharide solution

Entrapped polysaccharide-platelets and released platelets from kefiran in vitro were detected by the Coulter Counter. The results showed that the platelets count were changeable during the times (first the number of the platelets was reduced then, platelets were released from kefiran). In group I after 510 minutes, 14.5% of the platelets were reduced and then 4% of the platelets were released from kefiran (Table 1). In group II, after 870 minutes, 41.2% of the platelets were reduced, and then 50% of the platelets were released (Table 2), therefore entering and releasing of the platelets were observed following treatment with polysaccharide. So, encapsulated platelet – polysaccharide probably could be used as a new drug for surface bleeding due to releasing of platelets.

Microscopic study

These data were also determined by light microscopic examination. The Crystals made from kefiran and polysaccharide was organized and neatly regulated (Figure 2a). The connection of the platelets and kefiran by optical microscope is shown in Figure 2b.

Analysis of plasmid extraction products by agarose gel and spectrophotometry

Kefir was enriched in M17 and Eliker media then plasmid extraction was done. Plasmid profile extracted from kefir enriched in M17 media have been showed in Figure 3. The amounts of extracted DNA were detected by spectrophotometer (Bio Photometer, Eppendorph, Germany).

The proportion of maximum lambda at 260/280 of plasmid DNA extracted from the M17 and Eliker media were 1.48 and 1.35 respectively. So, the amounts of extracted DNA in M17 and Eliker media were similar.

PCR analysis

The plasmid profiles resulting from the extraction of plasmid DNA were shown by agarose gel electrophoresis (Figure. 3). Also amplification of 899 bp fragment of the plasmid genes for nisin was detected by PCR (Figure.4). This data reveals that this product is a lantibiotic. The existence of nisin in probiotic kefiran could be useful as an antimicrobial drug that induces an increase of both CD4 and CD8 T-lymphocyte cell counts in immune system. Agarose gel electrophoresis showed a non-specific band that it could be due to the type of PCR (touch up PCR). Touch up PCR requires minimum optimization using a temperature range throughout the amplification process (25).

Anti-microbial activities

The crude form of kefiran and purified polysaccharide showed anti-microbial activities against *Rizoctonia* (Fig 5a). This property was more active in the crude form of kefiran. The same results were obtained with *Pseudomonas* sp. isolated in Microbiology research Laboratory of the University of Isfahan as well as *S. aureus* (Figure 5b). In another experiment, anti-bacterial activities of the crude form of kefiran, protein and the purified polysaccharide were determined against *S. aureus* (Figure 5b). The results showed that the anti-microbial effect of the crude form of kefiran was more than the purified polysaccharide and protein. Purified polysaccharide, the crude kefiran and protein showed a weak anti-microbial activity against *Klebsiella*, *E. coli* and *P. aeruginosa*. So, kefiran could be useful as antibacterial drug especially for gram positive bacteria.

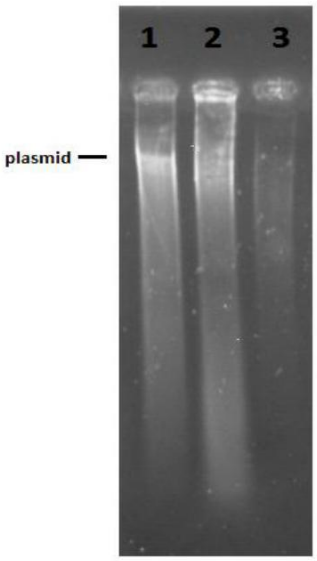

Figure 3. Analysis of the extracted plasmid products by agarose gel electrophoresis. Lane 1: plasmid extraction products by Fermentase kitof samples grown in M17 broth; Lane 2: plasmid extraction products by the Fermentase kit of samples grown in Eliker broth, Lane 3: negative control (without plasmid).

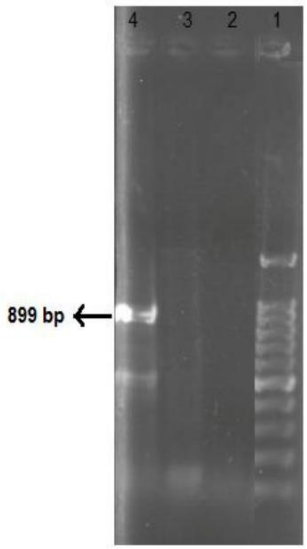

Figure 4. Analysis of the PCR products by agarose gel (1.5%) electrophoresis. The 899 bp DNA corresponds to the nisin DNA sequence. Lane 1 100 bp DNA ladder, lane 2 negative control, lane 3 PCR in the samples grown in eliker broth, lane 4 PCR products by the samples grown in M17 broth.

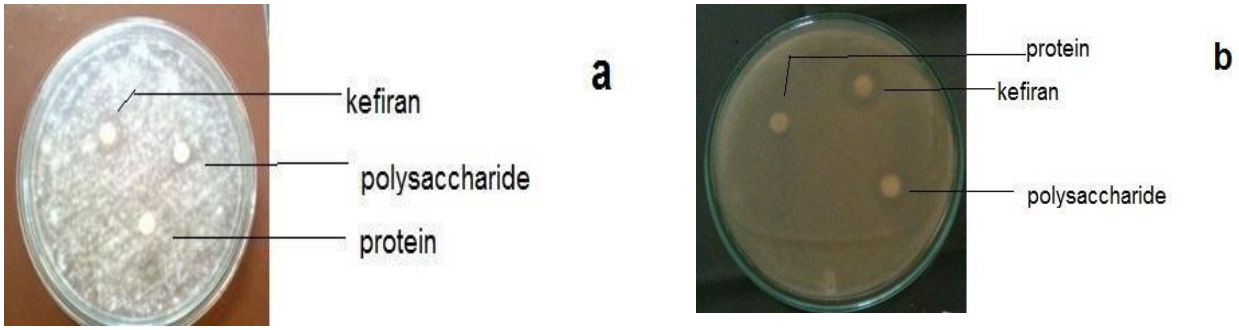

Figure 5. Antimicrobial activity of kefiran, polysaccharide and protein were analyzed by disc diffusion. The crude form of kefiran and purified polysaccharide showed anti-microbial activities against Rizoctonia (Figure 5a). This property was more active in the crude form of kefiran. The same results were obtained with Pseudomonas sp. isolated in Microbiology Laboratory of Isfahan University as well as *S. aureus* (Figure 5b).

Discussion

FTIR Spectroscopy is a useful tool for showing structural changes in biopolymers. The FTIR analyses showed that platelet-polysaccharide encapsulated, have addition peaks compare to blanks (platelet and polysaccharide). The platelets have, for example, C = C and NH and some couriers to polysaccharide linked as C-C-O-C. The acetylated C-H bond are for platelet-polysaccharide, therefore, it is concluded that the polysaccharide may interact with platelets via polysaccharide receptors. On the other hand, it should be noted that 2800 -3000cm-1 area is the fatty acid, 1500-1700 cm-1includes the amide I and II bands of proteins and peptides. Area 1200-1500 cm-1 is the mixed region of the fatty acid, protein, and phosphorus compounds, Region 900-1200 cm-1 contains bands of carbohydrates such as polysaccharides and 700-900cm-1 includes finger print region (24). In Mixture of platelets and polysaccharide (encapsulated platelet-polysaccharide) and platelets was shown that the amide I and II did not change, but the peak 1140 of polysaccharide had changed. Changes in finger print area are less than the polysaccharide area. Thus, all of the changes are in the polysaccharide area. Since there are transmembrane glycoprotein receptors binding in platelet lead to aggregation of platelet to kefiran. According to the characteristics and structure of platelets and kefiran, the ligand between these two has been occurred. It was shown that glycoprotein receptors of platelets bound to collagen and platelet aggregation associated. This reaction can be occurred with platelet and kefiran too. Fischer and colleagues in 2012 reported that poly-N acetylglucosamine nanofibers activate platelets in sub-unit B3 (CD61) and CD42b. Their study reported that Glycoprotein Ib and Glycoprotein IIb / IIIa platelet have essential role in connection with the polysaccharide (11).

Chia et al in 2004 reported that the cell wall polysaccharide composed of rhamnosus-glucose which is derived from *Streptococcus mutans* can stimulate platelet aggregation (10). In addition, variation in platelet count showed the release of these cells from kefiran .These might be as result of connection and disconnection of the bonds between platelets and the polysaccharide. Many receptors are in platelet such as glycoprotein Ib-IX-V complex (GPIb-IX-V), glycoprotein Ia / IIa complex (GPIa / IIa = integrin α2β1), glycoprotein IIb / IIIa complex (GPIIb / IIIa = integrin αIIbβ3, GPV / IIIa (GPV / IIa = integrin α5β1, GPV / IIIa GPV / IIa = integrin α5β1) (8, 9, 26) could interact with the polysaccharide kefiran. Moreover, in platelet counting, increasing of platelet number counts in group i and ii of encapsulated platelet-kefiran could be due to the adhesion and disassociation of platelets and polysaccharide bonds. Our results showed that *S. aureus* was more susceptible to antimicrobial activity of kefiran than *E. coli*. Rodrigues et al. (2005), a range of bacteria consisting of *S. pyogenes*, *S. aureus*, *Streptococcus salivarius*, *Candida albicans*, *Salmonella typhimurium*, *P. aeruginosa* and *E. coli* were evaluated for sensitivity to kefiran. Rodrigues et al. (2005), proved that *S. pyogenes* is the most sensitive bacteria to kefiran. *S. aureus*, *Streptococcus salivarius*, *Candida albicans*, *Salmonella typhimurium* showed lower sensitivity to this product. *P. aeruginosa* and *E. coli* showed the least sensitivity to kefiran (19). In this study, the antimicrobial effect of kefiran to *S. aureus*, *E. coli*, *P. aeruginosa*, isolated *Pseudomonas* and *Rizoctonia* were studied. The rate of sensitivity of *P. aeruginosa*, *E. coli* and *S. aureus* are in accordance with the work done by Rodrigues et al (2005). It seems that the reason of the sensitivity of isolated *Pseudomonas* to kefiran could be due to the presence porins in the outer membrane of these bacteria which is not seen in other bacteria. Resistance of gram negative bacteria to kefiran might be related to the outer membrane structure of the bacteria.

In another study, Medrano et al. (2007) showed the protective effect of kefiran against

structural cell damages produced by some virulence factors of *Bacillus cereus* strain B10502 (27). Antibacterial, nisin is used as a food additive in more than 50 countries (28). Pablo and colleagues in 1999 reported that short-term administration of a diet containing nisin increase in the number of CD4 and CD8 (29). The presence of nisin in polymer kefiran, is promising for various applications. Also Plasmid extraction from enriched kefir in M17 and Eliker media showed that many genes for lantibiotics are included in kefir which was detected by the PCR. Rich environments such as MRS or M17 have been selected for genetic studies (30).

Conclusion

The encapsulation of living biological cells like platelets could be served as a model system in a broad range of biomedical applications including local and sustained drug delivery, immune protection of artificial tissues, and versatile artificial blood. Also, kefiran has antimicrobial, anti-tumor and antifungal properties. On the other hand, the existence of nisin in kefiran could be useful as an antibacterial lantibiotic and can induce the number of both CD4 and CD8 T-lymphocyte. Further studies are required to further explore these observations.

Conflict of interest

None declared conflicts of interest.

Financial disclosure

There is no financial disclosure.

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