

Kefiran biopolymer: Evaluation of its physicochemical and biological properties

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Hajer Radhouani^{1,2,3} , Cristiana Gonçalves^{1,2,3},
Fátima R Maia^{1,2,3}, Joaquim M Oliveira^{1,2,3}
and Rui L Reis^{1,2,3}

Abstract

Kefiran, an exopolysaccharide produced by lactic acid bacteria, has received a great interest due to a variety of health claims. In this study, we aim to investigate the physicochemical and biological properties of Kefiran polysaccharide extracted from Portuguese kefir grains. The kefir growth rate was about 56% (w/w) at room temperature and the kefir pH after 24 h was about 4.6. The obtained yield of Kefiran polysaccharide extracted from the kefir grains was about 4.26% (w/w). The Kefiran structural features were showed in the ¹H nuclear magnetic resonance spectrum. The bands observed in the infrared spectrum confirmed that the Kefiran had a β-configuration; and the X-ray photoelectron spectroscopy analysis confirmed the structure and composition of Kefiran and revealed a C/O atomic ratio of 1.46. Moreover, Kefiran showed an average molecular weight (Mw) of 534 kDa and a number-average molecular weight (Mn) of 357 kDa. Regarding the rheological data obtained, Kefiran showed an interesting adhesive performance accompanied by a pseudoplastic behavior, and the extrusion force of Kefiran was 1 N. Furthermore, Kefiran exhibited a higher resistance to hyaluronidase degradation than hyaluronic acid. Finally, Kefiran showed a lack of cytotoxic response through its ability to support metabolic activity and proliferation of L929 cells, and had no effect on these cells' morphology. Our research suggested that Kefiran polymer has attractive and interesting properties for a wide range of biomedical applications, such as tissue engineering and regenerative medicine.

¹3B's Research Group, I3Bs—Research Institute on Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, Guimarães, Portugal

²CVS/3B's—PT Government Associate Laboratory, Braga, Portugal

³The Discoveries Centre for Regenerative and Precision Medicine, University of Minho, Guimarães, Portugal

Corresponding author:

Hajer Radhouani, 3B's Research Group, I3Bs—Research Institute on Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, Parque de Ciência e Tecnologia, Zona Industrial da Gandra, Barco, 4805-017 Guimarães, Portugal.
Email: hajer.radhouani@i3bs.uminho.pt

Keywords

Exopolysaccharide Kefiran, cytotoxicity, drug delivery system, enzymatic resistance, physicochemical characterization, Portuguese kefir grains

Introduction

Kefir, a traditional cultured-milk beverage, is considered one of the oldest methods of both temporary and long-term food preservation.¹ It was originated in the Middle and Far East of Asia, 1000s of years ago.^{2,3} This natural probiotic, with a range of reported health benefits, has been extremely popular in Eastern Europe, where it is regularly administered as probiotic food to patients in hospitals and recommended for infants. It is beginning to gain a worldwide acceptance and popularity as a healthy probiotic beverage; being commonly home fermented from shared grains, it also has been recently manufactured on a commercial scale.²

Kefir grains, the starter for obtaining the fermented milk kefir, are elastic, slimy, varying from white-to-light yellow in color and with an irregular and lobed-shaped cauliflower-like structure of different sizes (1 and 3 cm) in length.⁴ These grains are composed of proteins and polysaccharides, containing lactic acid bacteria (LAB), acetic acid bacteria, and yeast, held together by a matrix of protein and polysaccharide and yeast involved in the fermentation⁵ LAB such as *Lactobacillus* are generally recognized as safe and are known to produce extracellular polysaccharides (EPS), which contribute to the texture of the resulting fermented milk.⁶

The main exopolysaccharide of kefir grains, named Kefiran,⁷ is mainly produced by *Lactobacillus kefiranofaciens* but also by several other unidentified species of *Lactobacillus*.⁸ Kefiran, the clear or pale yellow slimy exopolysaccharide, is a water-soluble polysaccharide containing approximately equal amounts of glucose and galactose.⁹

Kefir can be considered as a remarkable probiotic resource due to its variety of health claims besides its nutritional status. It is important to highlight that, when compared with other polysaccharides, Kefiran has already several important advantages reported, such as antibacterial, antifungal, and antitumor properties.^{10–13} For commercial application, Kefiran must be produced at high levels of quality, in a low-cost media and the isolation procedure must be economically attractive. Taking into account these considerations, Kefiran polymer obtained from kefir grains is a promising alternative for several biomedical applications.

In this work, Kefiran exopolysaccharide, produced by kefir grains from Portugal, was characterized for its chemical and physical properties by different techniques, as well as its enzymatic resistance and cytotoxicity evaluation.

Materials and methods

Materials

Hyaluronic acid. Hyaluronic acid (HA) sodium salt from *Streptococcus equi* used in this research is a bacterial HA, which is a natural non-sulphated high molecular weight glycosaminoglycan. HA was provided by Sigma-Aldrich (St Louis, USA) (cat 9067-32-7, 53747).

Milk kefir production. Kefir grains (50 g), used as a starter culture in this research, were purchased from a household in Guimarães, Portugal. The grains were cultured overnight in skimmed milk in a closed sealed glass at room temperature, and the medium was exchanged daily for a new culture. This procedure was continued for 15 subsequent days in order to maintain the grain's viability.

Milk kefir grain mass. After opening the glass bottles, the culture milk-product kefir grains were sieved (in a plastic filter with 0.25 cm pore size) to separate the milk kefir grains from the fermented milk. The milk kefir grains were washed with 200 mL of sterile saline solution, and the total kefir grain mass (wet mass) was weighed. After determining the kefir grains mass, a certain amount of grains was used for the Kefiran isolation procedure.

Acidification kinetics. Throughout the fermentation, the pH's of Kefiran samples were regularly measured with a digital pH meter (Model HI5222-02, Hanna Instruments, Bedfordshire, UK). The pH meter was calibrated with standard buffer solutions of pH 4.0 and 7.0 before measuring the fermented kefir samples.

Isolation of Kefiran polysaccharide. Kefiran in the kefir grains was extracted by a method previously described¹⁴ with some modifications. Briefly, kefir grains (20 g) were treated in boiling water (1:10) for 30 min, with discontinuous stirring. The mixture was centrifuged at 18,300 G for 20 min at 20°C. The polysaccharides in the supernatant were precipitated by addition of two volumes of cold ethanol absolute and left at -20°C overnight. The mixture was again centrifuged at 18,300 G for 20 min at 4°C. Pellets were dissolved in hot water during 5 h and the precipitation procedure repeated three times. The precipitates were dissolved in water at 60°C and the resulting solution concentrated for a crude polysaccharide. Kefiran polysaccharides extracted were purified by dialysis (dialysis tubing cellulose membrane cut-off 14 kDa) (Sigma-Aldrich, St Louis, USA) for 1 week against distilled water to remove the impurities. Water was completely removed and exchanged at least three times per day. After dialysis and lyophilization, Kefiran polysaccharides were kept in desiccator to prevent adsorption of moisture. It is important to highlight that the yeast and bacteria in the filtrate were removed by centrifugation and filtration through a 0.22- μm filter (Millipore, Billerica, MA, USA) for cytotoxicity analysis.

Methods

¹H nuclear magnetic resonance spectroscopy. A solution of Kefiran was prepared (1% w/v) in deuterium oxide (Sigma-Aldrich, St Louis, USA) at room temperature, and 700 μL of this solution was transferred to an NMR sample tube. ¹H nuclear magnetic resonance (¹H-NMR) spectra were recorded on a Varian Unity Plus (Varian, Palo Alto, USA) spectrometer at 60°C using a resonance frequency of 400 MHz. Chemical shifts were reported in ppm (δ). MestReNova Software 9.0 (Mestrelab Research) was used for spectral processing.

Fourier-transform infrared spectroscopy. Powder samples of Kefiran were mixed with potassium bromide and were molded into a transparent pellet using a press (Pike, Madison, USA). Transmission spectra were acquired on IRPrestige-21 spectrometer (Shimadzu, Kyoto, Japan), using 50 scans, a resolution of 4 cm^{-1} , and a wavenumber range between 4400 and 400 cm^{-1} .

X-ray photoelectron spectroscopy. The chemical composition of a powder sample of Kefiran was determined by X-ray photoelectron spectroscopy (XPS) surface measurements using a Kratos AXIS Supra instrument equipped with aluminum $\text{K}\alpha$ -monochromatized radiation at 1486.6 eV X-ray source, within ESCApe software. The C1s, O1s and survey spectra were recorded using a Kratos AXIS Supra instrument (Manchester, UK). Monochromatic X-ray source Al- $\text{K}\alpha$ (1486.6 eV) was used for all samples and experiments. The residual vacuum in the X-ray analysis chamber was maintained at around 8.5×10^{-9} torr. The samples were fixed to the sample holder with double-sided carbon tape. The binding energies (BEs) positions were referenced to the C1s by setting the

binding energy of C1s photo peak at 285.0 eV C1s hydrocarbon peak. We have also employed an electron flood gun to minimize surface charging (Charge compensation). A wide-scan survey spectrum was used to identify and quantify the elements in the sample. High-resolution narrow scans are used to build the chemical state assessment, as well as to quantify the presence of the reference elements in the sample. Data analysis and atomic quantification were determined from the XPS peak areas using the ESCApe software supplied by the manufacturer Kratos Analytical (Manchester, UK). The effect of electric charge was corrected by the reference of the carbon peak (285 eV).

Gel permeation chromatography–size exclusion chromatography. Measurements were performed on a Malvern Viscotek TDA 305 using the refractometer and right angle light scattering detectors. Sample separation was achieved using four columns (PSS, Mainz, Germany): Suprema precolumn (5 μm , 8 \times 50 mm), Suprema 30 \AA (5 μm , 8 \times 300 mm), Suprema 1000 \AA (5 μm , 8 \times 300 mm), and Suprema ultrahigh (10 μm , 8 \times 300 mm). The system was kept at 30°C and phosphate-buffered saline (PBS) buffer (Sigma-Aldrich, St Louis, USA), 0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride, pH 7.4) was used as eluent at rate of 1 mL/min. Pullulan 21.1 kDa and polydispersity index (PDI) 1.09 (PSS standard services) was used for the calibration of the detectors. The d^*n^*/d^*C^* was measured on-line assuming that the input sample concentration is correct as recommended by Malvern and determined as 0.128.

Differential scanning calorimetry. Differential scanning calorimetry (DSC) experiments were conducted using TA-Q100 equipment, under a nitrogen atmosphere. The dry Kefiran powder samples (5–10 mg) were prepared and packed in aluminum pans. An empty aluminum pan was used as reference. The samples were heated in two stages at a constant heating rate of 20°C/min from 0°C up to 500°C, then were left at this temperature during 2 min and cooled at 20°C/min to the initial temperature. At this point, a second heating run was conducted.

Simultaneous thermal analyzer. The simultaneous thermal analyzer (STA) analysis was performed on the Simultaneous Thermogravimetric Analyzer (STA7200 Series, Hitachi, Tokyo, Japan) providing measurement of thermogravimetric analysis (TGA) with selected heating rates of 10°C/min in the range of 40°C–800°C. Samples of Kefiran powder, all of mass 2.5 mg, were put in alumina pan in an atmosphere of dry nitrogen and oxygen.

Rheology. Rheological analyses were performed using a Kinexus pro+ rheometer (Malvern Instruments, Malvern, UK), using the acquisition software rSpace. The measuring system used in these experiments was equipped with stainless steel (316 grade) cone-plate system: the upper measurement geometry cone, 40 mm of diameter and 4°, and a plate lower pedestal. The surface geometry was covered with dodecane to prevent water loss.

Rotational experiments. Rotational experiments were performed in order to obtain shear viscosity as a function of the shear rate, from 0.01 to 1000 s^{-1} , at 37°C. All plots are obtained by the average of at least three experiments. These experiments were conducted with Kefiran samples at different concentrations 1% and 10% (w/v) in H_2O .

Oscillatory experiments. Oscillatory experiments were performed to study viscoelasticity, by obtaining frequency sweep curves. All plots are obtained by the average of at least three experiments. These experiments were conducted with Kefiran samples at different concentrations 1% and 10% (w/v) in H_2O .

Pull away experiments. This experiment involved loading a sample and then pulling away the upper plate at a defined gap speed (1 mm s^{-1}), with a contact time of 2 s and a contact force of 1 N. The resultant normal force was then recorded as function of gap and was used to determine the adhesion properties (area under the force–gap curve).

Injectability (extrusion) assay. The injectability assay of the samples was performed using an injection measurement device (KD Scientific, Holliston, USA) commonly consisting of a syringe pump with a plastic syringe (1 mL) and a needle gauge of 21. The syringe was filled up with the different formulation solutions working in extrusion mode with a rate of 1 mL/min. Regarding the HA sodium salt from *S. equi* used in this research, it is a bacterial HA which is a natural non-sulphated high molecular weight glycosaminoglycan. HA was provided by (Sigma-Aldrich, St Louis, USA) (cat 9067-32-7, 53747).

Enzymatic resistance. Kefiran and HA were subjected to enzymatic reactions with hyaluronidase (EC 3.2.1.35; Hase), under pH 7 in a water bath at 37°C . The samples were cooled in an ice bath in order to stop the enzymatic reaction. The result was measured as the release of reducing sugars, using the DNS (dinitrosalicylic acid) method,¹⁵ since studied polysaccharides had reducing ends on the repeating units of the structure. Glucose was used as standard. Gel permeation chromatography–size exclusion chromatography (GPC-SEC) was also obtained for some experiments to confirm the reliable use of DNS method to monitor the enzyme action over the polysaccharides. HA was used as the negative control and non-substrate as positive control.

Analysis of Kefiran's cytotoxicity using a L929 cell line

Cell culture. Mouse L929 fibroblastic cell line was used to evaluate Kefiran cytotoxicity as described in the ISO 10993-5 (2009). Briefly, in the first day of experiment, 10,000 cells were seeded in each well of a 96-well plate. Then, in accordance with ISO 10993-12 (2012), the samples were prepared (4% w/v) of Kefiran diluted in 0.9% w/v of NaCl, and milk used as culture medium for kefir grains. In the next day, the culture medium was replaced for each of the previous solutions diluted in culture medium at final concentration of 1% v/v. The culture medium was composed of low-glucose Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St Louis, USA) supplemented with 10% v/v fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and 1% v/v antibiotic/antimycotic (Invitrogen, Carlsbad, CA, USA). Sample composed of culture medium was used as a negative control (Ctrl–). A positive control (Ctrl+) composed of Triton X-100 (Sigma-Aldrich, St Louis, USA) at a concentration of 1% v/v in culture medium was used. Cultures were maintained at 37°C under a humidified atmosphere of 5% v/v CO_2 in air. Finally, at 24, 48, and 72 h of culture, cell growth, cell proliferation, and cell damage were analyzed in this experiment.

Cell growth. Cell growth was assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS, Promega, Madison, WI, USA). At each time point (24, 48, and 72 h), cells were incubated with 20% v/v of MTS in culture medium without phenol red (Sigma-Aldrich, St Louis, USA) for the period of 3 h at 37°C . The supernatant was then transferred to a new 96-well plate and absorbance measurements were carried out using a microplate reader (Synergy HT, BioTek, Vermont, USA) at 490 nm.

Cell proliferation by dsDNA quantification. Cell proliferation was assessed by total double-stranded DNA (dsDNA) quantification. At each time point, cells were incubated for 1 h at 37°C in ultrapure H_2O . Then, cell lysates were transfer to a 1.5-mL tube and stored at -80°C until analyzed. Quant-iT PicoGreen dsDNA kit (Molecular Probes, Invitrogen, Eugene, USA) was used

according to manufacturer's instructions. Briefly, samples were transferred to a 96-well white plate and diluted in TE buffer. After adding the Quant-iT PicoGreen dsDNA reagent, samples were incubated for 10 min at room temperature in the dark, and fluorescence was quantified using a microplate reader (Synergy HT, BioTek) with Ex/Em at 480/530 nm. R.F.U. were converted into ng/mL using a standard curve of DNA in the range of 1–2000 ng/mL.

Cell damage. Cell damage was studied through F-actin staining. For that, cells were washed with phosphate buffer saline (PBS) (Sigma-Aldrich, St Louis, USA), fixed with 10% neutral-buffered formalin (Thermo Fisher Scientific, Michigan, USA) for 15 min and permeabilized for 5 min with 0.1% v/v Triton X-100 (Sigma-Aldrich, St Louis, USA) in PBS. Afterward, samples were incubated for 30 min in 1% w/v BSA (Sigma-Aldrich, St Louis, USA) in PBS to block unspecific binding. F-actin filaments were stained with Phalloidin–Tetramethylrhodamine B isothiocyanate 1:40 (Sigma-Aldrich, St Louis, USA) and nuclei were counterstained with 1:5000 of the stock of 4,6-Diamidino-2-phenylindole, dilactate solution (DAPI, 1 mg/mL) (Biotium, Hayward, USA). Samples were analyzed by fluorescence inverted microscope (Axio Observer, Zeiss, New York, USA).

Statistical analysis. All quantitative experiments were run in triplicate, and results were expressed as a mean \pm standard error for $n=3$. Statistical analyses were performed using GraphPad Prism 6.0 software. The non-parametric Mann–Whitney test was used to compare two groups, whereas comparison between more than two groups was performed using the Kruskal–Wallis test followed by Dunn's comparison test. The critical level of statistical significance chosen was $p < 0.05$.

Results and discussion

Milk kefir grain mass

The kefir grains (10% w/v) were cultured in skimmed milk, and a growth rate of the kefir grains of about 56% (w/w) was obtained after a 15-day culture period at room temperature.

Kinetics and mass Kefiran isolated

In our research, the pH of the kefir media after 24 h was acid ($\text{pH} = 4.62 \pm 0.618$) at room temperature. The kefir pH reported in the literature is usually reported between 4.2 and 4.6.¹⁶ Other studies reported lower values of pH that reached 3.9.^{8,17,18} This lower value of pH is possibly due to the presence of some components, such as carbon dioxide, acids, lactose, ethanol, proteins, and fat contents.^{16,19}

Regarding the obtained yield of Kefiran polysaccharide isolated from the kefir grains, it showed an interesting value of 4.26% (w/w). A slightly lower yield of extracted Kefiran (3.16% w/w) was observed in other report by Zajsek and Gorsek.¹⁸

¹H-NMR spectroscopy

¹H-NMR spectroscopy is a fundamental tool when studying the chemistry of polysaccharides. The use of ¹H-NMR spectroscopy, over other techniques such as chromatography, is characterized by some interesting advantages (i.e. easy sample preparation, easy equipment calibration, and fast obtained results).²⁰ Figure 1 shows the ¹H-NMR spectrum of the polysaccharide Kefiran in D₂O at 60°C.

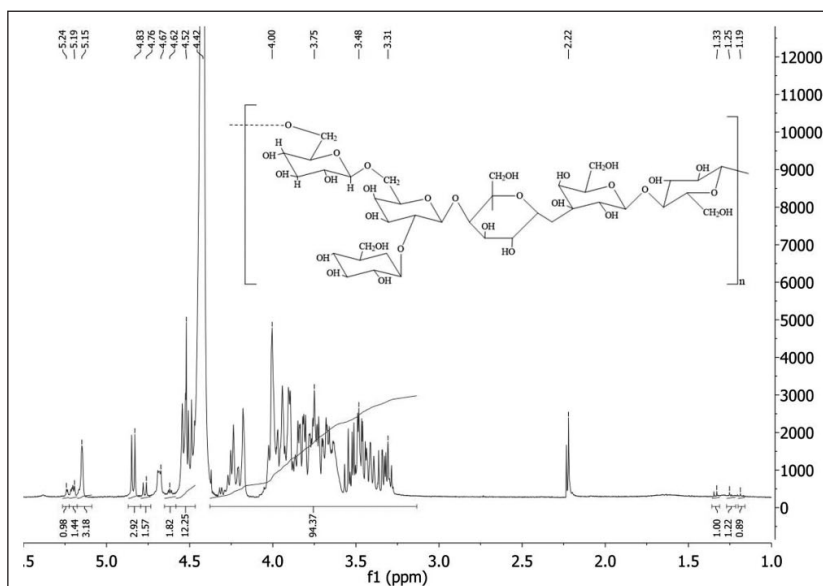


Figure 1. $^1\text{H-NMR}$ spectrum of Kefiran in D_2O at 60°C .

The $^1\text{H-NMR}$ spectrum revealed a peak at 5.15 ppm for an anomeric β hydrogen and showed also six signals at the chemical shifts of 4.85, 4.83, 4.78, 4.76, 4.67, and 4.62 ppm for several anomeric α hydrogens, assigned to a sugar on a lateral branch. With the results obtained by this technique, it was possible to identify the molecule structure of Kefiran. It is worth noticing that the $^1\text{H-NMR}$ data agree with those reported for a polysaccharide Kefiran produced by a ropy *Lactobacillus* and *L. kefirifaciens*.^{21,22}

Fourier-transform infrared spectroscopy

In order to further characterize Kefiran isolated from kefir grains and to identify the fundamental groups present in its structure, infrared radiation (IR) analysis was performed (Figure 2).

In the IR spectrum shown in Figure 2, a band at 3430 cm^{-1} that corresponds to the hydroxyl groups can be observed. In fact, this band region is attributed to the stretching vibration O–H in the constituent sugar residues.^{16,23,24} The band at 2930 cm^{-1} , which is associated with the stretching vibration of C–H in the sugar ring, can be assigned to methyl and methylene groups. The band at 1700 cm^{-1} is due the stretching vibration of O–H. It is important to highlight that reactive functional groups in polysaccharides make them easily flexible to several modifications, which is highly important for medical applications. Moreover, hydrogels can be quickly and easily obtained, which make them excellent candidates for tissue engineering and regenerative medicine.

The band around 1400 cm^{-1} is attributed to CH_2 and OH groups.^{25,26} The region of $1100\text{--}1150\text{ cm}^{-1}$ has showed intense absorptions, a characteristic of stretches C–O–C and alcohol groups in carbohydrates.¹¹ The presence of band at 900 cm^{-1} indicated β -configuration and also vibration modes of glucose and galactose.²⁷ According to several authors, these bands confirmed that the compound obtained is a polysaccharide.^{16,23–25} Besides chemical properties, applicability of polysaccharide in the tissue engineering field is largely dependent on its physical behavior.²⁸

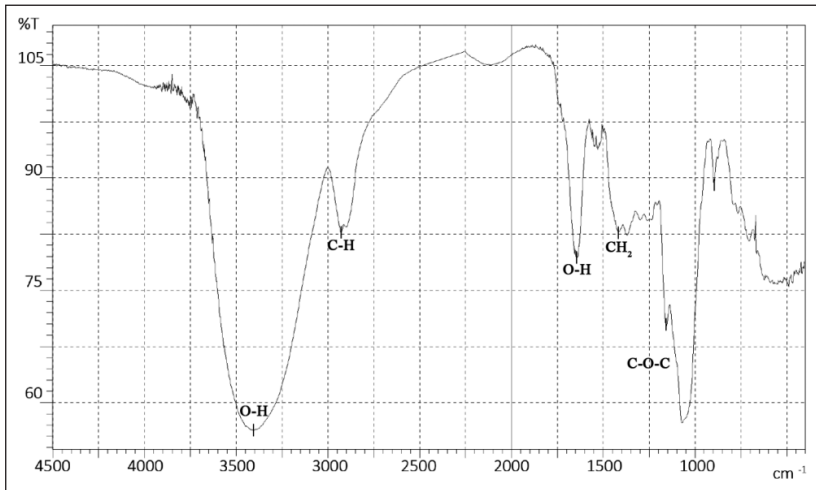


Figure 2. Infrared spectrum of Kefiran between 500 and 4000 cm^{-1} .

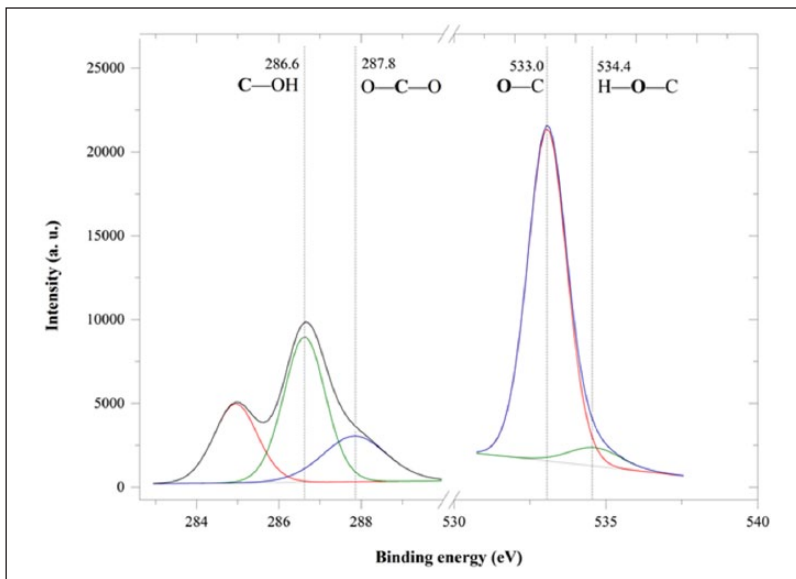


Figure 3. XPS spectra of Kefiran.

XPS

XPS analysis was carried out in order to evaluate the chemical bindings of Kefiran polysaccharide (Figure 3).

The C1s spectra of XPS spectra clearly revealed the presence of C–OH bond at 286.6 eV²⁹ and O–C–O bond at 287.8 eV.³⁰ Regarding the oxygen O1s peak, it was decomposed in two components attributed mainly to O–C bond at 533 eV³¹ and to oxygen bond to hydrogen [H–O–C] at 534.6 eV.³² Moreover, the XPS results revealed a C/O atomic ratio of 1.46. It is important to

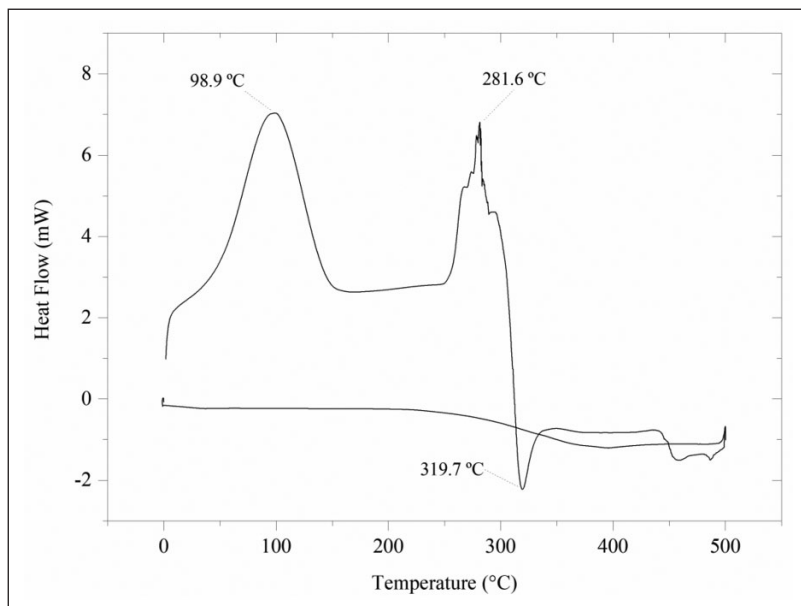


Figure 4. DSC curve of Kefiran (0°C–500°C).

highlight that XPS analysis has a depth of few nanometers and was performed in order to obtain supplementary information about the chemical structure of Kefiran polysaccharide.³³ In fact, the detailed data obtained regarding the bonds confirmed the results obtained through Fourier-transform infrared spectroscopy (FTIR) technique previously described.

In fact, FTIR and XPS results are complementary to each other. XPS helps identify the surface functional groups while FTIR analyses the overall availability of different functional groups.³⁴

GPC-SEC

The molecular weight of a substance, particularly a polymer, is a key chemical characteristic that can dramatically influence the material mechanical performance, especially the viscosity and the rheological behavior.³⁵ In this sense, size exclusion chromatography allowed determining the number-average molecular weight (M_n) and weight-average molecular weight (M_w) of Kefiran polysaccharide. Results showed an average of molecular weight of 534 kDa and a number-average molecular weight of 357 kDa, with a PDI of 1.49. The molecular weight has a significant impact on the polymer properties such as degradation, mechanical strength and solubility. It is important to highlight that production of low molecular weight biomaterial is highly interesting and desirable in modern medicine as it provides a shorter degradation rate.^{36,37}

DSC

DSC is a thermal analysis technique relevant in the characterization of the thermal properties and transitions of a polymer.²⁰ DSC was performed to Kefiran samples with two straight runs (Figure 4).

Kefiran thermogram (endothermic heat flow) exhibited a sharp endothermic peak at $98.7 \pm 0.2^\circ\text{C}$. This transition ($\sim 99^\circ\text{C}$) could be related to its melting point, being explained by the hydrophilic nature of Kefiran functional groups; the presence of this peak can also reveal the existence of water

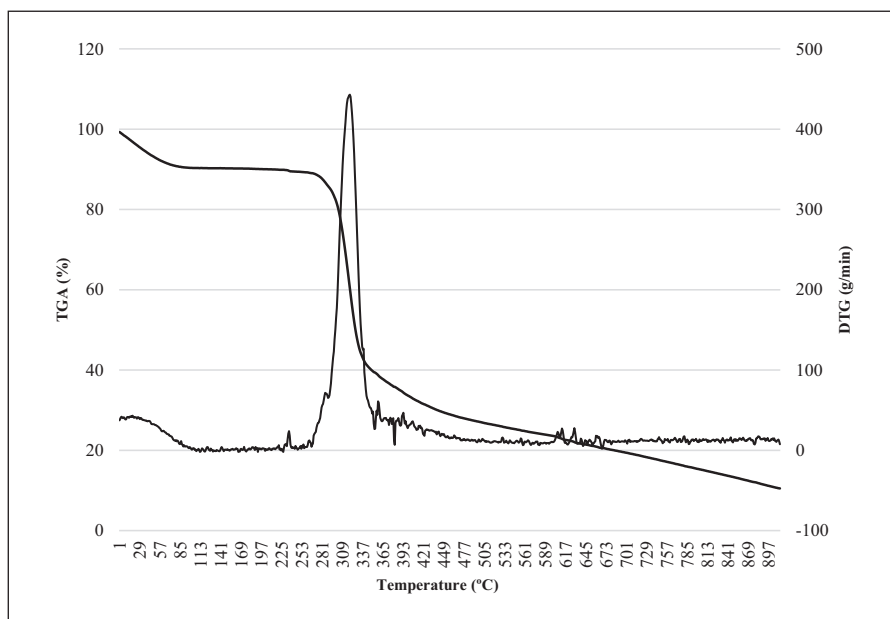


Figure 5. TGA and DTG curves of Kefiran (40°C–800°C).

bound. The appearance of several peaks at around 282°C was considered as just one peak. All these peaks were reproducible for all the replicates. The results showed in the DSC curve are in agreement with those presented in the TGA curve (Figure 5). In our research, Kefiran polysaccharide showed a similar thermal stability, compared to Kefiran extracted in other study that reported a degradation temperature of 352°C.¹⁶

STA

In the TGA figure (Figure 5), one event can be observed during the increasing of the temperature (40°C–106°C). This event occurred with the maximum mass loss (approximately 9%), which could be associated with the loss of moisture.¹⁶

The major weight loss (12%–65%) occurred in the second event in the range of 264°C–350°C, which was due to major degradation of Kefiran polysaccharide structure. This event was associated with an extensive enthalpy change as depicted in the DSC curve (Figure 4). Other studies of exopolysaccharides showed approximated temperature of degradation, with maximum at 352°C.¹⁶ The TGA profiles of the curves observed in this study were similar to those identified in a previous research, which reported the thermal properties of some natural polysaccharide materials.³⁸

It is important to point out the interesting thermal characteristics of Kefiran, reported in our research, for potential industrial applications where processing temperatures reach approximately 150°C.¹⁶

Rheology

Rotational experiments. Flow behavior of Kefiran solutions (1% and 10% w/v) was illustrated in Figures 6(a) and 6(b), respectively. Figure 6(a) presented the shear viscosity function to the shear rate.

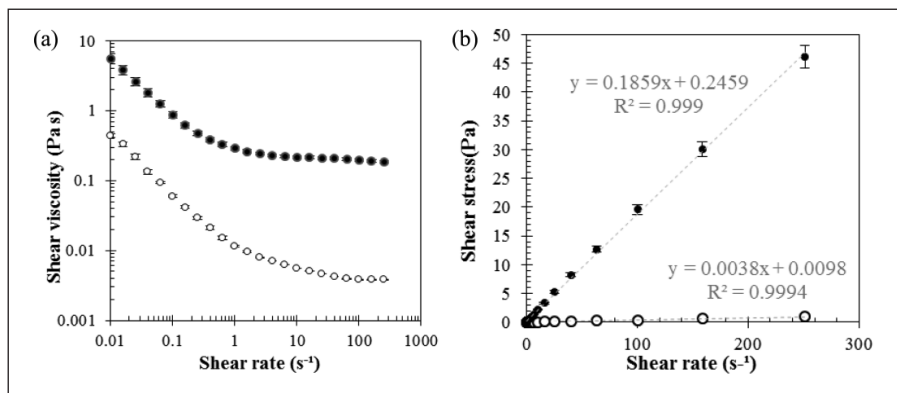


Figure 6. Curves of shear viscosity (a) and shear stress (b) versus shear rate of Kefiran samples at 1% (open symbol) and 10% (filled symbol) of concentration.

Shear viscosity of Kefiran samples at different concentrations decreased with the increment of shear rate (Figure 6(a)). Two main regions were noticed for both Kefiran solutions (1% and 10% w/v) at 37°C: (1) a *power law* region, showing shear-thinning (pseudoplastic) behavior and (2) a *Newtonian* region. This profile, with Newtonian fluid behavior at extreme shear rates, is frequent in many shear-thinning (pseudoplastic) fluids. Similar results were obtained and reported by others.^{14,23,39}

From the power law region the obtained indexes for 10% (w/v) and 1% (w/v) solutions were 0.24 and 0.29, respectively, which are typical values for pseudoplastic fluids. The pseudoplastic behavior and gelation ability of Kefiran point out the fact that this polymer could be exploited as matrix environments of different therapeutic agents, including proteins, stem cells, or genetically engineered cells.

Figure 6(b) presented the shear stress function to the shear rate. In these curves, the two regions were not so easily perceived, being even possible to adjust a linear regression model, where the slope is the average viscosity value. It demonstrated the relationship between the shear stress applied and the resulting shear rate. Kefiran samples (1% and 10% w/v) revealed an infinite viscosity until a sufficiently high stress as applied to initiate flow. Above this stress, our biomaterial showed simple Newtonian flow, which was identified as a Bingham plastic model. From the Newtonian region, where the viscosity is nearly constant, 10% (w/v) solutions stabilized at higher value of viscosity (approximately 0.3 Pa s) while 1% (w/v) solutions have just 0.004 Pa s of shear viscosity.

Oscillatory experiments. The mechanical spectra of 1% (w/v) and 10% (w/v) of Kefiran were obtained (Figure 7(a) and (b), respectively) at 37°C.

Storage and loss moduli are both functions of frequency and give an insight of the structure of polymer solutions. The different Kefiran concentrations studied (1% and 10% w/v) showed divergent behaviors, since that samples of 1% (w/v) started by a viscous behavior and samples of 10% (w/v) started by an elastic behavior. More particularly, Kefiran samples of 1% (w/v) had a cross over at approximately 8 Hz, changing from a phase angle of about 47° (viscous liquid) to approximately 31° (elastic/gel); while 10% (w/v) solutions presented a cross over at 1.6 Hz, changing from a phase angle of about 25.7° (elastic/gel) to an average of approximately 58° (viscous liquid). The different behavior between concentrations was not studied or reported by other authors, but it could justify the selection of a certain concentration based on its rheological response considering the therapeutic application. Further studies should be performed in order to better understand this

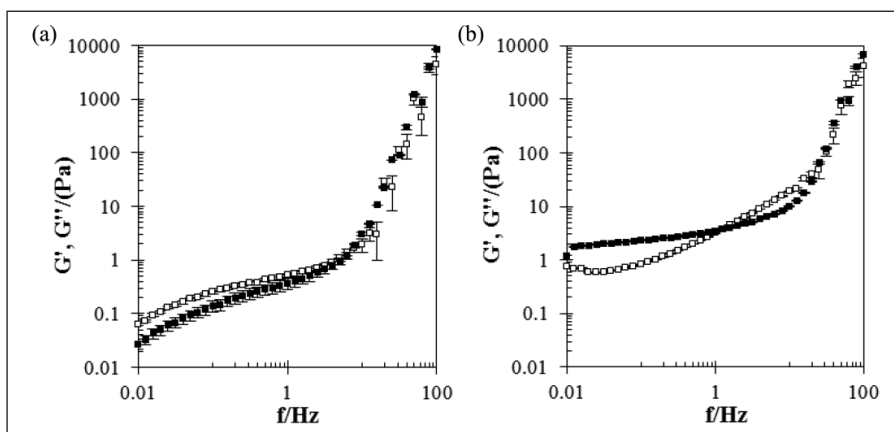


Figure 7. Frequency dependence of the viscoelastic moduli, loss modulus G'' (open symbol) and storage modulus G' (filled symbol), of 1% w/v (a) and 10% w/v (b) Kefiran samples, at 37°C.

statement. Piermaria et al.³⁹ demonstrated the same behavior in Kefiran samples at 1.08% w/v (10.8 g/L) that we observed in our 1% Kefiran samples. In this case (10% w/v of Kefiran solution), the mechanical spectra demonstrated the behavior of a polysaccharide solution at low frequency where the storage modulus was greater than the loss modulus. In fact, both moduli increased with frequency swept, but G'' increased more rapidly and tended to overcome G' at higher frequencies. This statement could be explained by the mechanism of an entrapped network system at low frequencies. At high frequencies, the polymer chains did not have sufficient time to uncoil and slide and, therefore, may behave like a gel ($G' > G''$).³⁹

The viscoelastic properties of Kefiran highlight its applicability in tissue engineering and regenerative medicine. For instance, it could be used in osteoarthritis treatment therapies (viscosupplementation) in order to restore the viscoelastic properties of the joint synovial fluid. This polysaccharide could be an economical and environmentally improved alternative to the well-known HA.

Pull away experiments. Preliminary pull away tests to 1% (w/v) and 10% (w/v) Kefiran samples allowed us to state that Kefiran polysaccharide was characterized by an adhesive performance. Being this effect 97% higher for 10% solutions, with an adhesion of 1.159 ± 0.018 N/s. Kefiran solutions of 1% (w/v) presented a similar adhesion value to water, 0.135 ± 0.049 N/s. In fact, polysaccharide such as Kefiran may help bacterial cells for adhesion to biological surfaces and biofilm formation.⁴⁰ It is important to point out that the advances in biofilm formation knowledge, coupled with emerging engineered biomaterials, provide many potential platforms and strategies to prevent or significantly reduce biofilm infections.⁴¹

These interesting results obtained through different analyses demonstrated that Kefiran is a relevant candidate in the field of biomaterials, especially for tissue engineering and regenerative medicine by replicating the tissues' mechanical and viscoelastic characteristics. The Kefiran may be used in the prevention or treatment of bone and cartilage disease or defects or also wound healing.

Injectability (extrusion) performance

Injectability is a key-product performance parameter of any parenteral dosage form. This assay included pressure or force required for injection, evenness of flow, and freedom from clogging

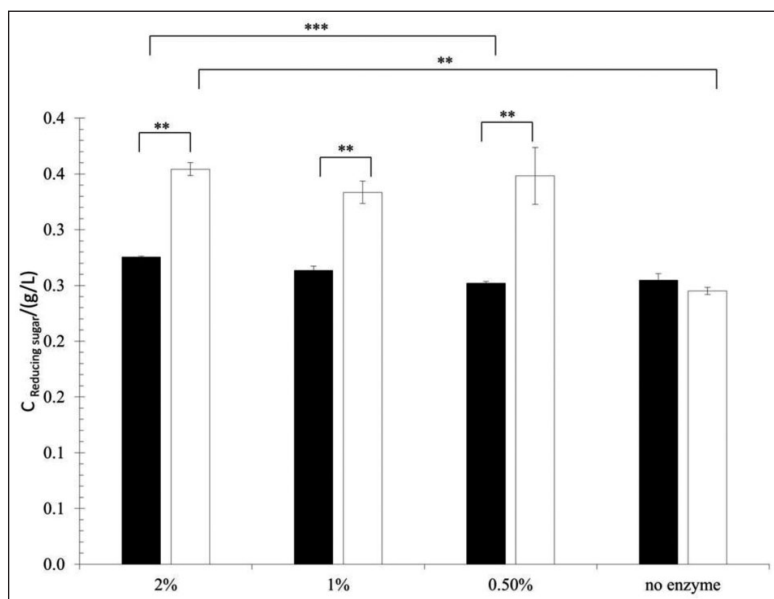


Figure 8. Enzymatic resistance to degradation of Kefiran (black) and HA (white) samples (2%, 1%, and 0.5% w/v).

*Symbols denote statistically significant differences ($p < 0.05$).

such as no blockage of the syringe needle. The extrusion force, measured by the injectable measurement device at room temperature, of Kefiran was 1 N. The Kefiran and HA samples presented different values of force required for injection. The extrusion force of HA (11.3 N), which presented the highest value, is clinically relevant data since the physician must inject the HA through a thin needle into soft tissue. It has been shown that Kefiran formulation could represent interesting viscosupplementation products, for example, over HA intra-articular injections since they offer a smooth injectability by low extrusion forces. It is important to point out that HA polysaccharide was considered as gold standard for viscosupplement, since it is currently used as a commercial injectable biomaterial (e.g. for osteoarthritis treatment).⁴² This polysaccharide was the first Food and Drug Administration (FDA)-approved viscosupplement, and in 1997, it became marketed and quickly growing in popularity due to its viscoelastic properties by re-establishing the elasticity and viscous properties of the articular joint improving pain and restoring function in osteoarthritis patients.⁴³ These injections provide a prompt pain relief, but their benefit appears to be short-lived. Thus, it will be interesting to create a new valid therapeutic alternative based on Kefiran polysaccharide that could have a great commercial interest and high-value therapy for osteoarthritis patient.

Enzymatic resistance

The enzymatic resistance of Kefiran polysaccharides obtained in this research was evaluated (Figure 8). The results achieved, in terms of reducing sugars release during the enzymatic reactions, for 48 h of reaction are presented in Figure 8. Kefiran exhibited a better resistance to hyaluronidase degradation as compared to HA that presented a degradation of 30%. GPC-SEC analysis confirmed the results obtained.

Generally, the hyaluronidase cleaves high-molecular-weight HA into smaller fragments. They are assumed to be the predominant catabolic mechanism involved in HA degradation throughout the vertebrate body. Thus, marketed viscosupplements based on HA are susceptible to hyaluronidases degradation action, resulting in a fast clearance.

The HA which is the responsible for the high viscosity and thixotropic behavior of synovial fluid, has a half-life varying from 1–3 weeks in inert tissues such as cartilage to 1–2 days in the epidermis of skin. The half-life in blood was much shorter (2–5 min) requiring several dosages.⁴⁴ HA modifications are being reported with higher half-life, but it is not sufficient and the HA production cost is still too high.

In this research, the evaluation of Kefiran resistance to HAase activity was an important step to confirm advantage over HA. So, it is important to highlight that Kefiran biopolymer could be a promising alternative viscosupplement to HA as it presents a better a resistance to hyaluronidase degradation. Further studies should be performed with other enzymes such as collagenase and proteoglycanase, in order to get a better knowledge of the enzymatic resistance property of Kefiran.

Analysis of Kefiran's cytotoxicity

To evaluate the cytotoxicity of Kefiran extract and its safety as an upcoming biomedical material, ISO 10993-5 was followed. The purpose of the study was to provide a test sample where the biological reactivity of any leachable could be detected.

In this experiment, cell growth, cell proliferation, and cell damage were evaluated (Figure 9). Figure 9(a) showed cell growth assessed by MTS assay, once the absorbance was directly proportional to the number of living cells within the culture. It is important to mention that along the culture, the number of living cells increased, as corroborated by dsDNA quantification and F-actin staining (Figure 9(b) and (c)). Finally, F-actin staining showed no damage on cells when submitted to any condition (Figure 9(c)). It is important to highlight that the L929 cells had an increasing cell metabolic activity level during the time after exposure to Kefiran polysaccharide.

The results of *in vitro* cytotoxicity are shown in Figure 9. The L929 cells showed to be metabolic active along the time of culture for all conditions (Figure 9(a)). Moreover, metabolic activity levels increased along the time, indicating that cells were proliferating, as corroborated by dsDNA quantification (Figure 8(b)). Nevertheless, comparing to the milk and control, Kefiran polysaccharide showed lower metabolic activity and dsDNA quantification levels after 72 h of incubation. Concerning the difference observed between Kefiran and milk, the difference could be explained by the fact that milk contains a certain amount of nutrients that can influence cells' behavior. In the case of the difference observed between Kefiran and control, it can be due to the fact that the extracts present new cues that cells need to adapt, delaying their development. Finally, it is important to point out that the cultured L929 cells showed no morphological differences between all the culturing conditions.

In our research, the *in vitro* evaluation of Kefiran cytotoxicity on L929 mouse fibroblast cells reveals the metabolic activity of our polysaccharide in a manner that is comparable to that on the negative control. It is imperative to perform a preliminary *in vitro* study before studying the tissue response of a material *in vivo* in order to obtain some knowledge about its performance in a physiological environment. In fact, cytotoxic responses can hinder the natural assimilation process that is needed for successful *in vivo* integration of a biopolymer such as Kefiran. The *in vitro* assessment should mimic perfectly the *in vivo* cell environment.^{44,45}

This research therefore chose L929 cells to represent tissues with Kefiran products, developed in the future, which will interact *in vivo* in various cartilage and bone tissue engineering therapies along with the cell line suggested per ISO 10993-5.

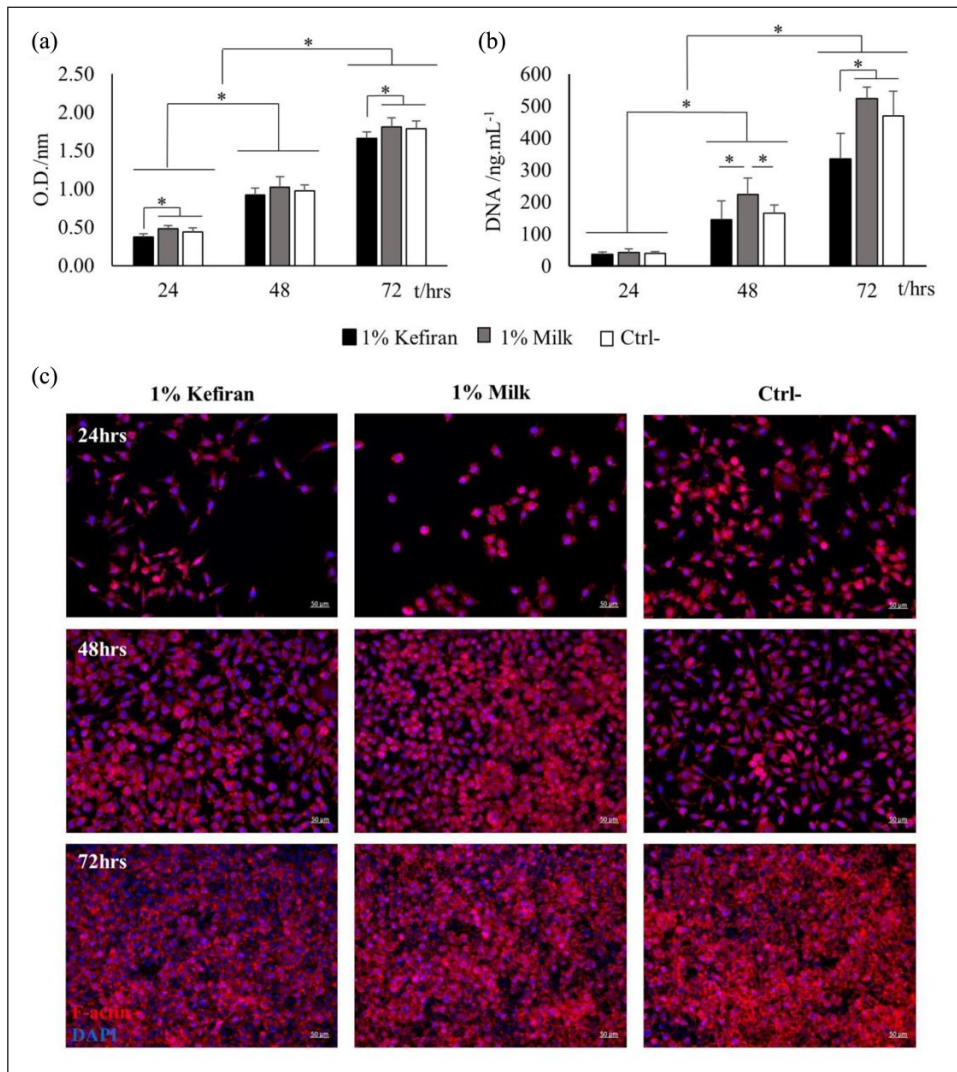


Figure 9. Kefiran cytotoxicity using a cell line: (a) cells growth assessed by MTS assay of L929 cells cultured during 72 h with Kefiran extract, (b) cells proliferation assessed by dsDNA quantification of L929 cells cultured during 72 h with Kefiran extract, and (c) cell damage assessed by F-actin staining (cytoskeleton, red) and counterstained with DAPI staining (nuclei, blue), during 72 h of culture (scale bar: 50 μm).

*denotes statistical significant differences along the time of culture ($p < 0.05$).

Furthermore, recent study investigated the potential cytotoxicity effect of Kefiran produced by *L. kefirnofaciens* ATCC 8007 cells on many cancer cell lines.⁴⁶ However, little information is present on the cytotoxic activity of the Kefiran on L929 cells. The results obtained through this research suggest that Kefiran is a suitable biomaterial for tissue engineering and regenerative medicine, as it showed a lack of cytotoxic response through its ability to support cell metabolic activity and cell proliferation, and had no effect on cells' morphology.

Conclusion

In this study, the physicochemical and structural characterizations of Kefiran polysaccharide isolated and purified from Portuguese kefir grains were performed. This research presented very important parameters to access the biopolymer structure and properties. These potential properties of Kefiran were obtained to show its competence and to clarify the highly application potential of Kefiran polysaccharide, through, for example, its physicochemical and rheological properties in tissue engineering and regenerative medicine. In fact, Kefiran polysaccharide with its several potential properties could be modulated to achieve, for example, an adequate structure as demanded for example by articular cartilage or bone defects applications. Further works will be performed to optimize scaling up for Kefiran industrial application and will comprise a set of new in vitro experiments in order to fully characterize its biological properties for therapeutic applications.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ORCID iD

Hajer Radhouani  <https://orcid.org/0000-0003-1007-4073>

References

1. Farnworth ER. Kefir: from folklore to regulatory approval. *J Nutraceuticals Functional Medical Foods* 1999; 1: 57–68.
2. Nielsen B, Gurakan GC and Unlu G. Kefir: a multifaceted fermented dairy product. *Probiotics Antimicrob Proteins* 2014; 6: 123–135.
3. Otlés S, Cagindi O and Akcecek E. Probiotics and health. *Asian Pac J Cancer Prev* 2003; 4: 369–372.
4. Garofalo C, Osimani A, Milanovic V, et al. Bacteria and yeast microbiota in milk kefir grains from different Italian regions. *Food Microbiol* 2015; 49: 123–133.
5. De Oliveira Leite AM, Miguel MA, Peixoto RS, et al. Microbiological, technological and therapeutic properties of kefir: a natural probiotic beverage. *Braz J Microbiol* 2013; 44: 341–349.
6. Rimada PS and Abraham AG. Comparative study of different methodologies to determine the exopolysaccharide produced by kefir grains in milk and whey. *Lait* 2003; 83: 79–87.
7. Prado MR, Blandon LM, Vandenberghe LP, et al. Milk kefir: composition, microbial cultures, biological activities, and related products. *Front Microbiol* 2015; 6: 1177.
8. Zajsek K, Gorsek A and Kolar M. Cultivating conditions effects on Kefiran production by the mixed culture of lactic acid bacteria imbedded within kefir grains. *Food Chem* 2013; 139: 970–977.
9. Kwon OK, Ahn KS, Lee MY, et al. Inhibitory effect of Kefiran on ovalbumin-induced lung inflammation in a murine model of asthma. *Arch Pharm Res* 2008; 31: 1590–1596.
10. De Moreno de Leblanc A, Matar C, Farnworth E, et al. Study of immune cells involved in the antitumor effect of kefir in a murine breast cancer model. *J Dairy Sci* 2007; 90: 1920–1928.
11. Rodrigues KL, Caputo LR, Carvalho JC, et al. Antimicrobial and healing activity of kefir and Kefiran extract. *Int J Antimicrob Agents* 2005; 25: 404–408.
12. Lopitz-Otsoa F, Rementeria A, Elguezabal N, et al. Kefir: a symbiotic yeasts-bacteria community with alleged healthy capabilities. *Rev Iberoam Micol* 2006; 23: 67–74.

13. Diniz RO, Garla LK, Schneedorf JM, et al. Study of anti-inflammatory activity of Tibetan mushroom, a symbiotic culture of bacteria and fungi encapsulated into a polysaccharide matrix. *Pharmacol Res* 2003; 47: 49–52.
14. Piermaria JA, Pinotti A, Garcia MA, et al. Films based on Kefiran, an exopolysaccharide obtained from kefir grain: development and characterization. *Food Hydrocolloid* 2009; 23: 684–690.
15. Gonçalves C, Rodriguez-Jasso RM, Gomes N, et al. Adaptation of dinitrosalicylic acid method to microtiter plates. *Anal Method* 2010; 2: 2046–2048.
16. Botelho PS, Maciel MI, Bueno LA, et al. Characterisation of a new exopolysaccharide obtained from fermented kefir grains in soymilk. *Carbohydr Polym* 2014; 107: 1–6.
17. Pop OL, Brandau T, Schwinn J, et al. The influence of different polymers on viability of *Bifidobacterium lactis* 300b during encapsulation, freeze-drying and storage. *J Food Sci Technol* 2015; 52: 4146–4155.
18. Zajsek K and Gorsek A. Experimental assessment of the impact of cultivation conditions on kefir production by the mixed microflora imbedded in kefir grains. *Chem Engineer Trans* 2011; 24: 481–486.
19. Kesenkas H, Dinkci N, Seekin K, et al. Physicochemical, microbiological and sensory characteristics of Soymilk kefir. *Afr J Microbiol Res* 2011; 5: 3737–3746.
20. Alves A, Caridade GS, Mano JF, et al. Extraction and physico-chemical characterization of a versatile biodegradable polysaccharide obtained from green algae. *Carbohydr Res* 2010; 345: 2194–2200.
21. Micheli L, Uccelletti D, Palleschi C, et al. Isolation and characterisation of a ropy *Lactobacillus* strain producing the exopolysaccharide Kefiran. *Appl Microbiol Biotechnol* 1999; 53: 69–74.
22. Maeda H, Zhu X, Suzuki S, et al. Structural characterization and biological activities of an exopolysaccharide Kefiran produced by *Lactobacillus kefiranofaciens* WT-2B(T). *J Agric Food Chem* 2004; 52: 5533–5538.
23. Ghasemlou M, Khodaiyan F and Oromiehie A. Rheological and structural characterisation of film-forming solutions and biodegradable edible film made from Kefiran as affected by various plasticizer types. *Int J Biol Macromol* 2011; 49: 814–821.
24. Ghasemlou M, Khodaiyan F, Oromiehie A, et al. Characterization of edible emulsified films with low affinity to water based on Kefiran and oleic acid. *Int J Biol Macromol* 2011; 49: 378–384.
25. Wang Y, Ahmed Z, Feng W, et al. Physicochemical properties of exopolysaccharide produced by *Lactobacillus kefiranofaciens* ZW3 isolated from Tibet kefir. *Int J Biol Macromol* 2008; 43: 283–288.
26. Wang YP, Li C, Liu P, et al. Physical characterization of exopolysaccharide produced by *Lactobacillus plantarum* KF5 isolated from Tibet kefir. *Carbohydr Polym* 2010; 82: 895–903.
27. Davidovic SZ, Miljkovic MG, Antonovic DG, et al. Water kefir grain as a source of potent dextran producing lactic acid bacteria. *Hem Ind* 2015; 69: 595–604.
28. Mano JF, Silva GA, Azevedo HS, et al. Natural origin biodegradable systems in tissue engineering and regenerative medicine: present status and some moving trends. *J R Soc Interface* 2007; 4: 999–1030.
29. Beamson G and Briggs D. High resolution XPS of organic polymers: the Scienta ESCA300 database. *J Chem Educ* 1993; 70: A25.
30. Hamer AD, Tisley DG and Walton RA. The X-ray photoelectron spectra of inorganic molecules—VIII[1, 2]: compounds of scandium(III), scandium(III) oxide and complexes with organic oxygen donor molecules. *J Inorganic Nuclear Chem* 1974; 36: 1771–1775.
31. Bhatia QS, Pan DH and Koberstein JT. Preferential surface adsorption in miscible blends of polystyrene and poly (vinyl methyl ether). *Macromolecules* 1988; 21: 2166.
32. Bournel F, Laffon C, Parent P, et al. Adsorption of some substituted ethylene molecules on Pt(111) at 95 K part 1: NEXAFS, XPS and UPS studies. *Surf Sci* 1996; 350: 60–78.
33. Rebelo R, Manninen NK, Fialho L, et al. Morphology and oxygen incorporation effect on antimicrobial activity of silver thin films. *Appl Surf Sci* 2016; 371: 1–8.
34. Kocaefe D, Sarkar A, Das S, et al. Review of different techniques to study the interaction between coke and pitch in anode manufacturing. In: Barry AS (ed.) *Light metals 2013*, John Wiley & Sons, Inc., Hoboken, New Jersey, USA; Part XXX: 1041–1050.
35. Addad S, Exposito JY, Faye C, et al. Isolation, characterization and biological evaluation of

- jellyfish collagen for use in biomedical applications. *Mar Drugs* 2011; 9: 967–983.
36. Yeh CC, Chen CN, Li YT, et al. The effect of polymer molecular weight and UV radiation on physical properties and bioactivities of PCL films. *Cell Polym* 2011; 30: 261–276.
 37. Ulery BD, Nair LS and Laurencin CT. Biomedical applications of biodegradable polymers. *J Polym Sci B Polym Phys* 2011; 49: 832–864.
 38. Iqbal MS, Massey S, Akbar J, et al. Thermal analysis of some natural polysaccharide materials by isoconversional method. *Food Chem* 2013; 140: 178–182.
 39. Piermaria JA, de la Canal ML and Abraham AG. Gelling properties of Kefiran, a food-grade polysaccharide obtained from kefir grain. *Food Hydrocolloid* 2008; 22: 1520–1527.
 40. Vu B, Chen M, Crawford RJ, et al. Bacterial extracellular polysaccharides involved in biofilm formation. *Molecules* 2009; 14: 2535–2554.
 41. Bryers JD. Medical biofilms. *Biotechnol Bioeng* 2008; 100: 1–18.
 42. Fakhari A and Berklund C. Applications and emerging trends of hyaluronic acid in tissue engineering, as a dermal filler and in osteoarthritis treatment. *Acta Biomater* 2013; 9: 7081–7092.
 43. Ondresik M, Maia FRA, Morais AD, et al. Management of knee osteoarthritis. Current status and future trends. *Biotechnol Bioeng* 2017; 114: 717–739.
 44. Wang MO, Etheridge JM, Thompson JA, et al. Evaluation of the in vitro cytotoxicity of cross-linked biomaterials. *Biomacromolecules* 2013; 14: 1321–1329.
 45. Thrivikraman G, Madras G and Basu B. In vitro/In vivo assessment and mechanisms of toxicity of bioceramic materials and its wear particulates. *RSC Adv* 2014; 4: 12763–12781.
 46. Elsayed EA, Farooq M, Dailin D, et al. In vitro and in vivo biological screening of Kefiran polysaccharide produced by *Lactobacillus kefiranofaciens*. *Biomed Res* 2017; 28: 594–600.