

## Article

# Biodegradable Films from Kefiran-Based Cryogel Systems

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**Abstract:** Kefiran, which was isolated from kefir grains, was used for the fabrication of cryogel-films in the presence of plasticizers, such as glycerol and sorbitol. Varying concentration ratios of the exopolysaccharide/plasticizer system were employed in the process of the cryogel-film formulation and their effect on the physical (film thickness, moisture content, and solubility) and the mechanical (tensile strength and elongation at break) properties of the films was monitored. Kefiran-film vapor adsorption isotherms were calculated, and a thermal analysis of the samples was also performed. The structural characteristics of the cryogel-films were observed using confocal laser scanning microscopy. The cryo-treatment, alongside the plasticizer addition, affected the physical and mechanical properties of the kefir films, as well as their morphology. Increasing kefir concentration resulted in increasing the film thickness, the moisture content, and the tensile strength, while decreased their solubility and their elongation at break. Kefiran-film adsorption isotherms were affected by the cryo-treatment, the kefir concentration, and the plasticizer use and concentration. The thermal analysis of the kefir films showed thermal stability. The presence of the plasticizers and their increment concentration resulted in decreasing the glass transition and the melting temperatures. The cryo-treatment and the presence of plasticizers resulted in the films appearing smoother. Glycerol proved to affect more than the sorbitol–water vapor adsorption and the morphology of kefir films.

**Keywords:** kefir; cryogel; biodegradable; edible; film; plasticizer



**Citation:** Exarhopoulos, S.; Goulas, A.; Dimitreli, G. Biodegradable Films from Kefiran-Based Cryogel Systems. *Macromol* **2022**, *2*, 324–345. <https://doi.org/10.3390/macromol2030021>

Academic Editor: Ana María Díez-Pascual

Received: 11 June 2022

Accepted: 11 July 2022

Published: 13 July 2022

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## 1. Introduction

The food and pharmaceutical industries, which are driven by the environmental, sustainability, and health awareness of consumers and customers, are constantly searching for new materials and ingredients with health promoting properties that are environmentally friendly but simultaneously have the characteristics that are required for the acceptable use of the marketed final product and/or application. Such applications range from biodegradable edible packaging biopolymers, to controlled drug delivery systems, wound dressing, and tissue-engineering scaffolds for medical uses [1–4].

On this line of research, a recently widely-studied biopolymer is kefir. It is the main polysaccharide that is found in kefir and is the primary structural component of kefir grains, which are the gelatinous irregular masses that form the symbiotic cultures of lactic and acetic acid bacteria, together with yeasts [5,6]. It is an extracellular polysaccharide that is composed of glucose and galactose, on equal ratios, which is formed and excreted during the metabolic activity of generally recognized as safe (GRAS) lactic acid bacteria with many functional, health-promoting, and therapeutic properties assigned to it [3]. Kefiran can be isolated to a high purity standard from kefir grains by using methods that have been described previously [7], however, its low formation rate during fermentation restricts its use, for the time being, to small-scale and high-margin applications [3].

In an evaluation report of the physicochemical and biological properties of kefir, by Radhouani and coworkers in 2018 [8], they reported the polysaccharide as possessing promising characteristics for biomedical applications, such as tissue engineering and regenerative medicine. The texture modification and edible film formation, as well as

the biodegradable hydrogel and cryogel production, have been studied by using this carbohydrate polymer [4,9–12] due to its acclaimed physical characteristics in stabilizing and emulsifying water-based systems, as well as its satisfactory mechanical and barrier properties.

The formulation of rigid and brittle membrane-like films using kefiran, at concentrations ranging from 5 to 10 g of kefiran/Kg film-forming solution, was initially reported by Piermaria and coworkers [9]. Subsequently, this research group [13], alongside others [14–16], went on to improve the mechanical properties of the kefiran films, especially their elasticity, by assimilating to their film-forming recipes several different plasticizers, such as glycerol, sorbitol, glucose, galactose, saccharose, and oleic acid.

Other research paths that have been pursued to improve the properties of the kefiran films include the incorporation of several different components, ranging from biodegradable biopolymers, such as starch, cellulose, pectin, and chitosan to other mostly inorganic materials, such as aluminum oxide, zinc oxide, nano-cellulose, titanium oxide, and montmorillonite [12].

Another technologically interesting property of kefiran is its ability to form cryogels [9,11], which can potentially open up new applications in the biotechnology, the medicine, the pharma, and the cosmetic industries [17,18]. Their formation, due to intramolecular interactions of the polymers' chains, is a consequence of their topological increased concentrations (occurring due to freezing), as ice crystals are formed during freezing. The cryogel structure is maintained after the thawing of the mixtures [17] and kefiran cryogels have been reported by Exarhopoulos and coworkers [19] as possessing significantly increased rheological properties in comparison to their mother liquor. Such intramolecular interactions as the ones that are observed in cryogels, alongside the presence of plasticizers, can have a significant impact on the kefiran membrane-film formation, but such a research initiative, to the best of our knowledge, has not been undertaken until now. In the herein presented work, the preparation of kefiran membrane-films from the polysaccharides cryogels is reported, in the presence of glycerol and sorbitol. The effects of the polymer and plasticizer concentration on the film properties and characteristics are also investigated.

## 2. Materials and Methods

### 2.1. Kefiran Extraction and Purification

Kefiran was isolated from kefir grains, as described by Exarhopoulos et al. [20]. In brief, kefir grain proliferation was facilitated by consecutive cultivations in skimmed ultra-high temperature (UHT) treated milk in batch laboratory and pilot-plant fermentations. Following inoculation, at a rate of 1.5% (*w/w*) of the grains in the milk, the fermentations proceeded under constant mild agitation, at 25 °C, in sealed fermenters with no aeration of the culture. The pH of the cultured milk was constantly monitored, and the fermentations were discontinued when it reached 4.5. Concomitantly, the grains were collected, washed with sterile distilled water, and were used for kefiran isolation.

Disintegration of kefir grains, and detachment of the exopolysaccharide from the bacterial cells, was affected by heating at 80 °C in distilled water under constant vigorous agitation until the grains were completely dissolved and no residue was collected when sieving with a sieve with 1 mm openings. Following the previously described methods [3,20,21], the crude kefiran solution was treated with trichloroacetic acid (Merck KGaA, Darmstadt, Germany), or hyperchloric acid (Carlo Erba Reagents SAS, Val de Reuil France), for facilitating protein and bacterial cell removal via centrifugation. The efficacy of both acids in the removal of proteins was equal, as determined by the protein concentration in the kefiran preparations, with the latter being preferably used at a large scale, due to its lower cost. The polysaccharide remained in suspension in the supernatant liquid and was collected and purified from low molecular weight contaminants by at least three consecutive ethanol or acetone precipitation steps, separated by intermediate dissolutions in distilled water.

The purity of the resulting freeze-dried kefiran preparation was monitored by determining the moisture (drying at  $102 \pm 1$  °C to constant weight), total nitrogen (Kjeldahl), and total sugar (phenol–sulfuric acid assay) [22]. The H-NMR spectrum (data not shown) of the carbohydrates' purified preparation, in D<sub>2</sub>O at 60 °C, taken on a 500 MHz NMR spectrometer (Agilent 500 DD2, Agilent Technologies, Inc., Santa Clara, CA, USA), showed to be in agreement with those reported previously by Maeda et al. [23] and Radhouani et al. [8] and confirmed the polymers' identity.

## 2.2. Kefiran-Film Preparation and Experimental Design

Varying concentrations of kefiran, ranging from 0.5 to 4% (*w/w*) of the total film-forming solution, were used in the preparation of cryo-films. The use of the plasticizers, glycerol and sorbitol, at concentrations of 150, 120, 90, 60, and 30% (*w/w*), calculated on the basis of dry kefiran, were also studied at a 3% (*w/w*) kefiran concentration of the total film-forming solution.

For the preparation of the films, the polysaccharide was dissolved in the quantity of water required by heating and mild agitation at 80 °C, followed by the addition of the respective plasticizer, either glycerol or sorbitol (Sigma-Aldrich Corp., St. Louis, MO, USA). The film-forming solutions were subsequently transferred to petri dishes and subjected to cryo-treatment by slow freezing at  $-18$  °C for 24 h, followed by defrosting at 4 °C for 24 h. Finally, the films were dried to a constant weight in a laminar air flow oven at 40 °C and were stored at 25 °C with 55% relative humidity conditions for at least 2 days prior to any further analysis.

The effect of the cryo-treatment on the characteristics of the prepared films was accessed in comparison to similar films, based on the previously described compositions, by emitting the cryo-treatment step and by casting and drying them in each case directly from the corresponding solution.

## 2.3. Evaluation of the Films' Physical Properties

The film thickness reported herein is the average value of at least 8 thickness measurements per film, performed at varying areas of it, using a digital micrometer with 0.001 mm resolution.

Film moisture content was determined in triplicate by oven drying at  $102 \pm 1$  °C to a constant weight [22].

Water solubility was accessed as described by Ghasemlou et al. [15]. In brief, pieces of film samples with an area of 3 (1 × 3) cm<sup>2</sup> were dried at  $50 \pm 1$  °C for 24 h and their initial dry weight was recorded in an analytical balance. Subsequently, the films were immersed in 50 mL distilled water and were kept for 6 h at 25 °C, being subjected periodically to mild agitation. When the 6 h holding period was completed, the remaining film pieces were dried at  $102 \pm 1$  °C for 24 h, their final dry weight was measured, and the percentage of total soluble matter (TSM %) of the films was calculated as follows:

$$\text{TSM (\%)} = [(\text{Initial dry weight} \times \text{Final dry weight}) / \text{Initial dry weight}] \times 100 \quad (1)$$

Water solubility tests were carried out in triplicate samples.

## 2.4. Kefiran-Film Vapor Adsorption Isotherms

Pieces of the films with an area of approximately 3 (1 × 3) cm<sup>2</sup> were placed in pre-weighed glass dishes and dried at  $50 \pm 1$  °C for 24 h. The dried samples were then weighed in an analytical balance and placed above over-saturated salt (Merck KGaA, Darmstadt, Germany) solutions with different relative humidity (RH) values in hermetically sealed glass containers (2 L). The RHs employed were as follows: LiCl of 11%, CH<sub>3</sub>COOK of 23%, MgCl<sub>2</sub> of 33%, K<sub>2</sub>CO<sub>3</sub> of 43.2%, Mg(NO<sub>3</sub>)<sub>2</sub> of 54%, NaCl of 75%, KCl of 85%, and KNO<sub>3</sub> of 95%. The containers were then stored at 30 °C for 7 days (whereupon preliminary tests have shown that equilibrium is achieved), before the sample weight was recorded again and the adsorption isotherm for each sample was calculated in a diagram showing g water/g dry matter versus water activity. Where g water is the weight difference of each

sample before and after equilibrium was achieved in each case and  $g$  dry matter is the weight of the dry sample.

### 2.5. Evaluation of the Films' Mechanical Properties

The mechanical properties of the films were evaluated at 25 °C by using the TA-XT Plus Universal Texture Analyzer from Texture Technologies Corp. (Hamilton, MA, USA), according to the standard method D882 [24]. Dog-bone strips of 40 mm in gauge length (each with grips of 10 mm) and 10 mm wide were cut, fixed at the tensile grip of the instrument, and stretched at a cross-head speed of 50 mm/min. From the derived stress–strain curves, the tensile strength and elongation at break (%) were determined. The reported data was derived from a six-fold analysis of each sample.

### 2.6. Film Thermal Analysis

#### 2.6.1. Thermogravimetric Analysis (TGA)

The thermal stability of films was studied using a TA Instruments TGA, model Q50 (TA Instruments, New Castle, DE, USA). A small piece of film, with a weight of about 5 mg, was used, which was placed in a platinum pan and heated from 30 to 600 °C with a heating rate of 10 °C/min in an inert atmosphere (50 mL/min flow of N<sub>2</sub>) and the mass loss, as well as its first derivative, was recorded.

#### 2.6.2. Differential Scanning Calorimetry (DSC)

The thermal analysis measurements were carried out using a DSC, model Q2000 (TA Instruments, New Castle, DE, USA). The instrument was calibrated with indium for the heat flow and temperature. Nitrogen gas flow of 50 mL/min was purged into the DSC cell. The sample mass was kept at around 5 mg. The Al sample, and the reference pans, were of identical mass with an error  $\pm 0.01$  mg. The method included equilibration at  $-50$  °C and heating up to 200 °C, with a heating rate of 10 °C/min.

### 2.7. Surface Morphology of the Kefiran Films

The topological film surface morphology was examined using a Carl Zeiss LSM 700 confocal laser scanning microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). The samples were mounted on glass slides, using double-sided adhesive tape in order to stabilize them, and their surface was observed. The observations were performed using a 405 nm laser in reflectance mode, without filters and without staining of the samples, in order to display the relief of the films' surface. The objective lenses used were  $\times 10$  and  $\times 20$  magnifications, in conjunction with digital zoom of the scanned area.

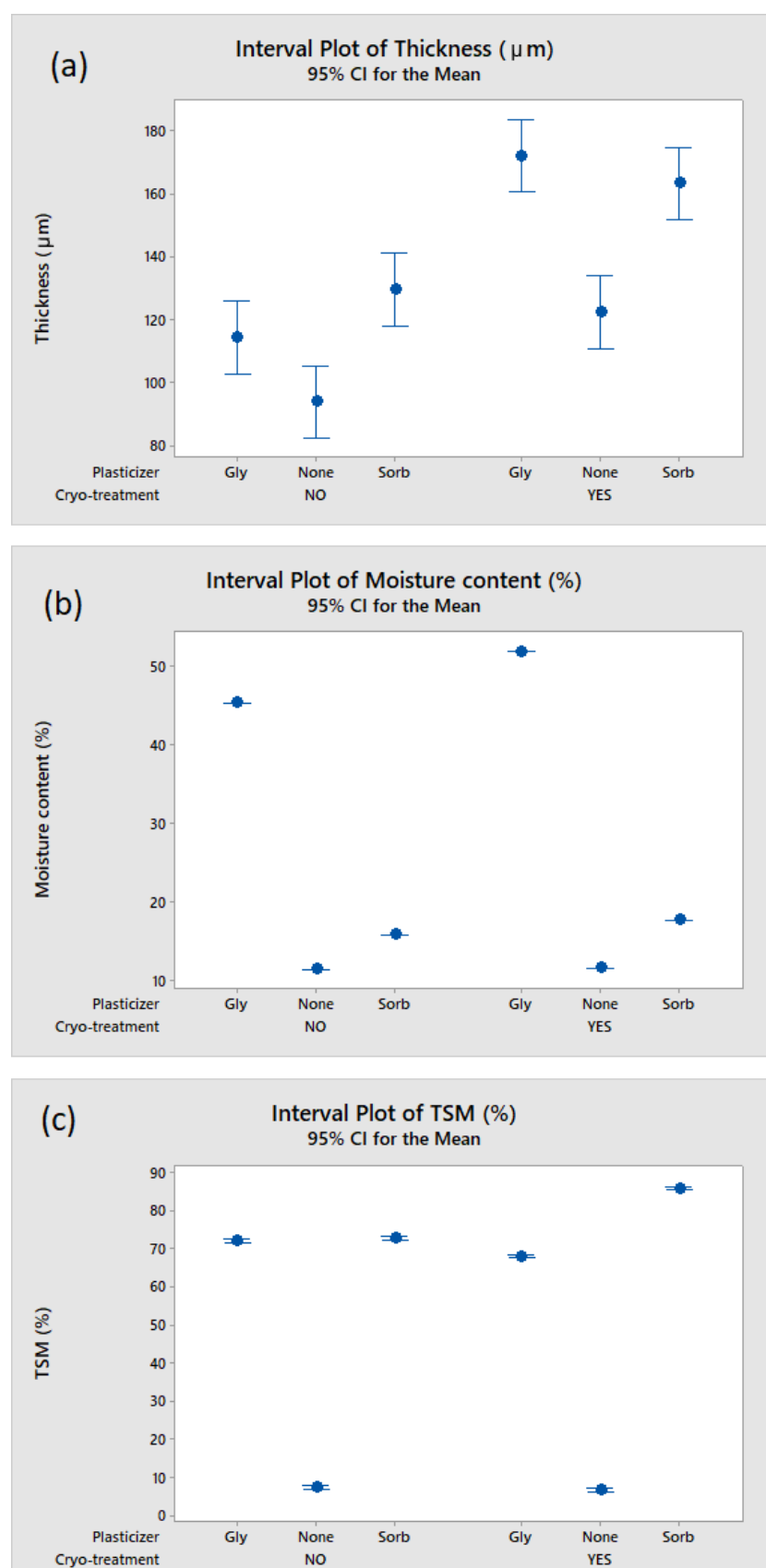
### 2.8. Statistical Analysis

The experimental data was subjected to one- or two-way ANOVA, depending on the factors examined each time as follows: effect of cryo-treatment with or without plasticizer addition, two factors; effect of kefir concentration, one factor; effect of plasticizer addition and plasticizer concentration, two factors. The results are displayed as the mean values of measurements with 95% confidence limits.

## 3. Results

### 3.1. Effect of Cryo-Treatment—With or without Plasticizer Addition

The effect of cryo-treatment on the physical properties of the films that were produced in the presence or absence of the plasticizer is shown in Figure 1. According to the two-way ANOVA results, the films that were fabricated with prior cryo-treatment of the formulations exhibited increased thickness compared to those with the same recipe but without the treatment, irrespective of the presence or absence of the plasticizer in the films (Figure 1a).



**Figure 1.** Effect of cryo-treatment with or without plasticizer (Gly: glycerol; Sorb: sorbitol), on kefiran films' thickness (a), moisture content (b), and total soluble matter (TSM) (c).

During the cryogel preparation, when a system in which large molecules are present is being cooled down to temperatures that are lower than the freezing point of the mixture's solvent, due to the solution composition and the presence of macro-molecules, as the temperature reduces to below freezing, a heterogeneous system is formed, which is composed of the solvent crystals and the non-frozen liquid micro-phase. This micro-phase occurrence is affected by the frozen solvent-crystal formation, that considerably elevates the macromolecule concentration (cryo-concentration) topologically, where the solvent is still in the liquid phase, provoking interactions between the solutes [17]. These increased concentrations of large molecules result in a forced alignment of their polymeric chains and the formation of side-chain bonds and interactions that remain even after the thawing of the system [25]. The three-dimensional structure of the cryogels, which is formed due to the polysaccharide molecule interactions as a function of the cryo-treatment, increases the cohesion and the hydrodynamic volume of the formed matrix, eventually leading to the increased thickness that is observed during cryo-film fabrication as the solvent (water) is evaporated.

As can be seen in Figure 1b, the cryo-treatment in the presence of plasticizers, especially glycerol, increased the moisture content of the prepared films/membranes. Glycerol is a more effective plasticizer compared to sorbitol because, due to its chemical nature, it has, by its weight in a films' recipe, the capability of binding more water molecules, which eventually results in the increased moisture contents that are observed in the kefiran films in which it was added. Moreover, with the cryo-treatment, higher moisture contents in the prepared films were seen in all cases where glycerol and sorbitol were used.

The film solubility was dependent on the cryo-treatment only when the plasticizers were used in their recipe. Glycerol, along with the cryo-treatment, effected a slight decrease in the solubility of the films, as depicted by the total soluble matter values (TSM) (Figure 1b), compared to the films without cryo-treatment. The opposite was observed for sorbitol.

In general, the occurrence of a plasticizer in such a system can diminish the interactions between the biopolymer molecules and thus increase the solubility of the existing matrix. This is due to the hydrophilic nature of the plasticizer, giving the polymer molecules a greater ability to attract water [15]. This is the cause for the great increase in the solubility when the plasticizers are added in the kefiran films (Figure 1c).

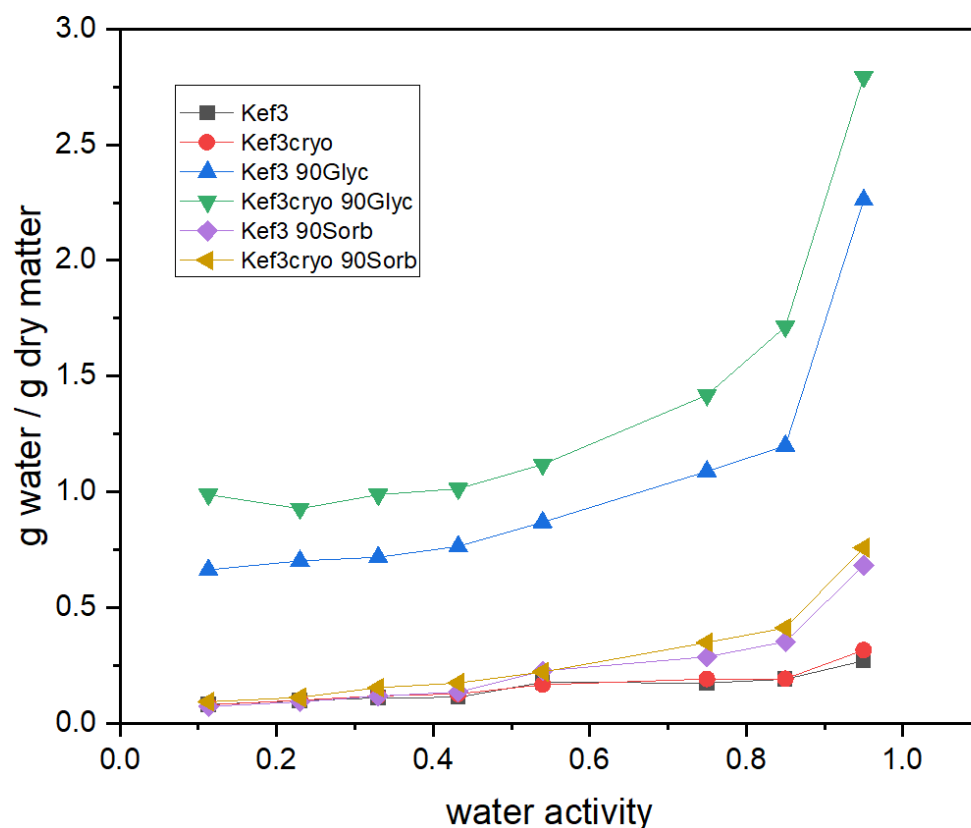
However, glycerol and sorbitol, due to the differences in their molecular mass and their ability to attract water molecules, appear to influence the solubility of the cryo-films in different ways. Glycerol, when cryo-treatment is used prior to the film fabrication, due to its more hydrophilic nature compared to sorbitol, acts antagonistically to kefiran in binding water, which allows the less hydrated kefiran molecules to form stronger interactions between them and reduces the solubility, whereas in the case of sorbitol the opposite is observed.

From Figure 2 it can be seen that the kefiran-film adsorption isotherms appear to be sigmoidal and the application of cryo-treatment alongside glycerol has increased the water vapor adsorption capacity throughout the whole range of the relative humidities that have been studied. In the case of sorbitol, this effect was not so pronounced, compared to glycerol, and an increase in water adsorption was observed only at the higher RH values. The more prominent water vapor adsorption that can be seen in the glycerol samples is directly related to its high hygroscopicity, which is much higher than sorbitol's on a weight basis. This is in agreement with the film-solubility results that have been reported herein. In all cases, comparisons were made with the control plasticizer-free samples, in which no differentiation was noticed between the cryo- and non-cryo-treated samples.

With regards to the mechanical properties of the kefiran films, Figure 3 shows that only a slight drop in the tensile strengths of the cryo-treated samples was observed compared to the control, irrespective of the use of plasticizers. Conversely, the use of plasticizers negatively affected the measured elongation at break values of the films. This effect on the mechanical properties of the films can be explained based on the hygroscopic nature of the plasticizers and the resulting moisture content of the films that were produced with them,



as it is related to the interconnections that are formed between the polysaccharide chains in such a system.



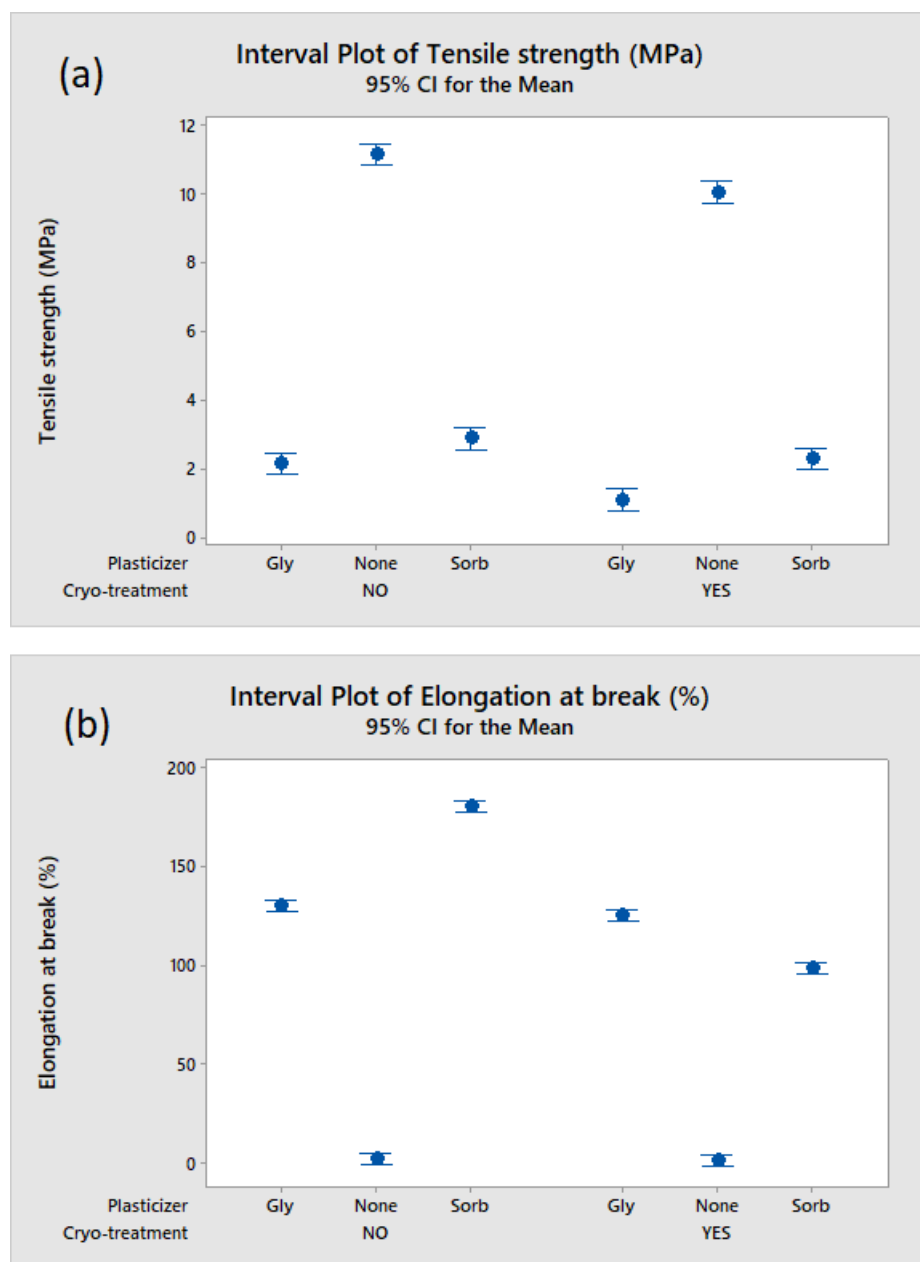
**Figure 2.** Adsorption isotherms of kefir films as an effect of cryo-treatment application and plasticizer addition (Kef3: kefir 3%; Kef3cryo: kefir 3% with cryo-treatment; Kef3 90Glyc: kefir 3% and 90% glycerol on dry kefir basis; Kef3cryo 90Glyc: kefir 3% with cryo-treatment and 90% glycerol on dry kefir basis; Kef3 90Sorb: kefir 3% and 90% sorbitol on dry kefir basis; Kef3cryo 90Sorb: kefir 3% with cryo-treatment and 90% sorbitol on dry kefir basis).

The tensile strength reduction with cryo-treatment, which is seen in the absence of plasticizers, is probably due to the three-dimensional structure that is formed during the freezing and thawing process. These films, as previously mentioned, have exhibited increased thickness, which logically leads to a more loose, swollen grid within the film structure and thus to worsened mechanical characteristics.

In Figure 4, a characteristic thermo-gravimetric analysis diagram of the kefir films is shown, in which the percentage film-weight change is depicted with increasing temperatures, along with its first derivative in terms of temperature. The main point of disintegration occurs between 220 °C and 380 °C, showing that the kefir films demonstrate an adequate thermal stability for their nature. This observed disintegration is due to kefir degradation, which is the main structural component of the films.

The glass transition temperature ( $T_g$ ) and the melting temperature ( $T_m$ ) of the kefir films (3% *w/w*) with or without the application of the cryo-treatment, and the use of plasticizers, are shown in Table 1. As can be seen, the  $T_g$  temperatures of the kefir films, which represent the temperature at which material undergoes a structural transition from an amorphous solid state to a more viscous rubbery state [15], ranged from −3.9 to −8.2 °C, whereas the  $T_m$  ranged from 79.63 to 92.67 °C. These values that are reported herein are somewhat greater than corresponding values that are mentioned in the literature for similar kefir films, as follows: −14 to −21 °C [15] and −15 to −20 °C [26] for  $T_g$  and for  $T_m$  68.36 to 73.29 °C [15] and 80 to 84 °C [26]. This variation to the reported values may be the outcome of differences in the kefir isolation and the purification methods that were

used since Ghasemlou et al. [15] and Montoille et al. [26], contrary to our method, did not use trichloroacetic or hyperchloric acid for facilitating the removal of the protein and bacterial cells.



**Figure 3.** Effect of cryo-treatment with or without plasticizer (Gly: glycerol; Sorb: sorbitol) on kefir-film tensile strength (a) and elongation at break (b).

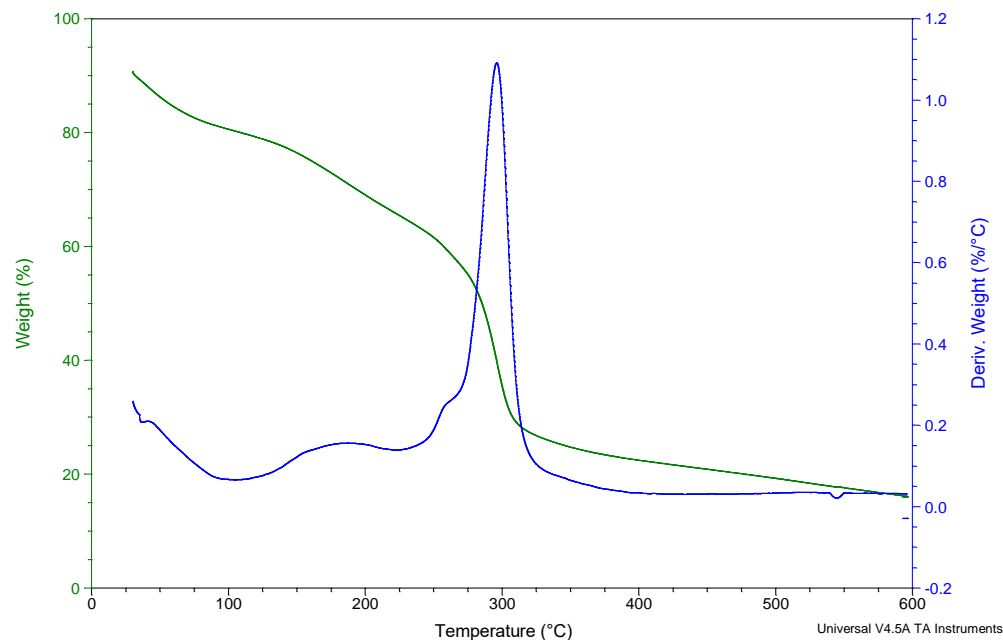
As can be seen from Table 1, the cryo-treatment did not affect the  $T_g$  or the  $T_m$  of the kefir films. In general, the  $T_g$  values of the kefir films suggest that they exist in a rubbery state at room temperature.

In the confocal microscopy pictures of the film's surface that are shown in Figure 5, the kefir films without the cryo-treatment appear to be rough when compared to the films where the specific process has been used. This phenomenon, with regards to film-surface roughness, becomes even more pronounced in the presence of plasticizers (Figures 6 and 7) with the surface of the cryo-treated films appearing smoother. These differences in the appearance of the film surface are possibly the effect of the macromolecule alignment that occurs during the freezing process, as the solvent diminishes. In addition, a less compact



and more uniform structure is achieved when water-binding plasticizers are used in the film recipe.

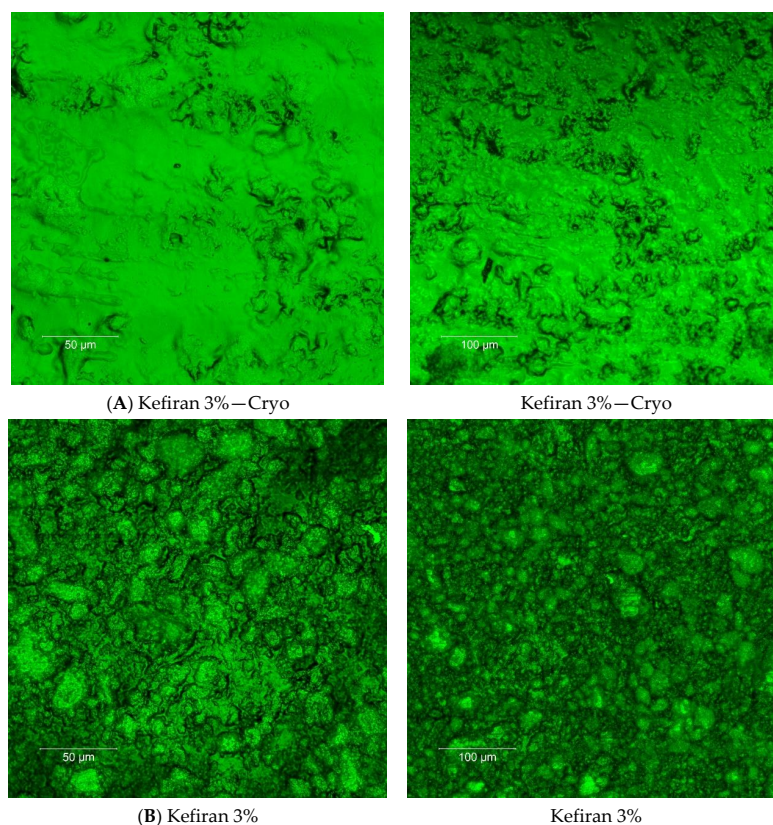
In the case of glycerol, the film-surface appeared to be smoother compared to sorbitol, which was possibly due to the differences on their hydrophilicity.



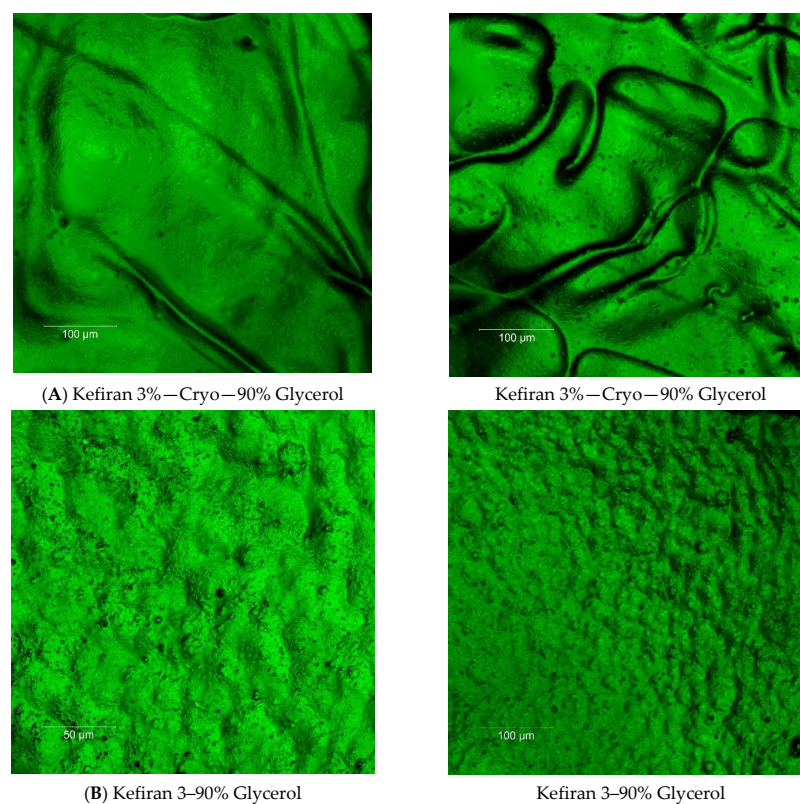
**Figure 4.** Characteristic TGA curves of kefir films (3% kefir and 30% glycerol on dry kefir basis).

**Table 1.** Glass transition temperature (T<sub>g</sub>) and melting temperature (T<sub>m</sub>) of kefir films (at 3% *w/w* kefir) with and without the application of cryo-treatment and plasticizer addition (plasticizer concentration is based on dry kefir content).

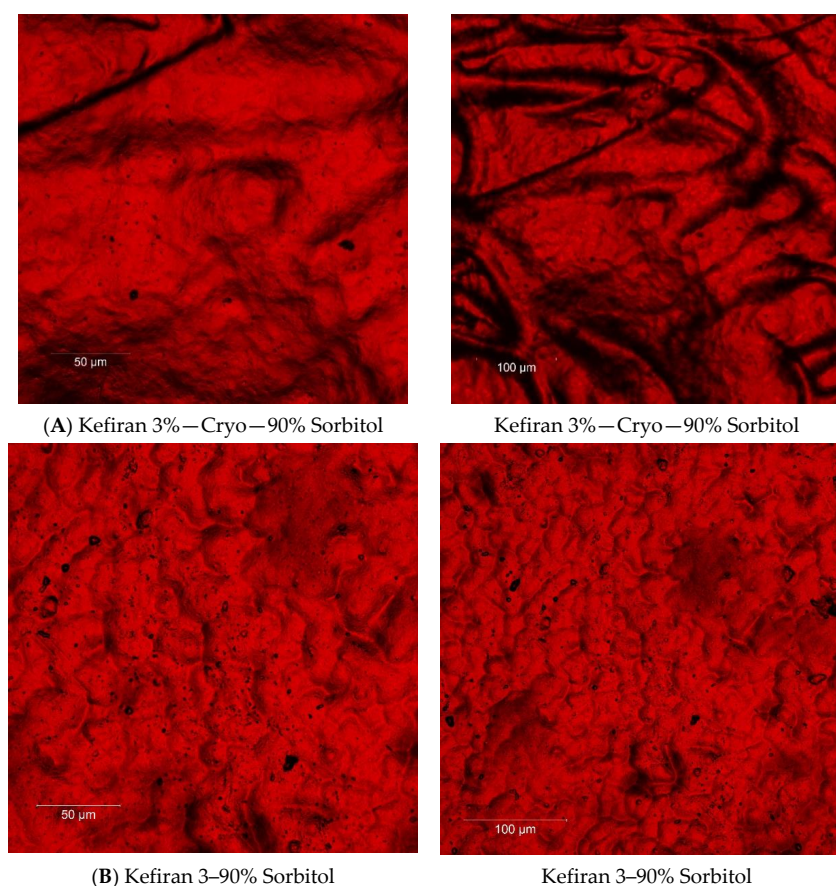
Sample	T <sub>g</sub> (°C)	T <sub>m</sub> (°C)
Kefiran 3%	−4.23	92.67
Kefiran 3%—Cryo	−3.90	92.31
Kefiran 3%—90% Glycerol	−7.43	84.02
Kefiran 3%—90% Sorbitol	−8.20	83.96
Kefiran 3%—Cryo—30% Glycerol	−5.94	89.50
Kefiran 3%—Cryo—60% Glycerol	−6.54	86.83
Kefiran 3%—Cryo—90% Glycerol	−7.08	83.97
Kefiran 3%—Cryo—120% Glycerol	−7.42	81.93
Kefiran 3%—Cryo—150% Glycerol	−8.05	80.09
Kefiran 3%—Cryo—30% Sorbitol	−6.19	89.25
Kefiran 3%—Cryo—60% Sorbitol	−6.53	86.92
Kefiran 3%—Cryo—90% Sorbitol	−6.44	84.01
Kefiran 3%—Cryo—120% Sorbitol	−7.44	82.02
Kefiran 3%—Cryo—150% Sorbitol	−7.63	79.63



**Figure 5.** Surface morphology of kefiran (3% *w/w*) films, with (A) and without (B) cryo-treatment at magnifications of  $\times 100$  (left) and  $\times 200$  (right).



**Figure 6.** Surface morphology of kefiran (3% *w/w*) films with glycerol (90% *w/w* on dry kefiran basis), with (A) and without (B) cryo-treatment at magnifications of  $\times 100$  (left) and  $\times 200$  (right).



**Figure 7.** Surface morphology of kefiran (3% *w/w*) films with sorbitol addition (90% *w/w* on dry kefiran basis), with (A) and without (B) cryo-treatment at magnifications of  $\times 100$  (left) and  $\times 200$  (right).

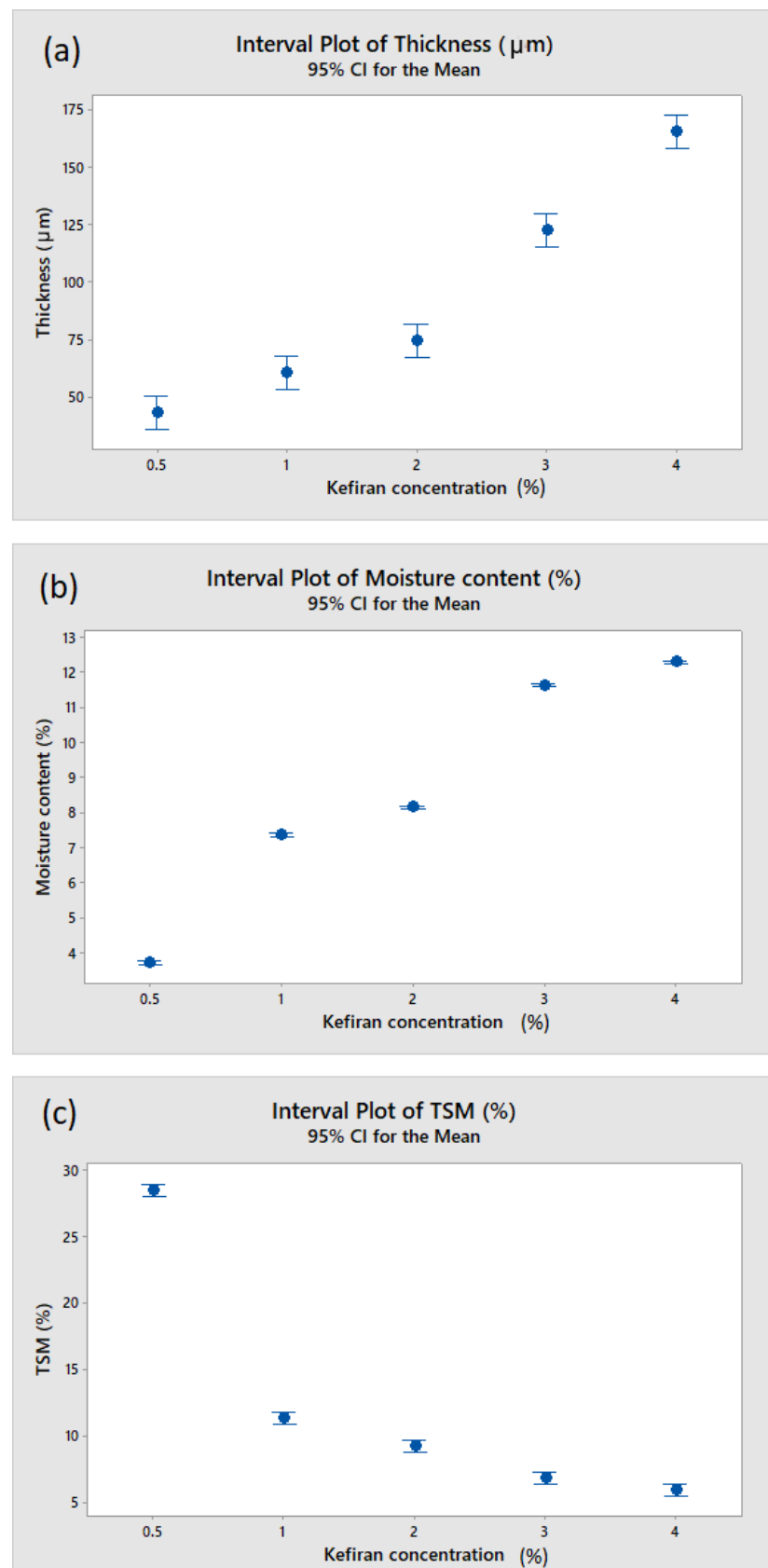
### 3.2. Effect of Kefiran Concentration

The film thickness increased concomitantly to the kefiran content (Figure 8a), with this observation being more pronounced in the polysaccharide concentrations that were greater than 2%. The higher kefiran concentrations cause more interactions between the polymeric chains during cryo-treatment, leading to more cohesive and strengthened matrices that maintain their structure and shape to a significant degree, even after water evaporation. Alongside the thickness, the moisture content of the films increased with the kefiran concentrations (Figure 8b), caused by the polysaccharide's capability to bind and withhold the water molecules within the film systems.

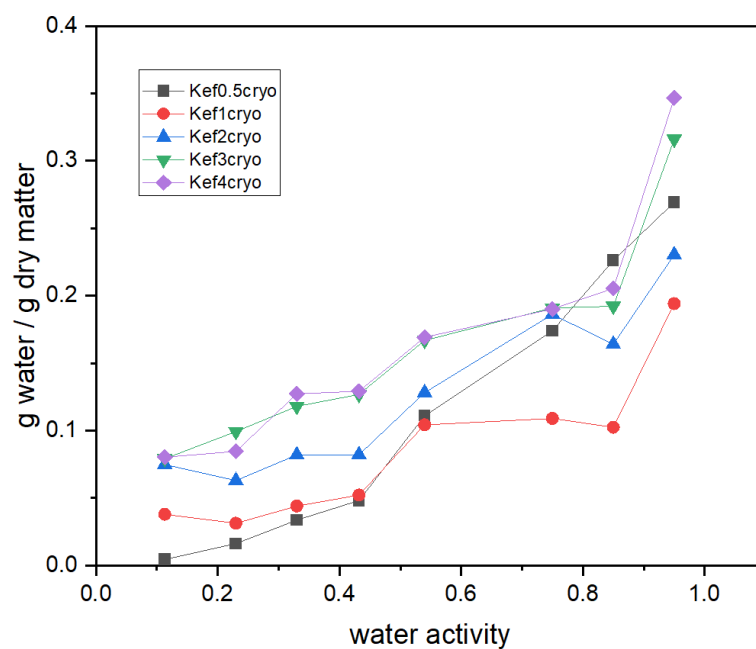
On the contrary, as shown by the results that are presented in Figure 8c, the cryo-film solubility decreased significantly with increased kefiran concentrations since, as mentioned previously, elevated concentrations of the polysaccharide lead to more cross-chain interactions and more dense structures that dissolve less.

Due to the water binding nature of kefiran, the water vapor adsorption isotherms showed increasing values alongside the kefiran concentration throughout the RHs range that was studied (Figure 9).

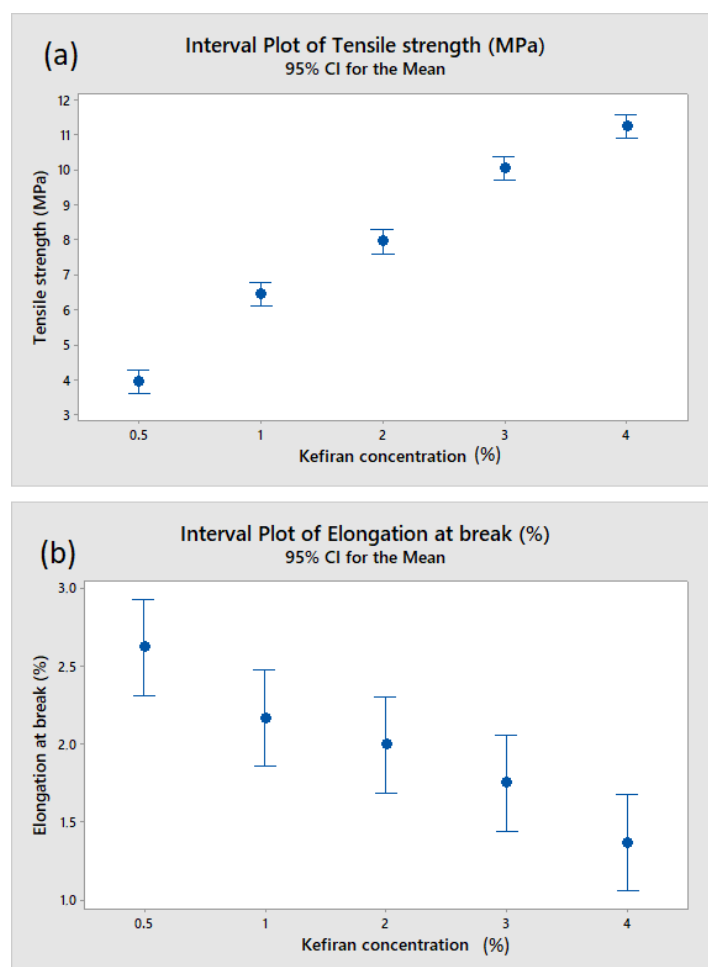
As expected, the increasing kefiran concentrations, and thus the dry mater, resulted in increased tensile strengths and reduced elongation at break values for the manufactured films (Figure 10). This is the result of the denser structures that are formed with more intermolecular interactions at higher polysaccharide concentrations, leading to more durable films, which are more brittle with less elasticity. The films without plasticizer were too brittle and fractured easily.



**Figure 8.** Effect of kefir concentration on cryo-films thickness (a), moisture content (b), and total soluble matter (TSM) (c).



**Figure 9.** Adsorption isotherms of kefir cryo-films at different concentrations (Kef0.5cryo: kefir 0.5% with cryo-treatment; Kef1cryo: kefir 1% with cryo-treatment; Kef2cryo: kefir 2% with cryo-treatment; Kef3cryo: kefir 3% with cryo-treatment; Kef4cryo: kefir 4% with cryo-treatment).



**Figure 10.** Effect of kefir concentration on cryo-film tensile strength (a) and elongation at break (b).



### 3.3. Effect of Plasticizer Addition

The use of plasticizers in the fabrication of the films, especially above certain concentrations, caused the membrane thickness to increase. Precisely, as can be seen from Figure 11a, this effect of glycerol and sorbitol on the film-thickness was observed for concentrations above 90 and 60% (on dry kefiran basis), respectively. The presence of the plasticizer in such matrices acts as a filler between the polymeric chains, which, especially above certain concentrations, causes the film thickness to rise.

The greater water binding ability of glycerol, in comparison to sorbitol, is clearly depicted in Figure 11b, in which the moisture content of the films is shown to concurrently increase with the glycerol concentration. In the case of sorbitol, the film moisture was not affected when compared to the control sorbitol-free samples.

The rise in the film solubility (as TSM), alongside the plasticizer concentration of the films, is portrayed in Figure 11c. This effect of the plasticizers, as discussed previously, is the consequence of reduced biopolymer molecular interactions, with the maximum in solubility being observed at 90%. At greater concentrations, lower solubilities were seen, which is possibly due to the formation of a more compact structure that intensifies the interactions between the kefiran chains.

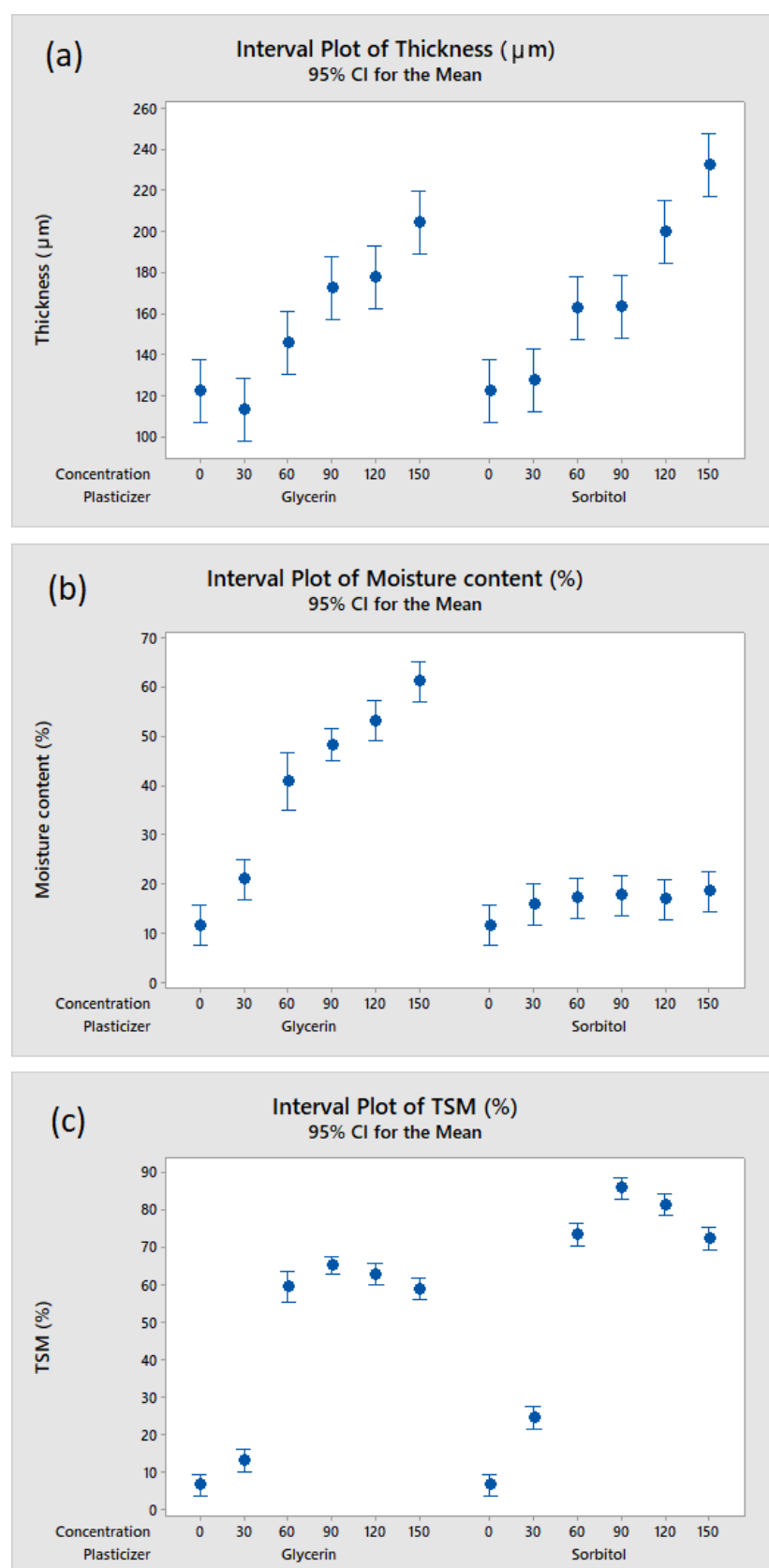
The adsorption capacity of the films rose as expected with the RHs that were used (Figures 12 and 13). A greater water vapor adsorption (>75%) was seen in the samples with plasticizers with increasing values together with their concentration. Glycerol, compared to sorbitol, had a more pronounced effect on the water vapor adsorption throughout the range of RHs that were studied, whereas, for the latter, this effect was more noticeable in the higher water vapor content environments.

The use of plasticizers in the kefiran-film matrix, as well as increasing their concentrations, caused the tensile strength to improve and lowered the elongation at break that the films could withstand (Figure 14). The tensile strength and the elongation at break values appeared to reach a plateau at concentration levels above 120% for glycerin and 90% for sorbitol (*w/w* on dry kefiran). Any further increase in the plasticizer concentration did not show any significant alteration in the mechanical properties of the films. This effect of glycerol (up to 35%) was also reported by [15]. According to the authors, the plasticizer molecules can be easily inserted between the polymer chains and they produce a “cross-linking” effect that decreases the free volume of the polymer, which causes the mechanical strength of the films to be decreased and the extensibility to be enhanced.

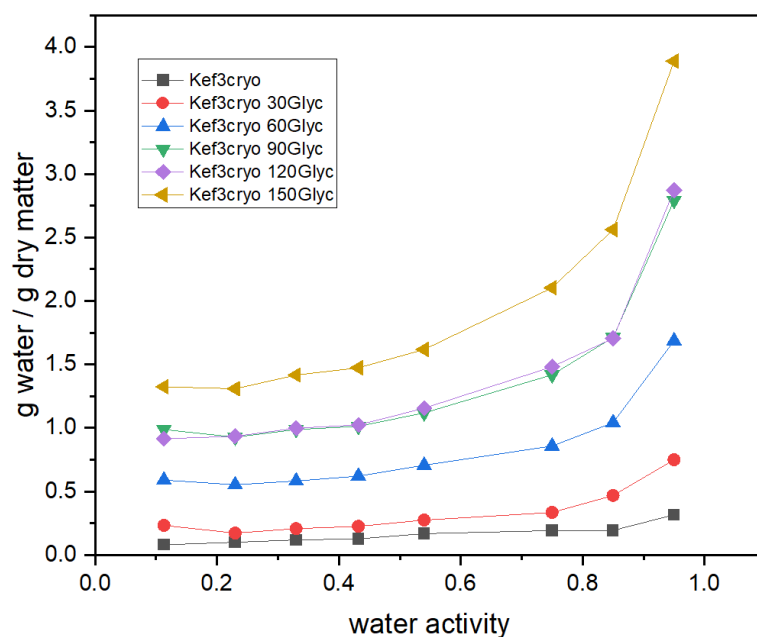
The DSC measurements of the plasticizer-containing samples, and its increasing concentrations, in agreement with the existing literature [15], lowered the shown  $T_g$  and  $T_m$  values due to the plasticizers’ water-retentive effect.

The surface roughness was affected by the presence of the plasticizers (Figures 15 and 16). In both cases, the film surface appeared to be smoother as the concentration increased, but this effect, in the case of sorbitol, appeared to culminate at a 90% concentration (on dry kefiran basis). The greater concentrations of sorbitol led to rougher film surfaces, which was possibly due to plasticizer crystallization.

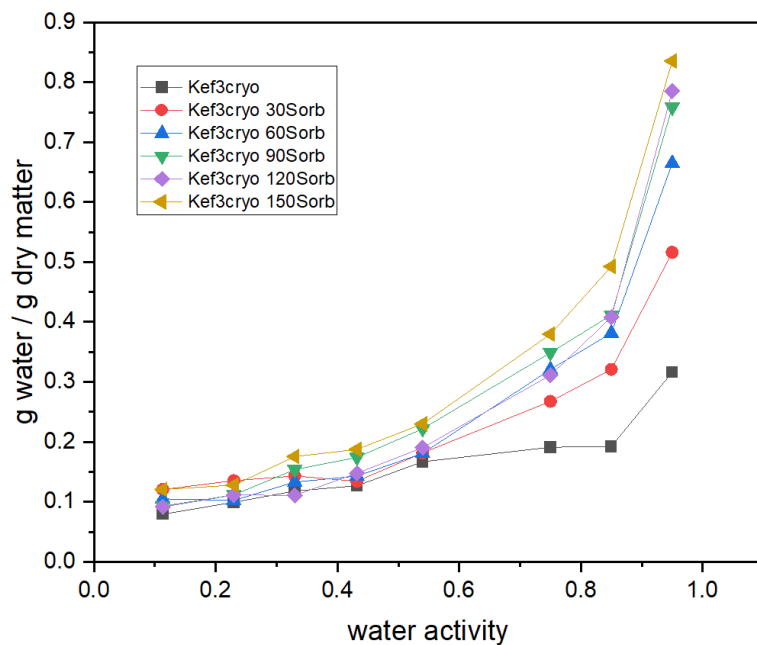




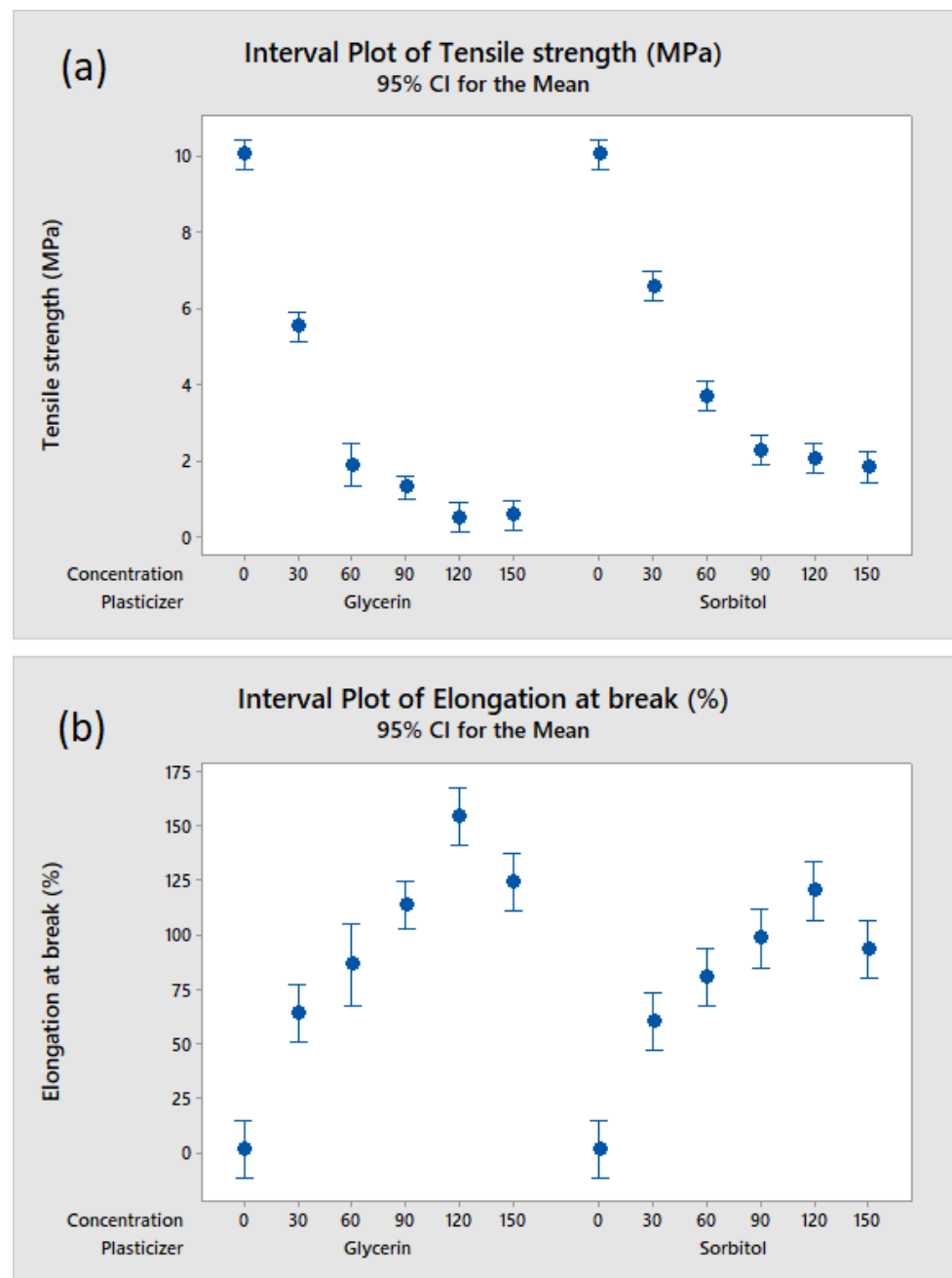
**Figure 11.** Effect of plasticizer addition on kefir cryo-films thickness (a), moisture content (b), and total soluble matter (TSM) (c).



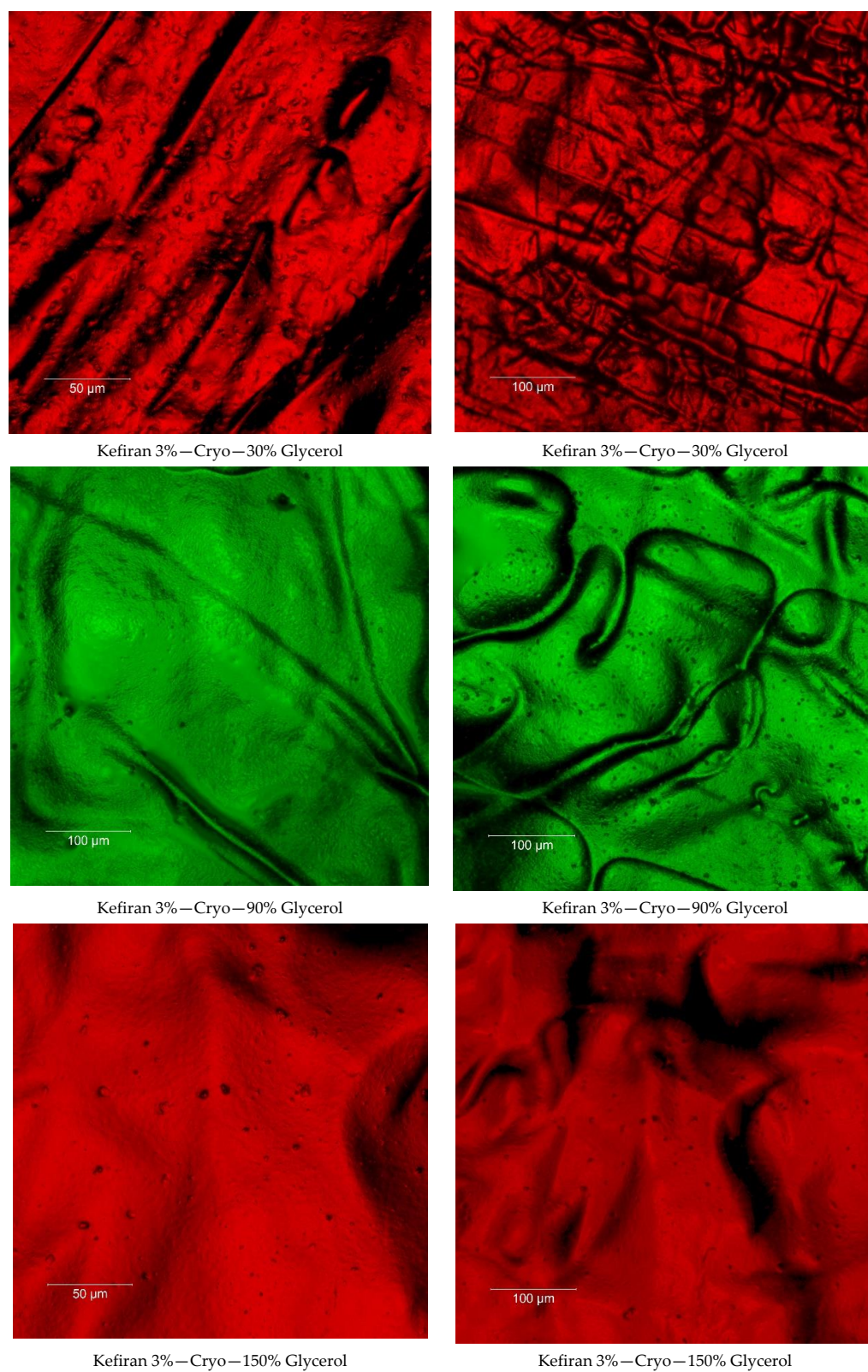
**Figure 12.** Adsorption isotherms of kefir cryo-films with or without glycerol addition at various concentrations (Kef3cryo: kefir 3% with cryo-treatment; Kef3cryo 30Glyc: kefir 3% with cryo-treatment and 30% glycerol on dry kefir basis; Kef3cryo 60Glyc: kefir 3% with cryo-treatment and 60% glycerol on dry kefir basis; Kef3cryo 90Glyc: kefir 3% with cryo-treatment and 90% glycerol on dry kefir basis; Kef3cryo 120Glyc: kefir 3% with cryo-treatment and 120% glycerol on dry kefir basis; Kef3cryo150Glyc: kefir 3% with cryo-treatment and 150% glycerol on dry kefir basis).



**Figure 13.** Adsorption isotherms of kefir cryo-films with or without sorbitol addition at various concentrations (Kef3cryo: kefir 3% with cryo-treatment; Kef3cryo 30Sorb: kefir 3% with cryo-treatment and 30% sorbitol on dry kefir basis; Kef3cryo 60Sorb: kefir 3% with cryo-treatment and 60% sorbitol on dry kefir basis; Kef3cryo 90Sorb: kefir 3% with cryo-treatment and 90% sorbitol on dry kefir basis; Kef3cryo 120Sorb: kefir 3% with cryo-treatment and 120% sorbitol on dry kefir basis; Kef3cryo150Sorb: kefir 3% with cryo-treatment and 150% sorbitol on dry kefir basis).

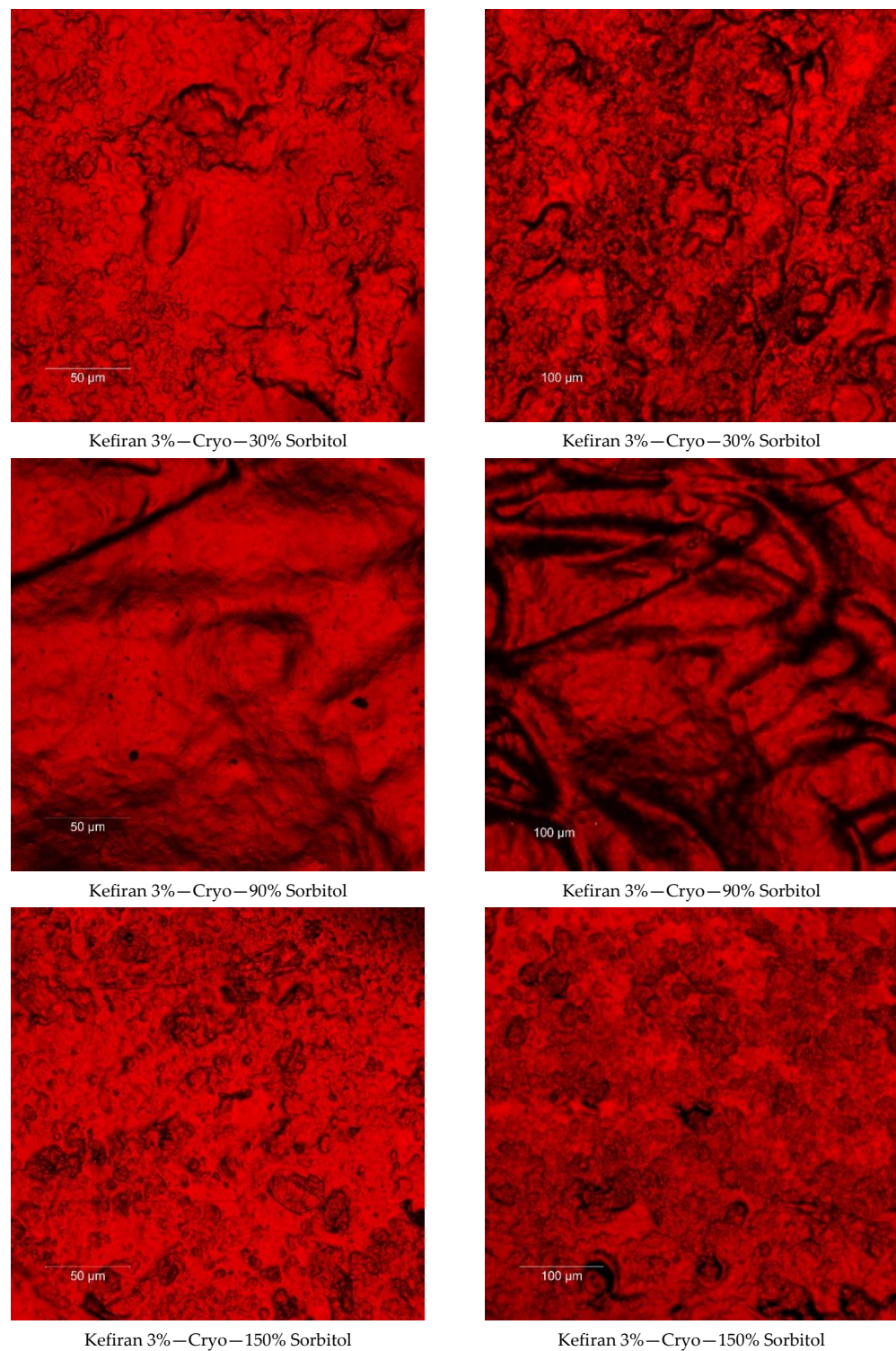


**Figure 14.** Effect of plasticizer addition on kefiran cryo-film tensile strength (a) and elongation at break (b).



**Figure 15.** Surface morphology of kefiran (3% *w/w*) films with increasing glycerol addition (30, 60, 90, 120, and 150% *w/w* on dry kefiran basis) and cryo-treatment at magnifications of  $\times 100$  (left) and  $\times 200$  (right).





**Figure 16.** Surface morphology of kefirin (3% *w/w*) films with increasing sorbitol addition (30, 60, 90, 120, and 150% *w/w* on dry kefirin basis) and cryo-treatment at magnifications of  $\times 100$  (left) and  $\times 200$  (right).

#### 4. Conclusions

Kefiran cryogel-films were prepared (Figure 17) with and without cryo-treatment and, to a certain extent, the effect of the plasticizers was elucidated, which allowed the formulation of films based on the application of defined requirements.



**Figure 17.** Selected images of the kefir cryogel-films prepared with or without plasticizers, in contrast to films prepared without cryo-treatment.

The cryo-treatment and the plasticizer addition affected the kefir-film properties and morphology, increasing the film thickness and the moisture content, but slightly lowering their mechanical sturdiness. Glycerol, along with the cryo-treatment, resulted in a slight decrease in solubility, while the opposite was observed for sorbitol.

The film thickness, the moisture content, and the tensile strength increased, while their solubility and elongation at break values decreased with increasing kefir concentration.

The use of plasticizers in the fabrication of the films, especially above certain concentrations (90% for glycerol and 60% (on dry kefir basis) for sorbitol) caused the membrane thickness to increase. The films' moisture content was concurrently increased with the glycerol concentration, but this was not the case for sorbitol. The films' solubility was increased alongside the plasticizer concentration, with the maximum solubility being observed at 90%.

The use of plasticizers, and increasing their concentrations, caused the tensile strength to increase and the elongation at break to decrease. However, the tensile strength and the elongation at break values appeared to reach a plateau at concentration levels above 120% for glycerol and 90% for sorbitol.

The water vapor adsorption capacity increased with the application of the cryo-treatment and glycerol addition and concentration, while, in the case of sorbitol, this



effect was not so pronounced throughout the range of RHs that were studied. Increasing the kefir concentration resulted in increasing the water vapor adsorption isotherms.

The thermal analysis (TGA) showed the kefir films to be thermally stable to a satisfactory level, whereas the DSC measurements elucidated that the presence and the concentration of the plasticizers reduced the measured Tg and Tm values.

The cryo-treatment and the plasticizers (in a concentration-dependent manner) resulted in the films appearing smoother. In the case of sorbitol, greater concentrations (90%) led to rougher film surfaces.

**Author Contributions:** Conceptualization, S.E., A.G. and G.D.; methodology, S.E., A.G. and G.D.; software, S.E., A.G. and G.D.; validation, S.E., A.G. and G.D.; formal analysis, S.E., A.G. and G.D.; investigation, S.E., A.G. and G.D.; resources, S.E., A.G. and G.D.; data curation, S.E., A.G. and G.D.; writing—original draft preparation, S.E., A.G. and G.D.; writing—review and editing, S.E., A.G. and G.D.; visualization, S.E., A.G. and G.D.; supervision, A.G. and G.D.; project administration, G.D.; funding acquisition, S.E. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was co-financed by Greece and the European Union (European Social Fund—ESF) through the Operational Programme «Human Resources Development, Education and Lifelong Learning» in the context of the project “Reinforcement of Postdoctoral Researchers—2nd Cycle” (MIS-5033021), implemented by the State Scholarships Foundation (IKY).



**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author.

**Conflicts of Interest:** The authors declare no conflict of interest.

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