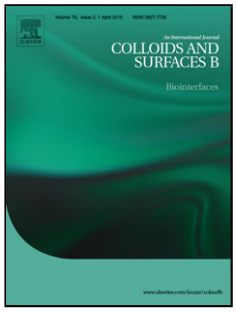

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Kefiran-alginate gel microspheres for oral delivery of ciprofloxacin

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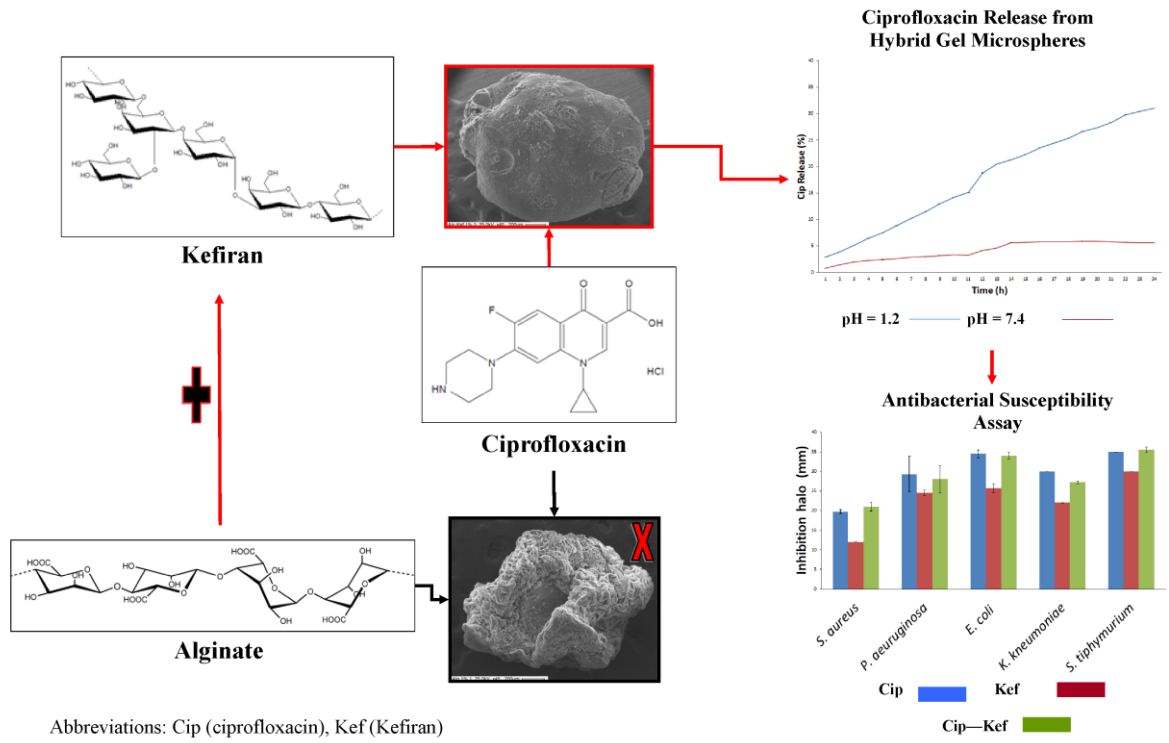
Graphical abstract



Highlights

- Hybrid gel microspheres composed by alginate and kefiran were developed.
- Kefiran-alginate microspheres protect the antibiotic from acid gastric conditions.
- FTIR and thermogravimetry suggest non-covalent interactions between the matrix components.
- Kefiran microspheres showed controlled release of ciprofloxacin.
- Kefiran and ciprofloxacin showed complementary antimicrobial activities.

Abstract

Ciprofloxacin is a broad-spectrum antibiotic associated with gastric and intestinal side effects after extended oral administration. Alginate is a biopolymer commonly employed in gel synthesis by ionotropic gelation, but unstable in the presence of biological metal-chelating compounds and/or under dried conditions. Kefiran is a microbial biopolymer able to form gels with the advantage of displaying antimicrobial activity. In the present study, kefiran-alginate gel microspheres were developed to encapsulate ciprofloxacin for antimicrobial controlled release and enhanced bactericidal effect against common pathogens. Scanning electron microscopy (SEM) analysis of the hybrid gel microspheres showed a spherical structure with a smoother surface compared to alginate gel matrices. *In vitro* release of ciprofloxacin from kefiran-alginate microspheres was less than 3.0% and 5.0% at pH 1.2 (stomach), and 5.0% and 25.0% at pH 7.4 (intestine) in 3 and 21 h, respectively. Infrared spectroscopy (FTIR) of ciprofloxacin-kefiran showed the displacement of typical bands of ciprofloxacin and kefiran, suggesting a cooperative interaction by hydrogen bridges between both molecules. Additionally, the thermal analysis of ciprofloxacin-kefiran showed a protective effect of the biopolymer against ciprofloxacin degradation at high temperatures. Finally, antimicrobial assays of *E. coli, K. pneumoniae*, *P. aeruginosa*, *S. typhymurium,* and *S. aureus* demonstrated the synergic effect between ciprofloxacin and kefiran against the tested microorganisms.

Keywords: Hybrid gel microspheres, Kefiran, Alginate, Ciprofloxacin, antimicrobial activity, controlled release, Kefiran-ciprofloxacin complex

1. Introduction

Ciprofloxacin (Cip) is a broad-spectrum antibiotic belonging to the family of fluoroquinolones. Cip is the fifth generic antibiotic in the world accounting for 24% of sales in the therapeutic market (close to USD 4,340 million per year in 2014). Fluoroquinolones are commonly used for the treatment of many microbial infections because DNA gyrase and topoisomerase IV inhibition causes bacterial cell death [1]. On the other hand, Cip is associated with gastric and intestinal disorders in humans when the antibiotic is orally administered during long periods [2]. Additionally, Cip possesses low solubility in physiological aqueous media and propensity to molecular stacking by the π-π interactions because of the presence of the aromatic rings when it is administered at high concentrations, which decreases the antibiotic bioavailability. In order to improve Cip oral delivery, the development of novel systems able to capture, transport, or deliver the molecule is desirable.

Alginate (Alg) is a linear polysaccharide produced by some brown algae (*i.e.*, *Macrocystis pyrifera*, among others) and some bacteria (*i.e.*, *Pseudomonas aeruginosa*). Alg is composed of β-D-mannuronic and α-L-guluronic acids; it can be cross-linked in the presence of multivalent cations such as Ca^{2+}, Zn^{2+}, etc. Alg is used in many biotechnology applications because the gel texture is similar to that of the extracellular matrix and considered GRAS (Generally Regarded as Safe) by the FDA [3, 4]. Also, Alg is biocompatible [5], of no thrombogenic nature [4], and low cost [6]. Nevertheless, Alg gels are unstable in the presence of cation chelating molecules such as phosphate, present in biological fluids. Likewise, the alginate tridimensional gel structure is lost after freeze-drying and rehydration in aqueous environments. In order to prevent a drastic Alg gel swelling and to produce matrix structure stabilization, one feasible strategy is to combine Alg with other polymers [7]

Kefiran (Kef) is a water-soluble glucogalactan produced by *Lactobacillus kefiranofaciens* and is present in kefir grains [8]. Kef has a Newtonian behavior in diluted solutions, becomes pseudoplastic at high concentration, and it is able to form gels as a result of cryogenic treatment [9]. On the other hand, it has been reported that Kef modulates the gut immune system [10], protecting epithelial cells against *Bacillus cereus* toxin [11], and has many beneficial activities, such as antitumor, antibacterial [12], anti-inflammatory [13], healing [14] and antioxidant [15]. Also, some studies reported the antibiotic activity of kefiran against Gram-positive, Gram-negative bacteria and the yeast *Candida albicans* [9, 16].

The aims of the present work were to develop hybrid Kef-Alg gel microspheres loaded with Cip, to examine the interaction and stability of ciprofloxacin in the matrix, in order to evaluate the antimicrobial activity of the formulation and the potential synergic or additive effect between Kef and Cip against pathogenic bacteria. Analyses of the gel microspheres

were performed by infrared spectroscopy (FTIR), scanning electron microscopy, thermogravimetry, and antimicrobial tests in agar and liquid media.

Materials and Methods

2.1. Materials

Cip (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid), low-viscosity sodium alginate, citric acid ($C_6H_8O_7$), sodium citrate ($C_6H_5Na_3O_7$), calcium chloride ($CaCl_2$), potassium chloride (KCl), hydrochloric acid (HCl), potassium phosphate (KH_2PO_4), potassium bromide (KBr), brain heart infusion broth, Mueller-Hinton medium and nutrient broth were purchased from Sigma-Aldrich (St. Louis, Mo, US).

2.2. Kefiran purification

The purification procedure was based on the protocol previously described by Piermaria et al. with some modifications [17]. A weighted amount of kefir grains was suspended in boiling water for 30 min with discontinuous stirring, after that, the mixture was centrifuged at 10,000xg for 20 min at 20°C to separate the biomass. The supernatant was precipitated by addition of three volumes of cold ethanol (left at -20°C overnight). Then, the mixture was centrifuged at 10,000xg for 20 min at 4°C, and the resulting pellets were dissolved in hot water. The analysis of protein content in the samples was performed by the Coomassie Blue technique using BSA (fraction V) as standard [18]. The precipitation procedure was repeated twice, and no protein content was detected in the samples after purification. Finally, the precipitate was dissolved in hot distilled water and freeze-dried.

2.3. Interaction analyses between ciprofloxacin and kefiran

2.3.1. Binding assay

Based on the previously described protocol with some modifications [19], solutions of 100 µg/mL Cip and 1.0% (w/v) Kef were made separately in citrate buffer (pH= 5.0), then 450 µL of the buffer solution (with or without the polymer (control)) was mixed with 50 µL of Cip solution and stirred for 1 h. Later, 1.0 mL of cold absolute ethanol was added to the aqueous solution, and the samples were centrifuged at 10,000xg for 20 min. Finally, the absorbance of the supernatant was measured at 279 nm. The binding percentage between Cip and Kef was obtained according to the following equation:

$$Binding\ (\%) = \frac{Drug\ concentration\ in\ the\ control - Drug\ concentration\ in\ the\ assay}{Drug\ concentration\ in\ the\ control} * 100 \quad (1)$$

2.3.2. Viscosity determinations

Kinematic viscosities were measured at 2.0% (w/v) Alg-Kef blends as follows: 0.0% Alg – 2.0% Kef, 1.5% Alg – 0.5% Kef, 1.0% Alg – 1.0% Kef, 0.5%Alg –1.5% Kef in a low temperature viscometer Herzog HVU482 (Integrated Scientific LTD, UK). The assays were performed according to the standard test method for kinematic viscosity of transparent and opaque liquids ASTM D445 at a constant temperature of 40°C and in a range of 2 mm^2/s to 10,000 mm^2/s.

2.3.3. *Fourier transform infrared spectroscopy (FTIR) analyses*

The mixture Kef-Cip (1:2) made for FTIR determination was prepared from an aqueous solution of kefiran (1.0%, w/v) and ciprofloxacin (2.0%, w/v) in citrate buffer (pH= 5.0) and kept for 12 h at room temperature under stirring until total dissolution of both components. After that, the sample was frozen and freeze-dried for further analysis. The FTIR spectra were obtained using KBr pellets. Samples were pressed into KBr (0.1%, w/v), and the FTIR spectra were recorded in a BOMEM-Hartmann & Braun MB-series spectrometer (Germany) with resolution of 4 cm^{-1}, 32 scans per minute and transmittance technique. The scanning range was from 400 cm^{-1} to 4,000 cm^{-1}. The data obtained were analyzed using the ACD/NMR processor academic edition.

2.4. Thermal properties

The thermal analyses of Kef, Cip and the mixture of Cip-Kef (1:2) samples were carried out using the Netzsch Sta 449 F3 *Jupiter* thermal analyzer (Germany). Five mg of each sample was placed in the equipment and scanned at a heating rate of 10°C/min at temperatures ranging from 20°C to 800°C.

2.5. Preparation of gel microspheres

The ionotropic gelation of 2.0% (w/v) Alg-Kef blends using different polymer ratios was studied. Microspheres were prepared by the jet technique, by dropping 2.0 mL of the blend solutions in 500 mM $CaCl_2$ at 0ºC. Later, the gel microspheres were washed with ultrapure water twice and kept at 5°C.

Aqueous solutions containing 100 µg/mL Cip, 1.0% (w/v) Kef and 1.0% (w/v) sodium alginate were prepared in citrate buffer (pH= 5.0) in an ice water bath (0ºC), as previously reported [19]. Alternatively, biopolymer blends containing only the antibiotic were made with 1.0% (w/v) sodium alginate or kefiran.

The percentage of encapsulation was calculated with the following equation:

$$Encapsulation\ (\%) = \frac{Initial\ concentration\ of\ the\ drug - Drug\ concentration\ in\ calcium\ solution}{Initial\ concentration\ of\ the\ drug} x\ 100 \quad (2)$$

The stability of Alg-Kef formulations using different polymer ratios up to 2.0% (w/v) was qualitatively assayed by incubating the gel microspheres in PBS (phosphate buffer saline) solution at room temperature, and determining the time for microsphere total dissolution. The results were expressed as gel strength.

2.6. Scanning Electron Microscopy (SEM) and roughness analysis

Gel microspheres were freeze-dried for 24 h before SEM observations. Furthermore, samples were prepared by sputtering the sample surface with gold using a Balzers SCD 030 metalizer, obtaining a layer thickness between 15 and 20 nm. Microsphere surfaces and morphologies were observed using Philips SEM 505 (Rochester, USA), and processed by an image digitizer program (Soft Imaging System ADDA II (SIS)).

SEM images were analyzed by ImageJ software (NIH, USA). The roughness of the surface was reflected by the standard variation of the gray values of all the pixels on the image. First, the SEM image files were opened by the software and converted to an 8-bit image. Then, all the pixels on the image were selected and statistically measured by a computer equipped with the software. The standard deviation values are directly proportional to the smoothness of the analyzed surface. The histograms of 710x SEM images were performed in duplicate.

2.7. Release at simulated gastric and intestinal conditions

Cip release from the microspheres was evaluated in simulated gastric and intestinal fluids. Briefly, 200 mg of microspheres was weighed and incubated in 50 mM KCl/HCl buffer solution (pH= 1.20, gastric conditions) at 37°C. Similarly, the same weight of microspheres was incubated in 50 mM potassium phosphate buffer solution (pH= 7.4) at 37°C for simulated intestinal fluid tests. Samples were taken at different times, and ciprofloxacin was measured at the maximum absorbance wavelength (277 or 270 nm based on the calibration curve in each buffer solution). In order to keep a constant vial volume of 1.5 mL of fresh media, the vial was refilled at each sample point.

2.8. Antimicrobial assays

Antimicrobial activities of 10 µg/mL Cip, 1.0% (w/v) kefiran and the Cip-Kef mixture (10 µg/mL Cip and 1.0% (w/v) Kef) were evaluated against *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae* (ATCC 70063), *Pseudomonas aeruginosa*, *Salmonella typhymurium* and *Staphylococcus aureus* using a modified agar disk diffusion method.

Bacteria were maintained in liquid cultures of brain heart infusion broth, until reaching a concentration of 0.5 McFarland scale. Later, the microorganisms were dispersed using sterile cotton swab in agar plates containing 25 mL of Mueller-Hinton agar medium in 100 mm diameter petri dishes. Furthermore, 30 µL of each sample solution was placed inside the cylinders, and the plates were incubated at 37°C for 24 h, and inhibition growth zones were determined. The agar assays were performed in duplicate.

Antimicrobial activity of Cip, Kef and Cip-Kef in liquid medium was performed using *E. coli* (ATCC 35218), *K. pneumoniae* (ATCC 70063), *P. aeruginosa*, *S. typhymurium* and *S. aureus*. The microorganisms were cultured in nutrient broth at 37°C for 24 h. After that, 1.0 mL of each culture was added to 40 mL of fresh culture medium contained in Erlenmeyer flasks and incubated at 37°C and 120 rpm. The flasks were marked as: (a) Control (bacteria alone), (b) bacteria plus 1.0 µg/mL Cip, (c) bacteria plus 0.05% (w/v)

Kef, (d) bacteria plus 1.0 µg/mL Cip and 0.05% (w/v) Kef. The antimicrobials were added when the bacteria reached half of the exponential growth phase. Bacterial growth was turbidimetrically monitored at 600 nm for 24 h. Experiments were carried out in duplicate.

Results and Discussion

3.1. Interaction analyses between ciprofloxacin and kefiran

The interaction between Cip and Kef was studied at acid pH (5.0), adjusted with citrate buffer because Cip precipitates and Kef becomes more viscous at alkaline pH. Precipitation of an aqueous mixture of Cip-Kef (1:100) showed 22.8% of Cip bound to the polymer. Furthermore, vibrational spectroscopy (FTIR) was used to determine the type of interaction between Cip and Kef. FTIR spectra of Cip, Kef and the mixture Cip-Kef are shown in **Figure 1**, and the relevant assignation bands are displayed in **Table 1S**.

The characteristic stretching vibrations of the carbonyl group and the phenyl framework conjugated to –COH of Cip were assigned at 1732 cm^{-1} and 1628 cm^{-1}, respectively, as previously reported [20]. The stretching vibration of the proton of the amine group in the piperazine moiety and of the C-F bond of Cip were assigned at 1395 $cm^{-1}$and 1295 cm^{-1}, respectively [19]. Regarding Kef, the broad band around 3380 cm^{-1} was assigned to the O-H stretching vibrations of the hydroxyl groups typically associated with polysaccharide structures [12]. Meanwhile, the weak stretching peak at 2924 cm^{-1} indicates the presence of methyl groups in Kef. The band at 1067 cm^{-1} could be possibly assigned to the ring vibrations overlapped with stretching vibrations of (C–OH) side groups and (C–O–C) glycosidic band vibration, which are characteristic of each polysaccharide [21]. Finally, the vibration bands around 890 cm^{-1} displayed in Kef and in Cip-Kef formulation indicate the presence of β-glycosidic linkages. The presence of ß-glycosidic linkages in Cip is relevant since the high biological activity of the biopolymer is preserved [15]. In **Figure 1**, the displacements of the typical bands of each component of the Cip-Kef formulation suggest interactions between the antibiotic and the biopolymer. Among them, the most relevant are the blue shift of the carbonyl stretching vibrations (shifted 14 cm^{-1}) and the amine group (shifted 10 cm^{-1}) of Cip that can interact together. On the other hand, the 37 cm^{-1} shift to lower wavenumbers of the Kef hydroxyl groups strongly suggests the presence of intermolecular hydrogen interactions, *i.e.,* hydrogen bridges. However, the absence of new Cip bands in the presence of the polysaccharide indicated that there was no obvious chemical reaction. Preservation of the biological activity of both components and their potential synergism could be a significant alternative to be used in biological systems.

For microsphere preparation, it is important to mention that pure kefiran is not able to produce gel microspheres by ionotropic gelation. Alg-Kef blends containing up to 2.0% (w/v, total polymer concentration) were analyzed by gel strength and viscosimetry (**Table 2S**). Kef concentrations higher than 1.0% did not show stable gels in physiological solutions at room temperature, and also displayed low viscosity solutions. On the other hand, aqueous solutions of 1.5% or higher Alg concentration produced hard gel microspheres by ionotropic gelation, but they were very viscous solutions difficult to be manipulated at room temperature. They had an unstable morphological gel structure after freeze-drying and also displayed low antimicrobial activity due to low kefiran concentration. Besides, the 1.0% Alg–1.0% Kef polymer blend provided manageable

viscous solutions and stable gel microspheres with good antimicrobial activity and morphology after intensive drying (see below). The 1.0% Alg–1.0% Kef polymer mixture was selected for further studies.

FTIR spectra of Alg and Alg-Kef microspheres showed characteristic absorption bands of alginate at around 1631 cm^{-1} (asymmetric stretching vibration of -COO) and 1440 cm^{-1} (symmetric stretching vibration of the COO group) with a slight shift due to calcium cross-linking, as previously reported (**Figure 1S**) [19]. In the FTIR spectrum of the Alg-Kef microsphere composites, the appearance of new peaks related to kefiran functional groups was observed. The characteristic peaks (previously described in **Table 1S**) at around 294 cm^{-1}, 895 cm^{-1} and 1029 cm^{-1} indicate the presence of the polymer in the alginate network and, even more, the wavenumber displacements in comparison with pure kefiran suggest its interpenetration by intermolecular interactions within alginate and not only due to a physical mixture.

3.2. Thermal analysis

Thermal properties of Cip, Kef and Cip-Kef (1:2) complex were determined (**Figure 2**). The Tg value of Kef was observed at 307°C with a sharp peak. Cip thermal degradation begins at 200°C approximately, as previously reported [22]. In **Figure 2B**, Cip does not show sharp peaks but broad small peaks in the range of 140°C to 500°C. The formulation displayed a broad peak between 200°C and 320°C. Cip and Kef decomposition began at 140°C, but the Cip peak was taller than the Kef peak. A small step of decomposition of the Cip-Kef complex began at 200°C, as is shown in **Figure 2A**, but after 500°C it became stable, suggesting that Kef provided some protection to ciprofloxacin from thermal degradation.

Particularly, the endothermic peak of the Kef-Cip complex was observed at 267.1°C, while Kef and Cip displayed peaks at 307.0°C and 270.3°C, respectively. The peak downshift was indicative of the complex formation between Cip and Kef, weakening the interaction between water and the molecules, particularly for the Kef polymer chains, which led to water release at lower temperatures.

The thermal analysis of Alg and Alg-Kef (1:1) microspheres showed similar weight loss profiles (**Figure 2S A**). However, the DTGA graph showed three distinctive endothermic peaks (**Figure 2S B** and **Table 3S**). The comparative analysis of Alg and Alg-Kef (1:1) endothermic peaks of the DTGA graph showed at least a $\Delta T \approx 5$°C difference between both samples in three peaks, confirming some interactions between alginate and kefiran even in the gelled structure of microspheres.

The first peaks at about 100°C indicate water loss from both polymers. Particularly, the lower endothermic peak at 97.5°C in the Alg-Kef sample could indicate the replacement of hydrogen bridges of water in alginate by the free hydroxyls of kefiran in the mixture with the release of free water molecules at lower temperature than in the alginate sample (102.1°C) (**Figure 2S B**, **Table 3S**).

The endothermic peak of alginate at 223.6°C was shifted up to 232.6°C for Alg-Kef (1:1). The +9°C difference between Alg-Kef and Alg could be interpreted as the result of a stronger gel structure with a closer matrix network provided by the presence of Kef. On the other hand, the endothermic peaks in the 270-280°C range in the DTGA graph showed a contrary trend, probably because of the thermal disruption of the weak nonionic interactions between the polymer chains in this temperature range (**Figure 2S B**, **Table 3S**).

3.3. Encapsulation of ciprofloxacin in microspheres and scanning electron microscopy analysis

Microspheres of Kef-Alg were obtained by gelation in the presence of calcium ion, and the gel matrix showed a ciprofloxacin encapsulation efficiency of 80%. The blended matrix showed an interesting increment in structural stability. Meanwhile, Alg- based gel microspheres showed a shrinkable matrix after the freeze-drying process, losing the microsphere morphology concomitantly with an irregular surface observed by SEM (**Figures 3A and 3B**). On the other hand, Kef incorporation into the formulation kept the spherical structure of the gel microspheres with a smoother surface rather than alginate microspheres (**Figures 3C and 3D**). The observations by SEM suggested that the addition of Kef in the Alg gel formulation stabilizes the matrix structure by avoiding Alg swelling, which disrupts the gel matrix structure.

The results observed by the SEM images were correlated with the ImageJ profiles (**Figure 4**). The analysis of Alg microsphere images showed a histogram with a wide range of gray values, due to the high roughness and folds on the surface. However, Alg-Kef microsphere composites showed a narrow distribution of the surface gray values due to a smooth surface. After Kef incorporation into the Alg based matrix, the standard variation (*i.e.*, standard deviation) decreased by about 35%, from 54 to 35, and displayed a smooth surface pattern that can be correlated with an increase in spheroid structural morphology.

3.4. Release at simulated gastric and intestinal conditions

Figure 5 shows the release curves of the Alg gel microspheres with and without Kef under gastric and intestinal stimulated conditions. Under gastric conditions, the rate of Cip release was slower in the case of microspheres composed of Alg-Kef compared to the Alg ones. The Cip release slopes from the gel microspheres were 0.46 h^{-1} and 0.26 h^{-1} (43% lower) for Alg and Alg-Kef microspheres, respectively. These results indicate a more stable gel network and could suggest an interpenetrated gel network between Alg and Kef. On the other hand, Cip releases from Alg and Alg-Kef microspheres under intestinal conditions were fast in both cases, displaying almost the same slopes of 1.30 h^{-1} (about 3-4 times higher compared to the acid conditions). These results confirmed the SEM observations displayed in **Figure 5**, indicating that microspheres made with Alg-Kef matrix showed better stability and their spherical morphology was preserved.

3.5. Antimicrobial assay

The antimicrobial assay, *i.e*., antibiogram, of Cip, Kef and Cip-Kef made against five pathogenic strains: *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* and *Salmonella typhymurium*, is shown in **Figure 3S** (**Supplementary Material**). Interesting antimicrobial activities of Cip and Kef alone with considerable inhibition halos were observed. These results not only confirm the expected Cip antimicrobial activity, but even the Kef bactericidal effect, as previously reported [16]. The Cip and the Cip-Kef mixture showed almost the same growth inhibition zone in all studied bacteria, which indicates the higher diffusion of the Cip molecules along the agar medium, but also the most relevant absence of interference within the Kef. Kef antimicrobial activity was lower than that of Cip in all tested cases. These facts can probably be attributed to the high molecular weight of Kef, which limits its diffusion across the agar medium. For this reason, another set of antimicrobial experiments were performed in liquid medium. The antimicrobial activity of Kef, Cip, and Cip-Kef against *E. coli, K. pneumonia, P. aeruginosa, S. aureus* and *S. typhymurium* was tested in liquid media (**Figures 6 to 8**). The antimicrobial activity of Cip was higher than that of Kef in most of the microbial cultures. However, the Kef-Cip complex showed additional antimicrobial activity over all bacterial cultures tested. Interestingly, the growth of *S. aureus* seemed to be more sensitivity to the Cip-Kef complex, which could suggest a synergic antimicrobial effect over the Gram-positive bacteria (**Figure 8**).

4. Conclusions

The development of hybrid gel microspheres composed of Alg and Kef for oral delivery provides not only a matrix able to encapsulate bioactive molecules, *e.g*., antibiotics, but also offers beneficial biological activity provided by Kef. Considering that the alginate tridimensional gel structure is lost after freeze-drying and rehydration in aqueous environments, the presence of Kef contributes to the matrix stability and prevents the shrinkage of the Alg matrix, keeping the spheroidal structure of microspheres.

The Kef-Alg hybrid gel matrix provides a protective effect to the load against harsh acid gastric environmental conditions but with the advantage of displaying proper molecular release at alkaline pH of the intestine. This property reduces the toxicity of Cip and increases the bioavailability of the antibiotic in the intestine.

In that type of application, it is indispensable to obtain Kef with a high degree of purity, because impurities like proteins could interfere with its solubility and biological activities, such as antimicrobial activity. Many methodologies have been reported to obtain Kef, some directly from kefir grains and others from the fermented media. In the present study, a high degree of purity was obtained by extracting Kef directly from kefir grains by a simple, reproducible and cheap methodology.

Importantly, the antimicrobial activity of Kef is preserved in the hybrid matrix and additionally, it stabilizes the encapsulated Cip against thermal degradation, which suggests some type of interaction between Kef and Cip. The interaction between Cip and Kef

studied by infrared spectroscopy demonstrates a noncovalent interaction, which is provided by hydrogen bridges, suggesting a complex formation between both molecules.

Antimicrobial assays of the Kef-Alg microspheres containing Cip against five microbial potential pathogens (*i.e.*, Gram-positive and Gram-negative bacteria) showed greater antimicrobial activity of the Cip-hybrid formulation compared to aqueous Cip. These results suggest the possibility of reducing the amount of Cip to be orally administered because of two reasons: (1) the enhanced bioavailability of the antibiotic in the intestine due to a controlled release profile, reducing undesirable side effects; (2) the presence of complementary antimicrobial activity of Kef, which is highly beneficial during chronic antimicrobial treatments and in potentially compromised populations such as the elderly and small children [23].

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Figures

Figure 1. FTIR spectra of Cip, Kef and the Cip-Kef (1:2 ratio) formulation at pH= 5.0.

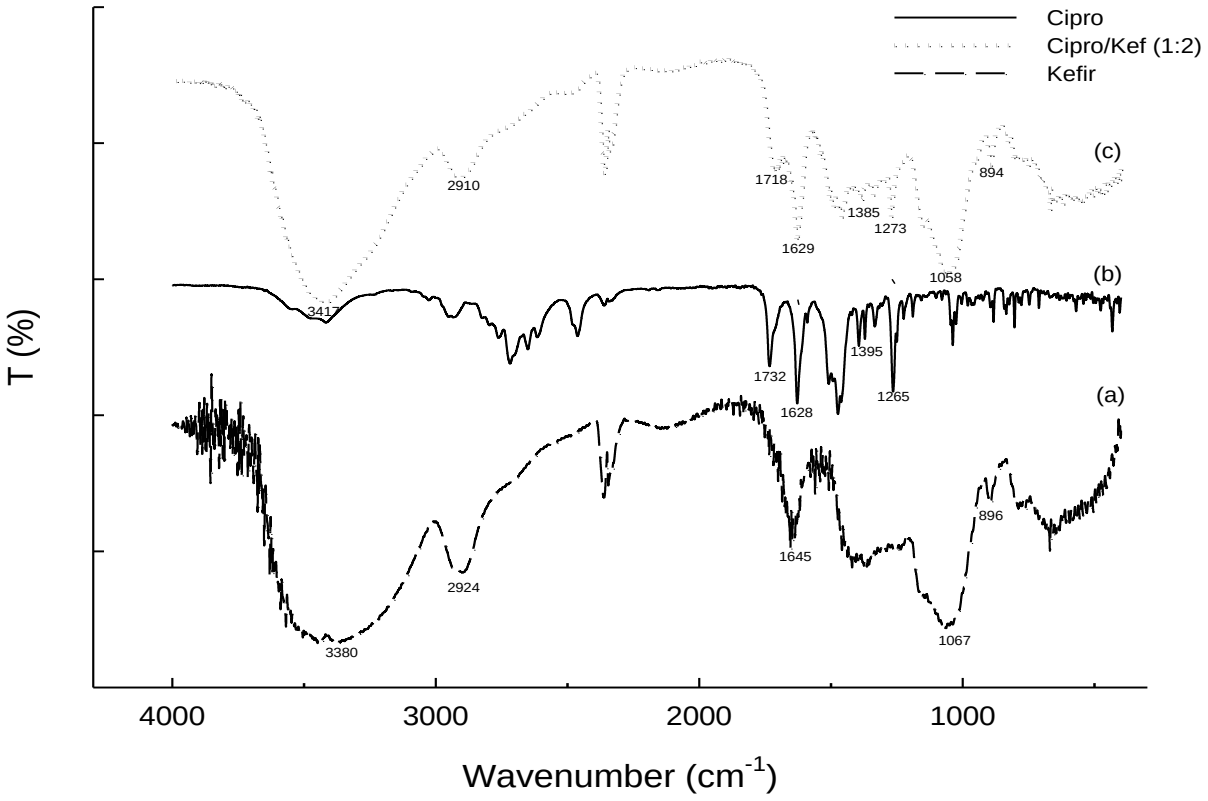

Figure 2. Thermogravimetric curves of Ciprofloxacin (·····), Kefiran (- - -) and Cip-Kef (——). TGA (A) and DTGA (B).

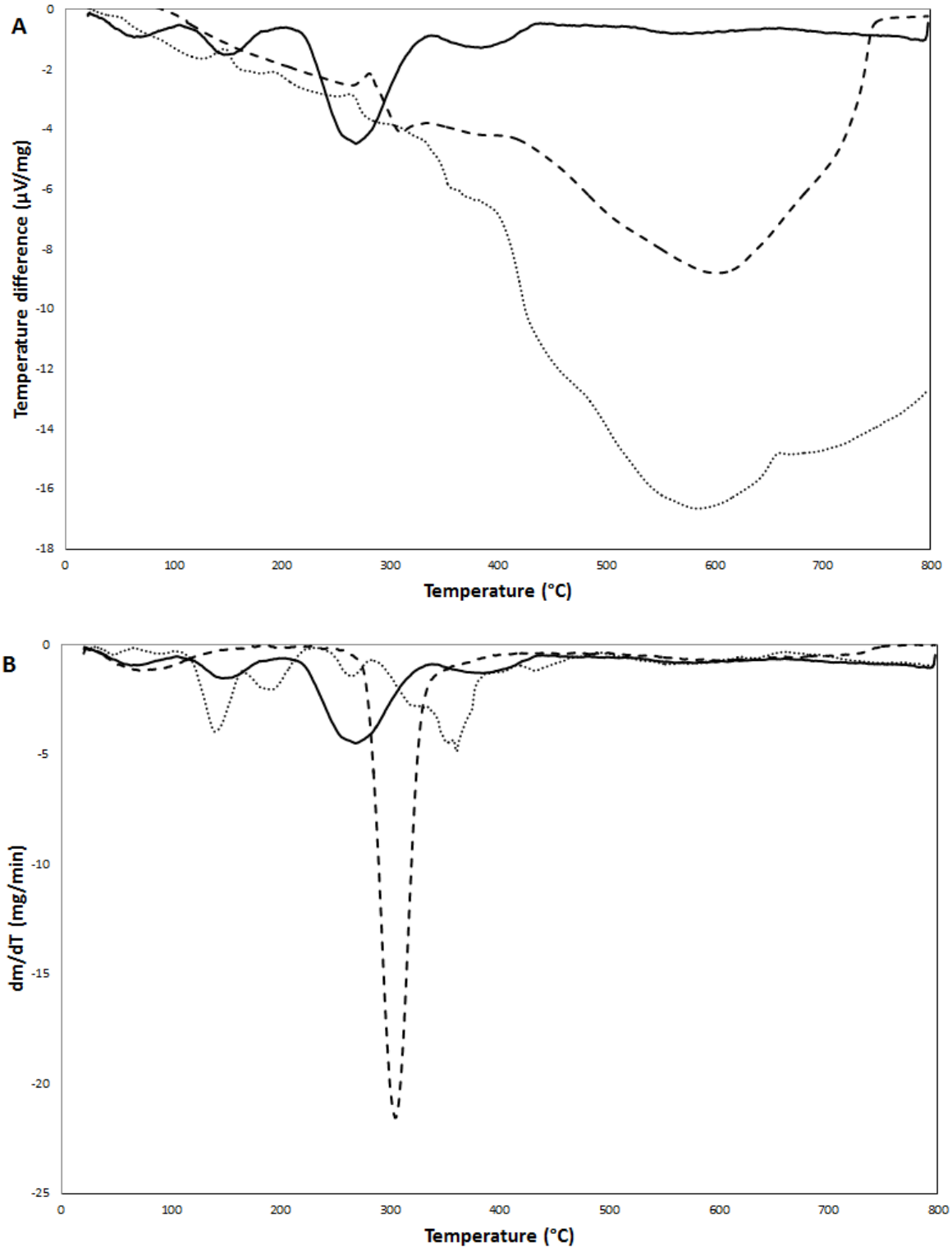

Figure 3. SEM images of microspheres composed of: 1.0% Alginate at 45x (a) and 350x (b); and 1.0% Alginate - 1.0% Kefiran at 40x (c) and 350x (d).

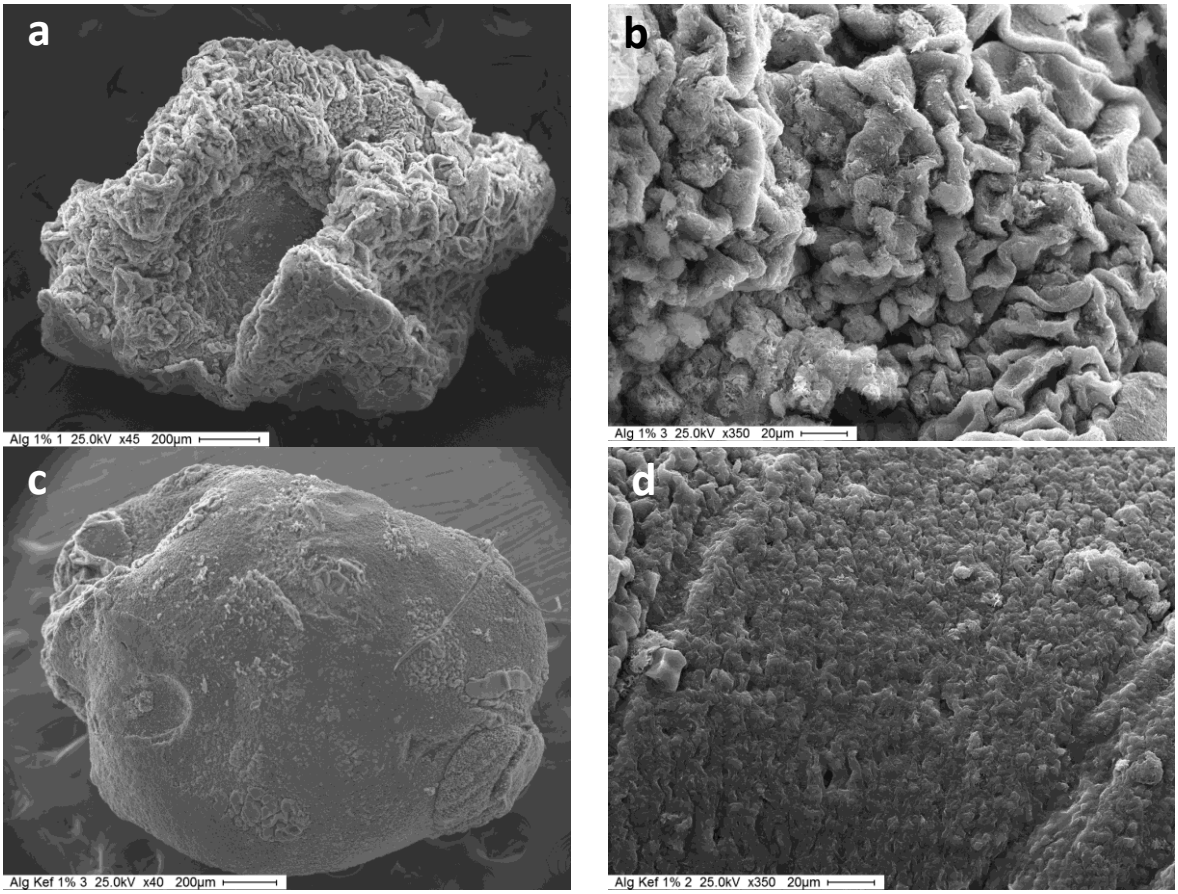

Figure 4. Roughness analysis of SEM images of microspheres composed of: 1.0% Alg at 45 (left) and 1.0% Alg – 1.0% Kef (right). At the top, the histograms of the beads surface are obtained and at the bottom the plot profile by ImageJ software.

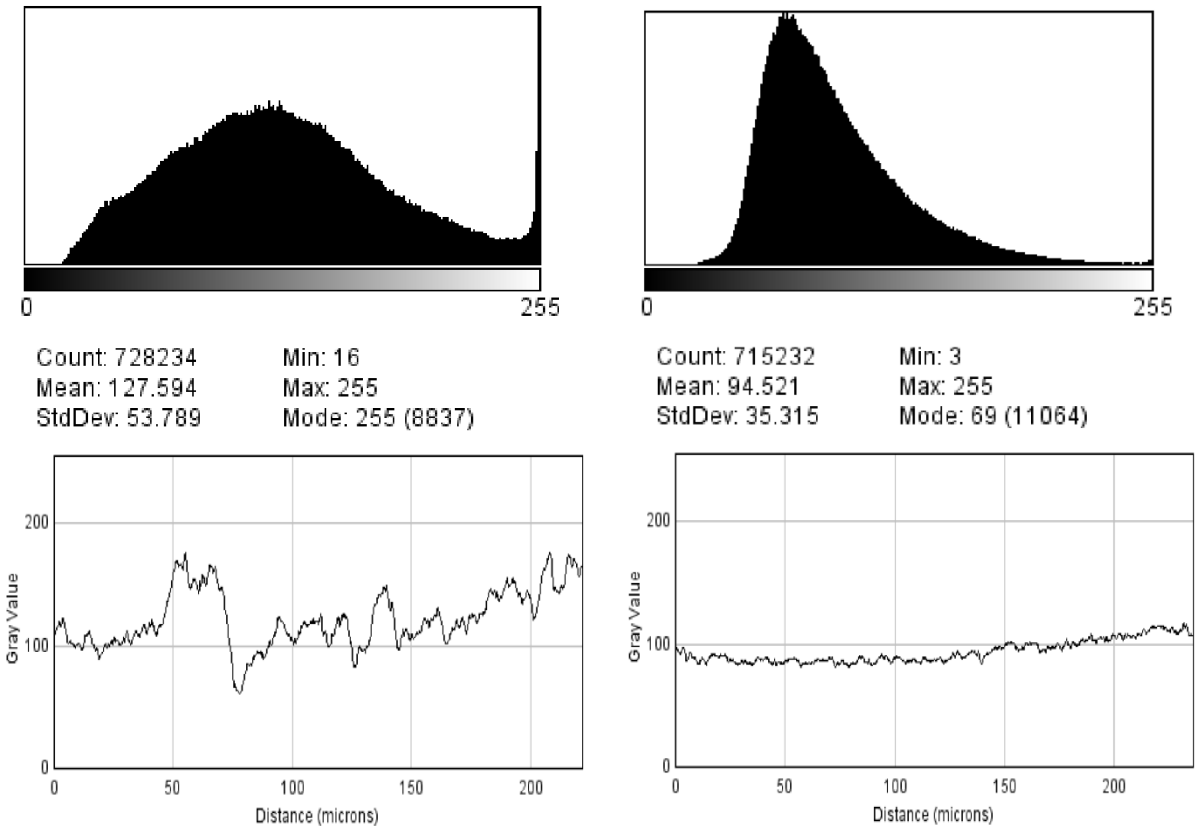

Figure 5. Kinetic of ciprofloxacin release from Alg and Alg-Kef gel microspheres at pH 1.2 (A), and pH 7.4 (B). (Errors: SD≤ 10%, n = 3)

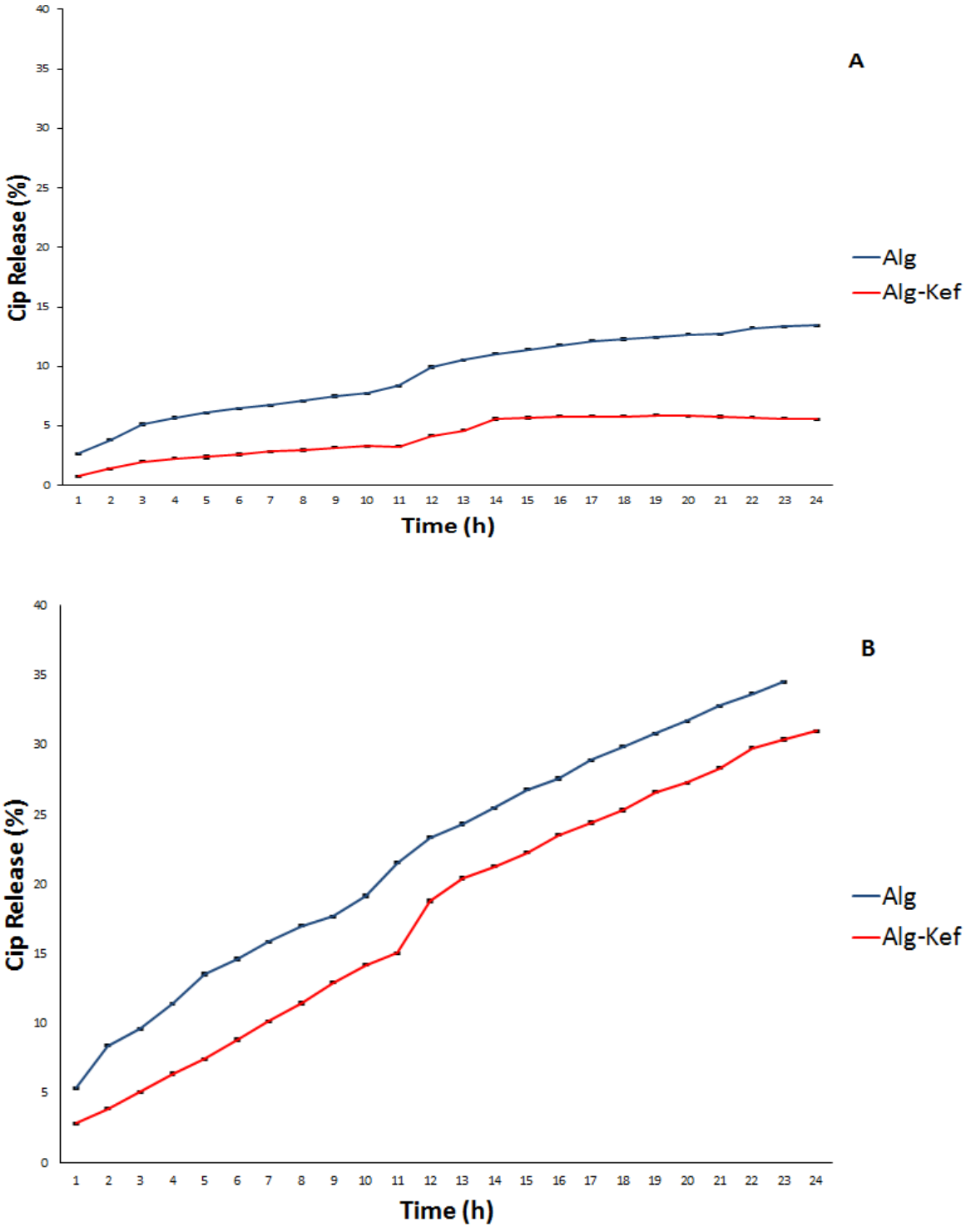

Figure 6. Antimicrobial assay of ciprofloxacin (Cip), kefiran (Kef), and the formulation of kefiran containing ciprofloxacin (Cip-Kef) against pathogenic bacteria: *E. coli* and *K. pneumonia*. (Errors: SD≤ 10%, n = 3).

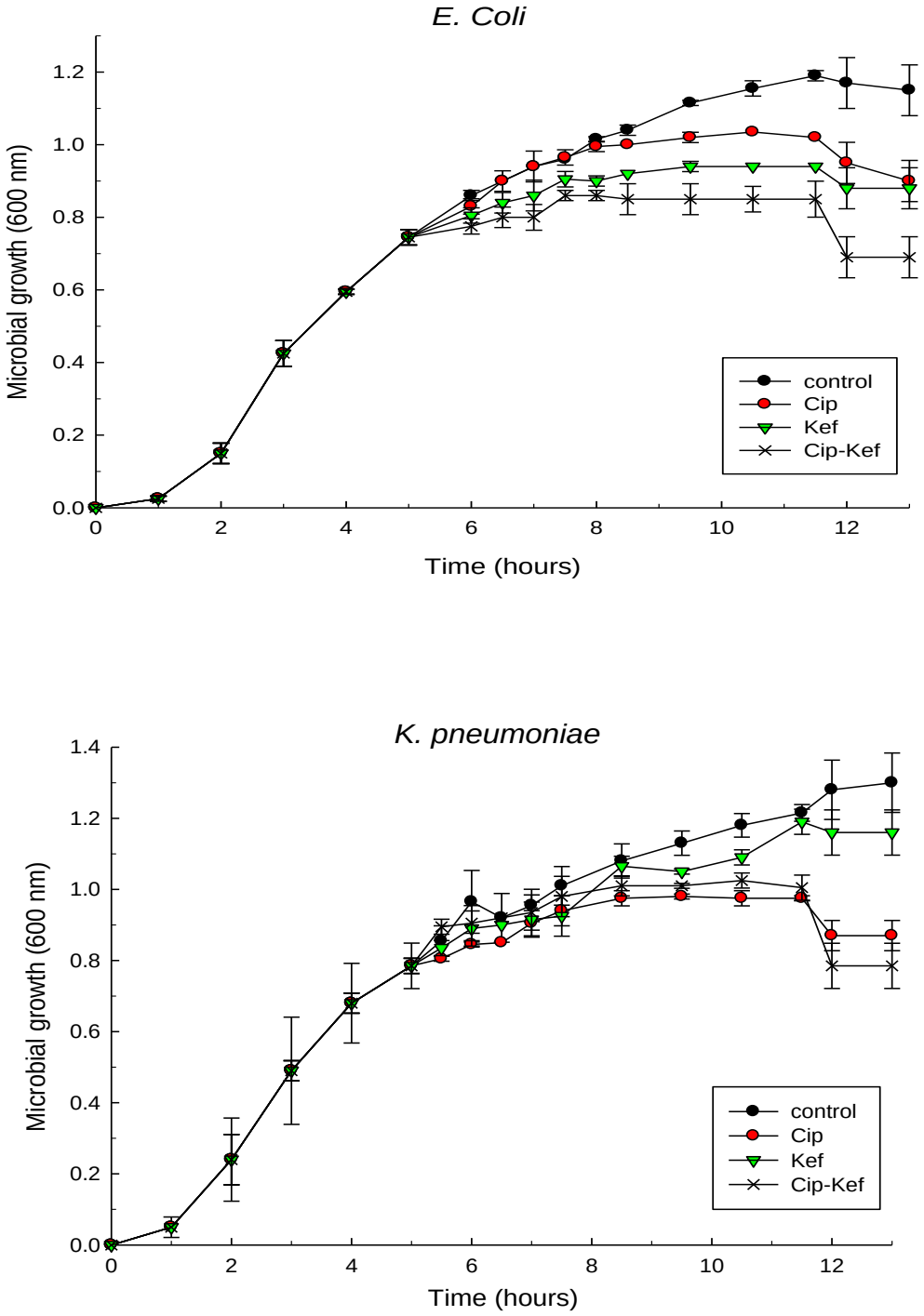

Figure 7. Antimicrobial assay of ciprofloxacin (Cip), kefiran (Kef), and the formulation of kefiran containing ciprofloxacin (Cip-Kef) against pathogenic bacteria: *P. aeruginosa* and *S. tiphymurium*. (Errors: SD≤ 10%, n = 3).

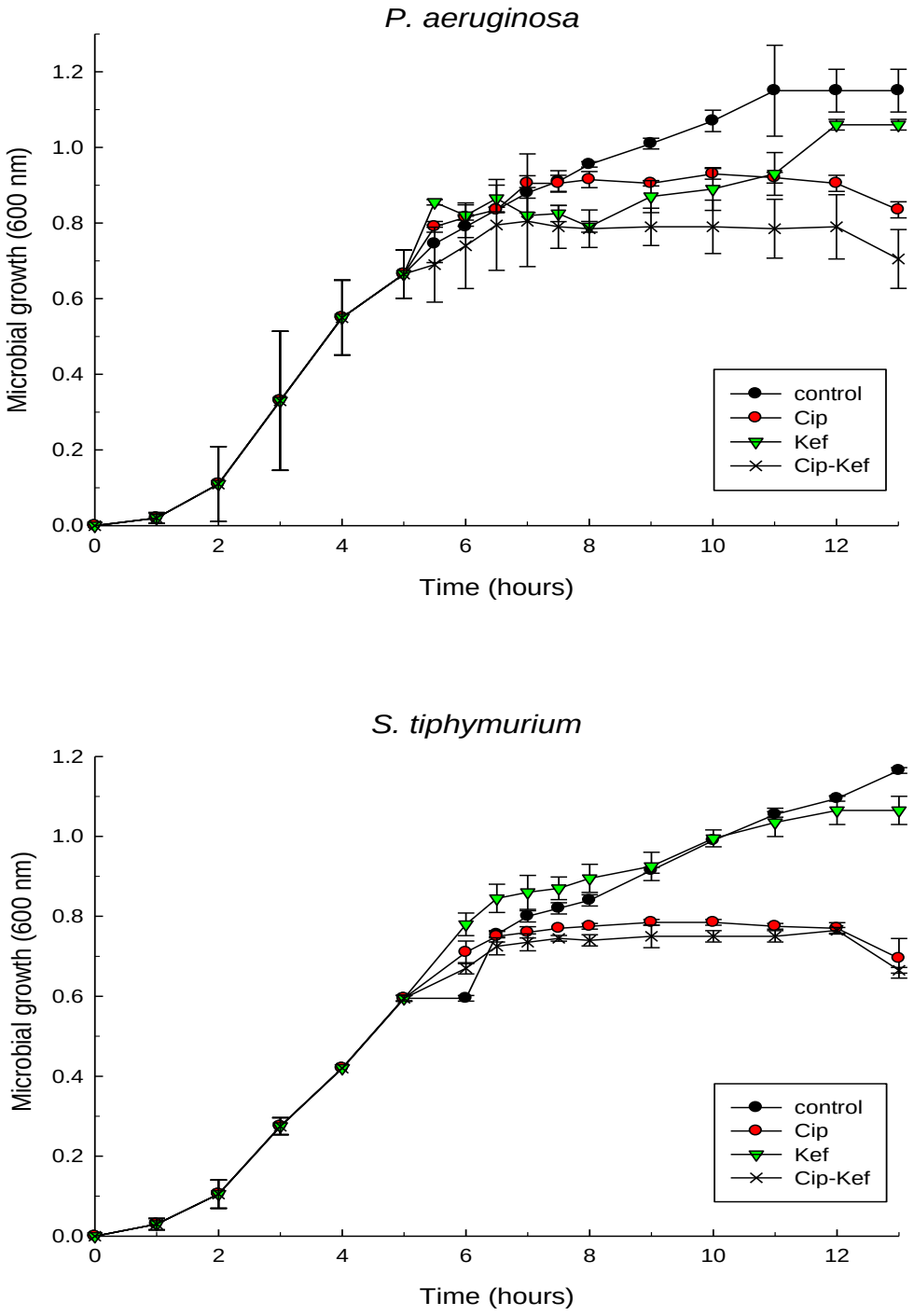

Figure 8. Antimicrobial assay of ciprofloxacin (Cip), kefiran (Kef), and the formulation of kefiran containing ciprofloxacin (Cip-Kef) against pathogenic bacteria: *S. aureus*. (Errors: SD≤ 10%, n = 3)