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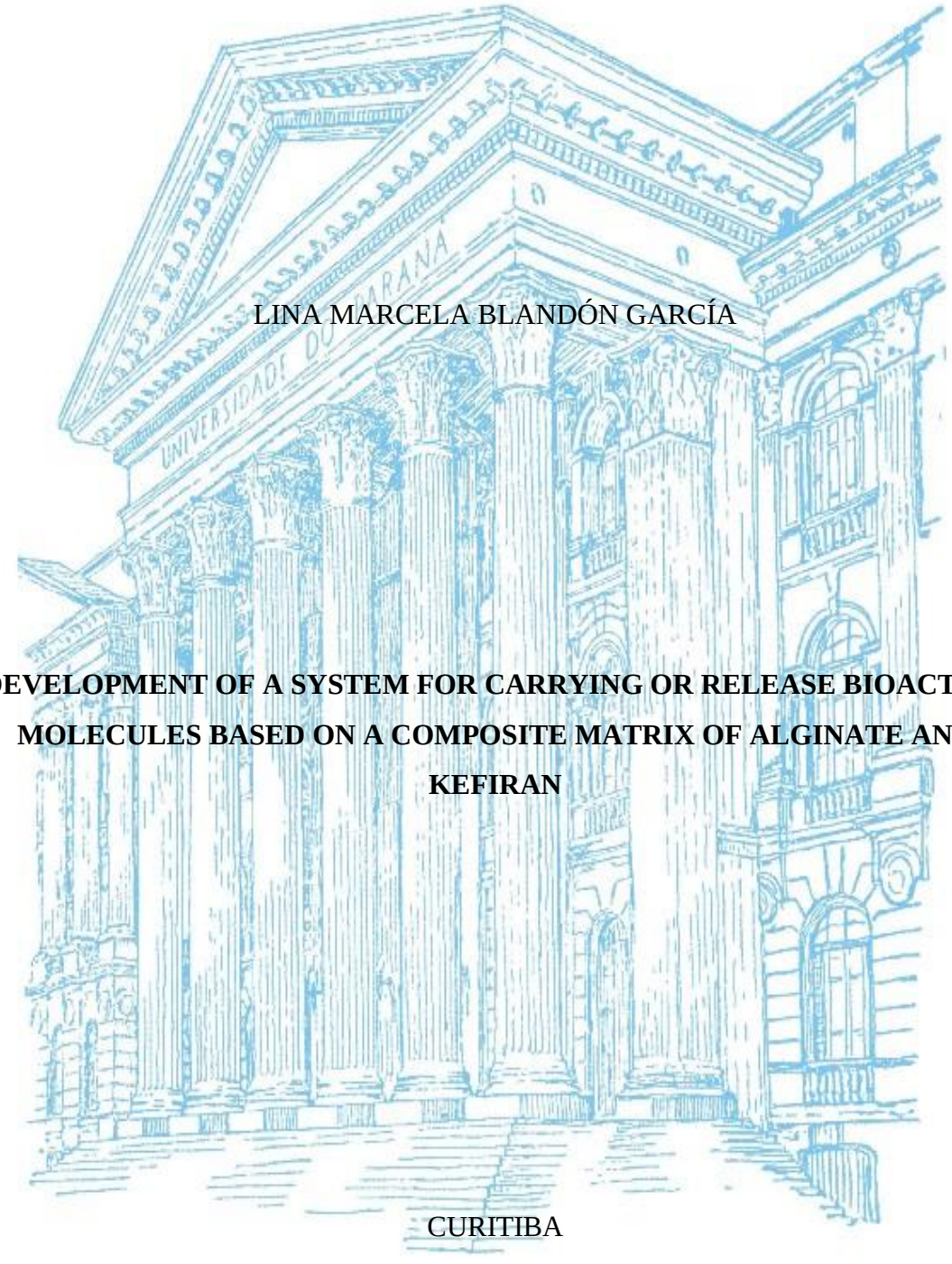

LINA MARCELA BLANDÓN GARCÍA

DEVELOPMENT OF A SYSTEM FOR CARRYING OR RELEASE BIOACTIVE MOLECULES BASED ON A COMPOSITE MATRIX OF ALGINATE AND KEFIRAN

CURITIBA

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DEVELOPMENT OF A SYSTEM FOR CARRYING OR RELEASE BIOACTIVE MOLECULES BASED ON A COMPOSITE MATRIX OF ALGINATE AND KEFIRAN

Thesis presented as partial requirement for the degree of Doctor in Bioprocess Engineering and Biotechnology, at the Bioprocess Engineering and Biotechnology course, Technology Sector, Universidade Federal do Paraná

Supervisor: Carlos Ricardo Soccol, PhD

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **LINA MARCELA BLANDON GARCIA** intitulada: **DEVELOPMENT OF A SYSTEM FOR CARRYING OR RELEASING BIOACTIVE MOLECULES BASED ON A COMPOSITE MATRIX OF ALGINATE AND KEFIRAN**, após terem inquirido a aluna e realizado e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO.

Curitiba, 29 de Dezembro de 2016.

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To accomplish great things, we must not only act, but also dream, not only plain, but also believe.

Anatole France

ABSTRACT

Biopolymers are polysaccharides found in nature with biological functions and promising useful applications in food and pharmaceutical industries. Two polysaccharides were used in this study alginate and kefiran. Alginate is a linear polysaccharide produced industrially by some brown algae (i.e., *Macrocystis pyrifera*, among others) and also found in some bacteria (i.e., *Pseudomonas aeruginosa*), is composed of β -D-mannuronic and α- L -guluronic acids and is commonly employed in gel synthesis by ionotropic gelation. Kefiran is a water-soluble glucogalactan produced by *Lactobacillus kefiranofaciens* present in kefir grains, is able to form gels with the advantage of displaying antimicrobial, antioxidant and others biological activities, but until now is not produced industrially. In that way, the physicochemical conditions for kefiran production were optimized, finding as the best fermentation medium whey supplemented with 15% (w/v) of glucose and the best physical conditions were 30°C, 10 h and without shaking. The resulting product was analyzed using High - Pressure Size Exclusion Chromatography (HPSEC), gas chromatography-mass spectrometry (GC-MS), nuclear magnetic resonance (NMR), Fourier - transform infrared (FTIR) and Raman spectroscopy, the polymer, was characterized as kefiran. Antimicrobial properties of kefiran against microbial pathogen (*Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus,* and four strains of *Salmonella typhimurium*) were tested; the results showed that the optimization process did not alter the biocidal properties of the polymer. On the other hand, a system for carrying or release bioactive molecules was developed based on a composite matrix of alginate and kefiran, in that way, 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. Fourier transform infrared spectroscopy (FTIR) of ciprofloxacin-kefiran showed the displacement of typical bands of ciprofloxacin and kefiran, suggesting a cooperative interaction by hydrogen bonds 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 *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhymurium*, and *Staphylococcus aureus* demonstrated the synergic effect between ciprofloxacin and kefiran against the tested microorganisms.

Key words: kefir, biological activity, polysaccharides, kefiran, alginate, hybrid gel microspheres, ciprofloxacin, controlled release, optimization, cheese whey, biocidal properties.

RESUMO

Os biopolímeros são polissacarídeos encontrados na natureza que apresentam funções biológicas e uteis aplicações na indústria farmacêutica e de alimentos. Neste trabalho fórum empregados dois biopolímeros o alginato e o kefiran. O alginato é um polissacarídeo lineal produzido industrialmente por algumas algas marrom (*Macrocystis pyrifera* a mais comum), além pode se encontrar em algumas bactérias (*Pseudomonas aeruginosa*), estruturalmente está composto pelo ácidos β -D-manurônico e α- L –gulurônico e é comumente utilizado na síntese de géis por gelação ionotrópica. Por outro lado, o Kefiran é um glucogalactano produzido pela bactéria *Lactobacillus kefiranofaciens* encontrada nos grãos de kefir, apresenta capacidade de formar géis e tem a vantagem de possuir atividades biológicas como antimicrobiana, antioxidante entre outras, más ainda não é produzido industrialmente, por conseguinte, fórum otimizadas as condições físico – químicas para a produção de kefiran, encontrando como as melhores, o uso de soro de leite suplementado com glicose ao 15% (p/v) como meio de fermentação, 30°C, 10 h e sem agitação; o produto resultante foi analisado com cromatografia de exclusão de tamanho de alta pressão (HPSEC), cromatografia gasosa - espectrometria de massa (GC – MS), ressonância magnética nuclear (RMN), espectroscopia de infravermelho com transformada de Fourier (FTIR) e espectroscopia RAMAN e foi confirmado que o polímero produzido é o kefiran. Do mesmo modo as propriedades antimicrobianas do kefiran fórum testadas contra microrganismos patógenos (*Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, e quatro cepas de *Salmonella typhimurium*), os resultados mostraram que o processo de otimização não altera as propriedades biocidas do mesmo. Aliás, um sistema para carregar ou liberar moléculas foi desenvolvido baseado numa matriz composta de alginato e kefiran, de tal forma que para o encapsulamento e liberação controlada do ciprofloxacino microesferas de alginato e kefiran fórum produzidas melhorando o efeito bactericida contra patógenos comuns. Contudo, a estrutura das microesferas foi analisada com microscopia eletrônica de varredura (SEM) encontrando uma forma esférica com uma superfície lisa em comparação com microesferas feitas só com alginato, logo a seguir a liberação do ciprofloxacino *in vitro* foi avaliada encontrando uma liberação do 3% e 5% num pH estomacal (pH 1.2) e 5% e 25%

num pH intestinal entre 3 e 21 horas respetivamente. O analise da espectroscopia infravermelha com transformada de Fourier para a matriz de alginato e kefiran sugere uma interação não covalente entre os polímeros formada por ligações de hidrogênio, consequentemente, o analise de degradação térmica mostrou um possível efeito protetor do kefiran contra a degradação do ciprofloxacino em altas temperaturas. Finalmente, fórum feitos testes antimicrobianos sob *Escherichia coli, Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhymurium*, e *Staphylococcus aureus*, demonstrando um possível efeito sinérgico entre o ciprofloxacino e o kefiran sob os microrganismos testados.

Palavras chave: Kefir, atividade biológica, polissacarídeos, kefiran, alginato, microesferas de gel hibridas, ciprofloxacino, liberação controlada, otimização, soro de leite, propriedades biocidas.

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LIST OF ACRONYMS

CFU	-	Colony-forming unit
EFSA	-	European Food Safety Authority
FDA	-	U S Food and Drug Administration
FTIR	-	Fourier transform infrared spectroscopy
GC-MS		Gas chromatography - mass spectrometry
GRAS	-	Generally recognized as safe
HPSEC	-	High Pressure Size Exclusion Chromatography
LAB	-	Lactic Acid Bacteria
NMR	-	Nuclear Magnetic Resonance
SEM	-	Scanning Electron Microscopy
TFA	-	Trifluoroacetic acid

LIST OF ABBREVIATIONS

ALG - Alginate

CIP - Ciprofloxacin

EPS - Exopolysaccharides

EX - Example

GLC - Glucose

GAL - Galactose

Gal*p* - Galactopiranose

Glc*p* - Glucopiranose

KEF - Kefiran

SUMMARY

1. THEORETICAL FRAMEWORK

1.1 BIOPOLYMERS

Biopolymers are polysaccharides found in nature with biological functions such as storage of energy (starch), cell wall architecture (cellulose) and cellular communication (glycosaminoglycans). Structurally polysaccharides can be homopolysaccharides (Composed by only one type of monomer) or heteropolysaccharides (Composed by two or more types of monomers) and its structure can be or not substituted by a non-sugar group (BADEL; BERNARDI; MICHAUD, 2011). Due to its chemical differences polysaccharides has differences in molecular properties (interactions, electrical charge, flexibility, structure, degree of branching and molecular weight), these molecular differences lead to differences in functional properties such as solubility, thickening, gelation, water holding capacity, digestibility, surface activity and emulsification (MATALANIS; JONES; MCCLEMENTS, 2011). Consequently, because of biopolymers properties they can be use in useful applications like food and pharmaceutical industries, especially as encapsulating agents, delivery systems, lightening agents and texture modifiers (JONES; MCCLEMENTS, 2011).

1.1.1 Biopolymers in pharmaceutical industry

Due to their biocompatibility, reasonable biological half-life and non-production of toxic metabolites biopolymers are a promising biomaterials for the use in drug formulations, drug delivery systems and encapsulating agents (JONES; MCCLEMENTS, 2011; SAFARI; ZARNEGAR, 2014). The used of polymers for conjugation of drugs initiated with Jatzkewitz in the early fifties who used a dipeptide (GL) spacer to attach a drug (mescaline) to polyviniylpyrrolidone. In the sixties and seventies Ushakov's group synthesized water – soluble polymer drug conjugates focusing on conjugates of antibiotics and polyvinylpyrrolione. After that, Mathé et al., began the conjugation of drugs to immunoglobulins then DeDuve discovered the lysosomotropism of macromolecules an important phenomena for the design of polymer drug conjugates and Ringsdorf presented a concept of the use of polymers

as drug carriers (KOPEČEK, 2013), finally, in the early 1990s the first polymer-protein conjugates (Zinostatin stimalmer) was market approval (DUNCAN, 2014).

Nowadays, pharmaceutical industry is continuously improving generating new technologies involving drug formulations, such as, liposomes, nanoparticles, biodegradable polymeric implants, immunoconjugates and others advanced drug delivery systems for oral delivery (DUNCAN; VICENT, 2013) an interesting examples of advances in this area are two polymer therapeutics Neulasta® and Copaxone® (DUNCAN, 2014). Neulasta (pegfilgrastim) is a PEGylated form of the recombinant human granulocyte colony – stimulating factor (GCSF), it helps to reduce the risk of infection and provides support through a chemotherapy cycle stimulating the level of white blood cells (https://www.neulasta.com/). Furthermore, Copaxone (glatiramer acetate) is a random polymer of four amino acids (glutamic acid, lysine, alanine and tyrosine) found in myelin basic protein and is an immunomodulator drug used to treat multiple sclerosis because it acts as a decoy for the immune system (https://www.copaxone.com/). Another interesting product is a mannonsylated dextran based sentinel lymph node imaging agent for melanoma and breast cancer patients called Lymphoseek, it was the first FDA approved receptor targeted lymphatic mapping agent (http://www.lymphoseek.com/).

There are two possible levels for a polymer – drug conjugates to be biorecognizable: at the plasma membrane, eliciting selective recognition and internalization by target cells and intracellularly, where lysosomal enzymes induce the release of drug from carrier, then the drug is transport across the lysosomal membrane into the cytoplasm and translocated into the adequate organelle for biological activity (KOPEČEK, 2013).

Controlled drug delivery systems (DDS) emerged in the early 1970s with the aim of transport proper amounts of drugs to specific sites (Tumors and diseased tissues). Besides minimize side effects of the drugs on healthy tissues reducing dosing frequency and toxic effects, increasing the residence time, improving patient compliance gaining efficacy with dosage requirements (SADAT HOSSEINI; HEMMATI; GHAEMY, 2016). In addition to, oral delivery is the most desirable way

for drug administration because the drug is absorbed in the gastrointestinal tract without the help of trained personnel (BOSIO et al., 2015).

Encapsulation is the process to entrap a substance (active agent, the core, fill, active, internal or payload phase) within another substance (wall material, the coating, membrane, shell, capsule, carrier material, matrix or external phase) (NEDOVIC et al., 2011). Drug encapsulation offers many advantages, such as the protection of the drug against *in vivo* degradation, the reduction of potential side effects associated with the drug doses, increase in patient comfort, more favorable pharmacokinetics and optional design of drug sustainable release (BOSIO et al., 2012). Specifically, as encapsulating agents, once a functional component had been encapsulated it is possible to design the particle properties to deliver it into a particular site of action such as colon, mouth, stomach, small intestine. Also, it is possible to use different kinds of proteins or polysaccharides to make the particles (JONES; MCCLEMENTS, 2011)

1.1.2 Biopolymers in food industry

One of the most common material employed in food applications are polysaccharides, such as syrups, cellulose, polydextrose, dextrins, maltodextrins, amylose, amylopectin gum arabic, gum tragacanth, gum karaya, mesquite gum galactomannans pectins, soluble soybean polysaccharides, carrageenans, alginate, dextran, quitosan, xanthan, and gellan (NEDOVIC et al., 2011). In **table 1.1**, a summary of the most common food – grade polysaccharide use for assembling biopolymer particles is show, the table characteristics are according to the industrial use.

Encapsulation is one of the principal applications of biopolymers in food industry. Functional compounds such as bioactive molecules (e.g. phytosterols, vitamins, minerals, antioxidants) and living cells (e.g. probiotics) are important components, which cannot be added in a free way to a food product. Because they are susceptible to environmental damage, processing and/or gastrointestinal conditions, in that way, encapsulation provides an effective protection of this components because they are completely enveloped and protected by a physical

barrier avoiding its protrusion and preventing undesirable interactions with food matrix. In addition to, encapsulation can modify physical characteristics of the original material to help to separate the components of a mixture, to allow easier handling and to provide an adequate concentration of an active agent (NEDOVIC et al., 2011).

TABLE 1.1 - . SUMMARY OF IMPORTANT MOLECULAR CHARACTERISTICS AMONG COMMON FOOD – GRADE POLYSACCHARIDES FOR ASSEMBLING BIOPOLYMER PARTICLES.

Name	Source	Main structure type	Major monomer	Gelation mechanism
Alginate	Algal	Linear	β – D Mannuronic Acid	Calcium cross – linking
Beet pectin	Sugar beet pulp	Branched coil with protein	Glucuronate (backbone)	Sugar/heat (HM); calcium (LM)
Carrageenan	Algal	Linear/helical	Sulfate galactane	Cooled set
Chitosan	Crustaceans, invertebrates	Linear	2 – amino – 2-deoxy – β – D glucose	No common application
Gum Arabic	Acacia sap	Branched coil domains on protein scaffold	Galactose	Concentration dependent
Inulin	Plants or bacteria	Linear with occasional branches	β – D Fructose	Concentration dependent
Methyl cellulose	Wood pulp	Linear	Methylated glucose	Heat – set (rev)
Pectin	Plant cell walls	Highly branched coil	Glucuronate (Backbone)	Sugar/heat (HM); calcium (LM)
Xanthan gum	*Xanthomonas campestris* exudate	Linear/helical (high MW)	β – D – glucose (backbone)	None; thickens with concentration

FONT: Adapted from MATALANIS; JONES; MCCLEMENTS. (2011)

1.2 BACTERIAL EXOPOLYSACCHARIDES

According to its cellular localization polysaccharides produced by bacteria are classified in three groups: first, cytosolic polysaccharides which are a carbon and energy source; second, polysaccharides that are part of the cell wall and third, polysaccharides that are excreted to the extracellular media known as exopolysaccharides (EPS) (DONOT et al., 2012).

Important advantages are related to EPS: It requires little time to be produced, industrial wasted can be used for their production, a high level of purity can be obtained and its used can be considered safe because they are produced *in situ* by GRAS bacteria (Generally recognized as safe) (BADEL; BERNARDI; MICHAUD, 2011; DONOT et al., 2012). **Table 1.2** shows important EPS, its most relevant physicochemical and functional properties, main areas of application and market assessment.

EPS biosynthesis can be done in three steps: i) Carbon source assimilation, ii) intracellular synthesis of the polysaccharides, and iii) the polysaccharides is expulsed outside of the cell (F**igure 1.1**). Initially, the substrate enters to the cell through specific mechanisms of transport such as facilitated diffusion, proton motive force, concentration gradient. Once inside the cell, substrate is catabolized through glycolysis until piruvate which under aerobic conditions is converted to acetil – coA entering to the krebs cycle. The primary metabolites are used as precursors for the biosynthesis of little biomolecules such as aminoacids, ribonucleotides, and hexoamines. On the other hand, phosphorylated sugars are converted in a monosaccharides font. Consequently, polysaccharides synthesis and polymerization occurs through two mechanisms: i) Wzx-Wzy dependent system. the repeat unit is synthesized for the sequential transfer of monosaccharides from nucleoside diphosphate sugars (sugars – NDP) toward the lipid charger polyprenylphosphate. Then, the mature repeating units are transport through the interior membrane by a flippase (Wzx) through the periplasmic face where the polymerization occurs by a polymerase action (Wzy). After that, the translocation route from the cell envelope is formed by a polysaccharide copolymerase (PCP) which determines the length of the polymeric chains and a polysaccharide export protein (OPX) that export the polysaccharides toward the extern membrane forming a channel. ii) In the ABC-transporter-dependent system, the polysaccharide is polymerized toward the citoplasmic face of the inner membrane through the sequential addition of residual sugars to the nonreducing end of the polymeric chain. It cross the inner membrane with the help of the ABC transporter, following by its traslocation through the

periplasm and the outer membrane with the help of the PCP and OPX proteins (DONOT et al., 2012).

1.3 KEFIR, KEFIR GRAINS AND KEFIRAN

Kefir is a naturally carbonated fermented milk beverage with a slightly acidic taste, yeasty flavor and creamy consistency originally made in Balkans, Eastern Europe and the Caucasus (ISMAIEL; GHALY; EL-NAGGAR, 2011). The beverage is obtained by an acidic - alcoholic fermentation of the fresh milk by the addition of kefir grains. Kefir grains are a kind of yogurt starter which are white to yellow – white, gelatinous and variable in size (Varying from 0.3 – 3.5 cm in diameter). Kefir is a complex microbial symbiotic mixture of lactic acid bacteria (108 CFU/g), yeast (106-107 CFU/g) and acetic acid bacteria (105 CFU/g) which are stick to a polysaccharide matrix principally composed by glucose and galactose called Kefiran (CHEN et al., 2015; GARROTE; ABRAHAM; DE ANTONI, 2010). Nevertheless the number of microorganisms found in kefir grains is variable depending on the origin of the grains, the culturing method and substrates used (HAMET et al., 2013). After successive fermentations, kefir grains can break up to new generation grains which have the same characteristics as old ones (GAO et al., 2012a).

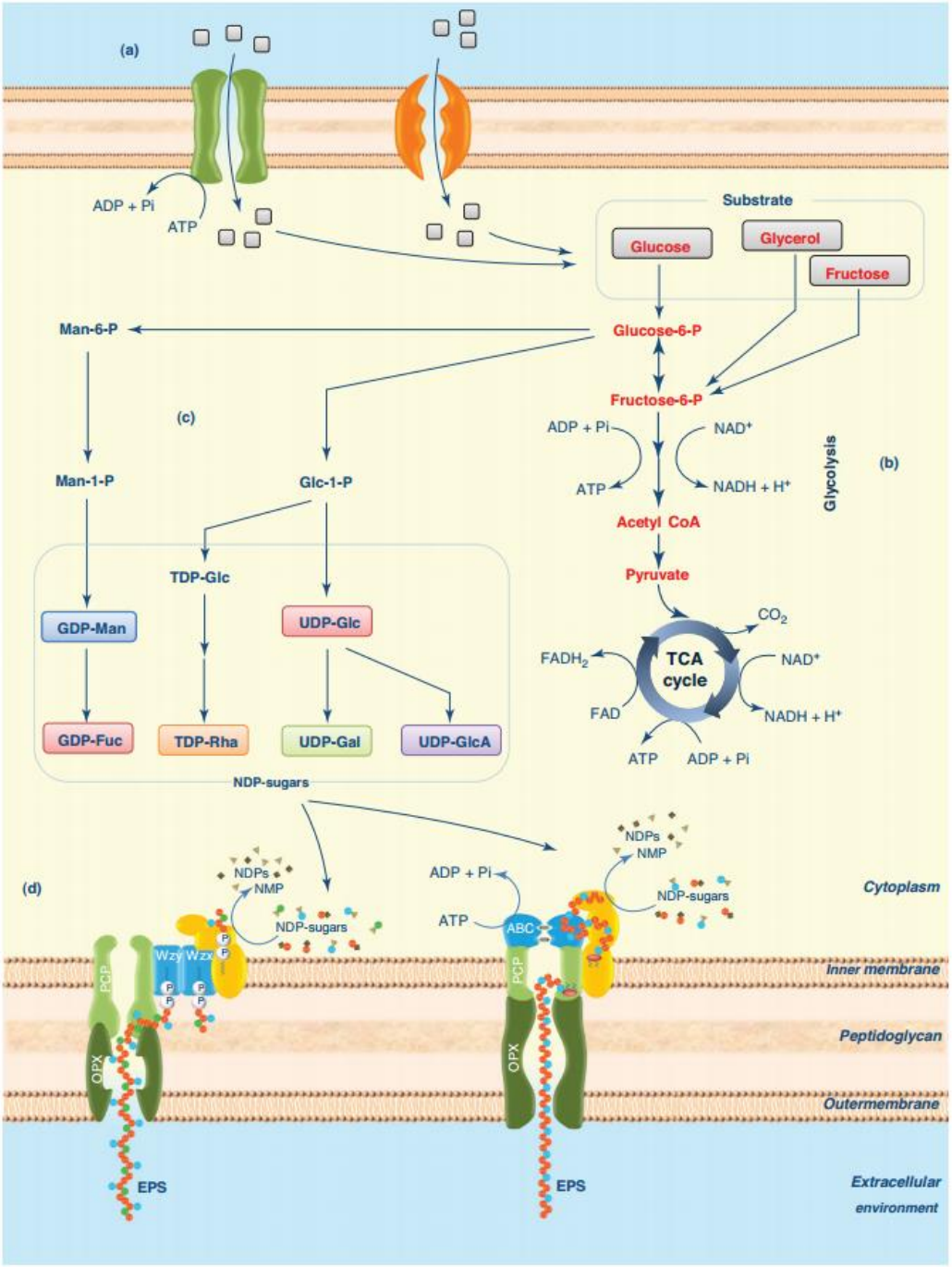
TABLE 1.2 - IMPORTANT BACTERIAL EPS, ITS MOST RELEVANT PHYSICOCHEMICAL AND FUNCTIONAL PROPERTIES, MAIN AREAS OF APPLICATION AND MARKET ASESSMENT.

EPS	Components	Main Properties	Main Applications	Market (metric tons)	Market value (US$)	Price (US$)/Kg
Xanthan	Glucose Mannose Glucuronic acid Acetate Pyruvate	- Hydrocolloid	- Foods - Petroleum industry - Pharmaceuticals - Cosmetics and personal care products - Agriculture	96000	235 millions	3 – 5
Gellan	Glucose Rhamnose Glucuronic acid Acetate Glycerate	- Hydrocolloid - Gelling capacity - Thermoreversible gels	- Foods - Pet food - Pharmaceuticals - Research	N.A	15 millions	55 - 66
Alginate	Guluronic acid Mannuronic acid Acetate	- Hydrocolloid - Gelling capacity - Film-forming	- Food hydrocolloid - Medicine	30000	N.A	5-20
Cellulose	Glucose	- High crystallinity - Insolubility in most solvents - High tensile strength - Moldability	- Foods - Biomedical	N.A	N.A	5,8-12
Dextran	Glucose	- Non-ionic - Good stability - Newtonian fluid behavior	- Foods - Pharmaceutical industry - Chromatographic media	2000	N.A	N.A
Curdlan	Glucose	- Gel – forming ability - Water insolubility - Edible and non-toxic - Biological activity	- Foods - Pharmaceutical industry - Heavy metal removal - Concrete additive	N.A	N.A	55

EPS	Components	Main Properties	Main Applications	Market (metric tons)	Market value (US$)	Price (US$)/Kg
Hyaluronan	Glucuronic acid Acetylglucosamine	- Biological activity - Highly hydrophilic - Biocompatible	- Medicine - Solid culture media	N.A	1 billion	100000
Succinoglycan	Glucose Galactose Acetate Pyruvate Succinate 3-hydroxybutyrate	- Viscous shear thinning aqueous solutions - Acid stability	- Food - Oil recovery	N.A	N.A	N.A
Levan	Fructose	- Low viscosity - High water solubility - Biological activity - Adhesive strength - Film-forming capacity	- Food (prebiotic) - Feed - Medicines - Cosmetics - Industry	N.A	N.A	N.A
GalactoPol	Galactose Mannose Glucose Rhamnose Acetate Succinate Pyruvate	- Viscous shear thinning solutions in aqueous media. - Film-forming. - Emulsifying capacity - Flocculating capacity	- Hydrocolloid - Coatings - Packages	-	-	-
FucoPol	Fucose Galactose Glucose Acetate Succinate Pyruvate	- Viscous shear thinning solutions in aqueous media. - Film forming. - Emulsifying capacity. - Flocculating capacity. - Biological activity due to fucose content	- Hydrocolloid. - Source of fucose and fuco-oligosacharides	-	-	-

FONT: Adapted from FREITAS; ALVES; REIS. (2011)

FIGURE 1.1 - SCHEMATIC DIAGRAM SUMMARIZING THE BIOSYNTHETIC PATHWAYS INVOLVED IN BACTERIAL EPS SYNTHESIS BY GRAM – NEGATIVE BACTERIA.



Font: Adapted from FREITAS; ALVES; REIS. (2011)

1.3.1 Microbial Composition and Grain Formation

The microbial composition in kefir grains may vary according to kefir origin, the substrate used in the fermentation process and the culture maintenance methods (PRADO et al., 2015). In that way, many microbiological studies and molecular approaches studies are used to identify the microbial composition of kefir grains from different sources, for example, MIGUEL et al.(2010) analyzed bacteria associated with milk kefir grains from Brazilian states, Canada and the United states of America finding variations in the microbial species composition. On the other hand, DOBSON et al.(2011) analyzed the bacterial diversity of the Irish Kefir an interest font of the lacticin 3147 a two peptide broad spectrum lantibiotic produced by *Lactococcus lactis* DPC3147 finding differences between the microbiota of the beverage and the starter grain. Nonetheless, it was not found differences on the chemical of the fermented milk made from different type of milk ZÜBEYDE ÖNER, (2015) but the quality of the beverage depends of the microbial quality of kefir grains (ALTAY et al., 2013).

As it was mentioned, Kefir grains are composed principally by bacteria and yeast, the majority of bacteria as much as 80%, belong to the genus *Lactobacillus* (*lactobacilli, lactococci, leuconostocs* and *acetobacteria*) (GONZÁLEZ-SÁNCHEZ et al., 2010) filamentous fungi have been described, but are only present in low number (POWELL et al., 2007). **Table 1.3** summarized some of the microorganisms found and identified in kefir and kefir grains from different origins.

Kefir grains microorganisms are classified into four groups, namely homofermentative lactic acid bacteria, heterofermentative lactic acid bacteria, lactose assimilating yeast and non-lactose assimilating yeasts. The nonlactose assimilating yeasts was assumed to survive by consuming galactose and lactic acid as a carbon source and energy source (CHEIRSILP; RADCHABUT, 2011). Some authors reported that interrelationships of bacteria and yeasts inside kefir grains may have significant influence on the activities of each strain and some authors also describe the association inside kefir grains as a symbiosis, in that way bacterial end – products could be used by yeasts as an energy source (CHEIRSILP; SHIMIZU; SHIOYA, 2007).

TABLE 1.3 MICROBIAL COMPOSITIONS FOUND IN KEFIR AND KEFIR GRAINS

Microorganism	**Source - Country**	**Author**
Lactobacillus kefir, Lactobacillus kefiranofaciens, Lactobacillus paracasei, Lactobacillus plantarum, Lactococcus lactis subsp. *lactis, Kluyveromyces marxianus, Lactobacillus parakefir, Saccharomyces cerevisiae, Saccharomyces unisporus, Leuconostoc mesenteroides, Acetobacter* sp., *Saccharomyces* sp., *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, *Lactococcus lactis, Lactobacillus kefiri, Lactobacillus parakefiri*	Kefir grains and beverage – Argentina	DIOSMA et al. (2014); HAMET et al. (2013); LONDERO et al. (2012); GARROTE et al. (2001)
Lactobacillus kefiri, Lactobacillus kefiranofaciens, Leuconostoc mesenteroides, Lactococcus lactis, Lactococcus lactis ssp. *cremoris, Gluconobacter frateurii, Acetobacter orientalis, Acetobacter lovaniensis, Kluyveromyces marxianus, Naumovozyma* spp., *Kazachastania khefir*	Kefir grains and beverage - Belgium	KORSAK et al.(2015)
Lactobacillus kefiri, Lactobacillus kefiranofaciens, Leuconostoc mesenteroides, Lactococcus lactis, Lactobacillus paracasei, Lactobacillus helveticus, Gluconobacter japonicus, Lactobacillus uvarum, Acetobacter syzygii, Lactobacillus satsumensis, Saccharomyces cerevisiae., Leuconostoc sp., *Streptococcus* sp., *Acetobacter* sp., *Bifidobacterium* spp., *Halococcus* spp., *Lactobacillus amylovorus; Lactobacillus buchneri; Lactobacillus crispatus; Lactobacillus kefiranofaciens* subsp *kefiranofaciens; Lactobacillus kefiranofaciens* subsp *kefirgranum, Lactobacillus parakefiri*	Kefir grains - Brazil	MIGUEL et al. (2010); LEITE et al. (2012); ZANIRATI et al. (2015)
Lactobacillus brevis, Lactobacillus delbrueckii subsp. *bulgaricus, Lactobacillus helveticus, Streptococcus thermophilus, Lactobacillus casei* subsp. *pseudoplantarum, Kluyveromyces marxianus* var. *lactis, Saccharomyces cerevisiae, Candida inconspicua, Candida maris, Lactobacillus lactis* subsp. *Lactis*	Kefir grains and beverage - Bulgaria	SIMOVA et al. (2002)

Microorganism	Source - Country	Author
Lactobacillus paracasei, Lactobacillus parabuchneri, Lactobacillus casei, Lactobacillus kefiri, Lactococcus lactis, Acetobacter lovaniensis, Kluyveromyces lactis, Kazachstania aerobia, Saccharomyces cerevisiae, Lachancea meyersii	Brazil - Kefir beverage	(MAGALHÃES et al. (2011)
Lactobacillus kefiranofaciens, Leuconostoc mesenteroides, Lactococcus lactis, Lactobacillus helveticus, Kluyveromyces marxianus, Saccharomyces cerevisiae, Pseudomonas sp., *Kazachstania unispora, Kazachstania exigua ,Lactobacillus kefiri, Lactobacillus casei, Bacillus subtilis, Pichia kudriavzevii, Leuconostoc lactis, Lactobacillus plantarum, Acetobacter fabarum, Pichia guilliermondii, Lactococcus* sp., *Lactobacillus* sp., *Acetobacter* sp., *Shewanella* sp., *Leuconostoc* sp., *Streptococcus* sp, *Acinetobacter* sp., *Pelomonas* sp., *Dysgonomonas* sp., *Weissella* sp., *Shewanella* sp.	Kefir grains (Tibet)- China	GAO et al. (2012b, 2013a); ZHOU et al. (2009)
Acetobacter acetic, Enterococcus faecalis, Enterococcus durans, Lactococcus lactis subsp. *cremoris, Leuconostoc pseudomesenteroides, Leuconostoc paramesenteroides, Lactobacillus brevis, Lactobacillus acidophilus, Saccharomyces* sp., *Brettanomyces* sp., *Candida* sp., *Saccharomycodes* sp., *Acetobacter rancens*	Kefir beverage - China	YANG et al. (2007)
Lactobacillaceae and *Streptococcaceae*	Kefir grains and beverage - Ireland	DOBSON et al. (2011)
Lactobacillus kefiranofaciens, Dekkera anomala, Streptococcus thermophilus, Lactococcus lactis, Acetobacter sp., *Lactobacillus. lactis, Enterococcus* sp., *Bacillus* sp., *Acetobacter fabarum, Acetobacter lovaniensis, Acetobacter orientalis*	Kefir grains - Italy	GAROFALO et al. (2015)
Leuconostoc sp. , *Lactococcus* sp., *Lactobacillus* sp., *Lactobacillus plantarum, Zygosaccharomyces* sp., *Candida* sp., *Candida lambica, Candida krusei, Saccharomyces* sp., *Cryptococcus* sp.	Kefir grains and beverage – South Africa	WITTHUHN; SCHOEMAN; BRITZ. (2005)

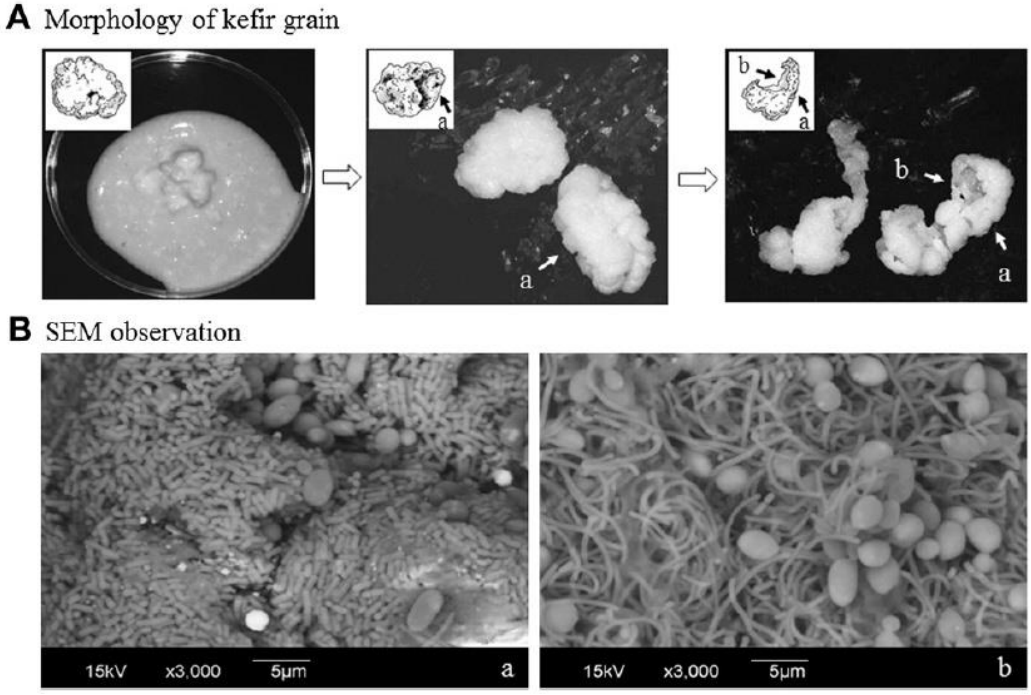
Microorganism	Source - Country	Author
Lactobacillus sp., *Leuconostoc* sp., *Lactococcus* sp., *Zygosaccharomyces* sp., *Candida* sp., *Saccharomyces* sp.	Kefir grains – South Africa	WITTHUHN; SCHOEMAN; BRITZ. (2004)
Lactobacillus kefiri, Lactobacillus kefiranofaciens, Leuconostoc mesenteroides, Lactococcus lactis, Escherichia coli, Pseudomonas spp., *Saccharomyces turicensis,*	Kefir grains - Taiwan	WYDER; MEILE; TEUBER, (1999); CHEN; WANG; CHEN .(2008); WANG et al. (2012);
Lactobacillus kefiri, Leuconostoc mesenteroides, Lactococcus lactis, Streptococcus thermophilus, Lactobacillus kefiranofaciens,, Lactobacillus acidophilus	Kefir grains and beverage - Turkey	GUZEL-SEYDIM et al.(2005); KESMEN; KACMAZ. (2011)
Lactobacillus helveticus, Lactobacillus buchneri, Lactobacillus kefiranofaciens, Lactobacillus acidophilus, Lactobacillus helveticus, Streptococcus thermophilus, Bifidobacterium bifidum, Kluyveromyces marxianus	Kefir grains - Turkey	TAŞ; EKINCI; GUZEL-SEYDIM. (2012); NALBANTOGLU et al.(2014;)
Lactococcus cremoris, Lactococcus lactis, Streptococcus thermophilus, Streptococcus durans	Kefir beverage - Turkey	YÜKSEKDAĞ; BEYATLI; ASLIM. (2004)

Font: The author

WANG et al. (2012) examined a section of a whole kefir grain by microscopy and photographed **(Figure 1.2**). They found that the outer layer of the grain contain *lactobacilli, lactococci* and yeasts, the inner layer of the grain was similar except that the *lactobacilli* were much longer and more yeasts cells were found in the inner layer of grain. The kefir grains can only grow from pre-existing grains and very little is known about the mechanism of grain formation. The same authors, had been investigated the relationship between different cell surface properties and interaction among kefir Lactic acid bacteria and kefir yeasts, they observed a great variability in the surface properties of kefir microorganisms finding that hydrophobicity and charge are the only nonspecific interactions between cell and surface. The same authors proposed a hypothesis to explain kefir grain formation (**Figure 1.3**). "Initially, *Lactobacillus kefiranofaciens* and *Sacharomyces turicensis* start to auto – aggregate and coagregated to small granules. The aggregation is enhanced at the pH drops. The

biofilm producers, *Lactobacillus kefiri, Klu. Marxianus* HY1 and *Pichia fermentants* HY3 then adhere to the surface of these small granules due to their cell surface properties and their strong aggregation ability, which gives rise to thin biofilms. After biofilm formation the kefir yeasts and lactic acid bacteria continue to co-aggregated with the granule strains and associate with the granule biofilm to become a three dimensional microcolony. As the cell density due to the growth of kefir yeasts and lactic acid bacteria increases, cells and milk components present in the liquid phase accumulate on the granule surface and the kefir grains are formed".

FIGURE 1.2 KEFIR GRAINS MORPHOLOGY (A) AND MICROSTRUCTURE (B) OF KEFIR GRAINS. THE OUTHER LAYER OF THE GRAIN (a), THE INNER LAYER OF THE GRAIN (b).

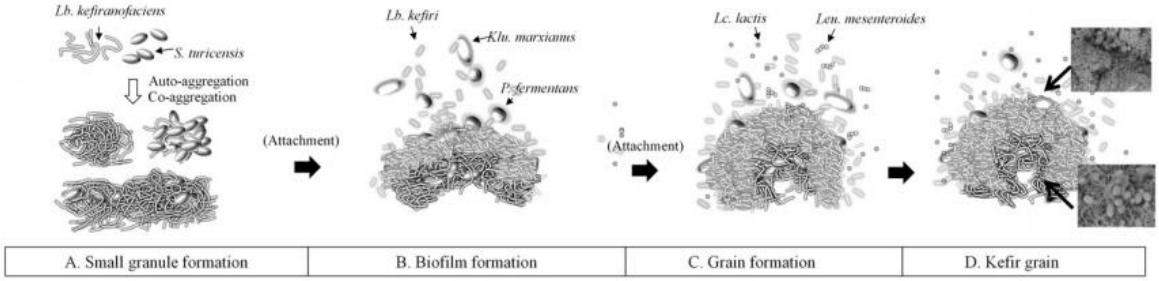


Font: Adapted from WANG et al. (2012)

1.3.2 Biological activity of kefir

Due to its composition, kefir is mainly considered a probiotic resource (SOCCOL et al., 2014). “Probiotics are microbial cells preparations or components of microbial cells that have a beneficial effect on the health of the host” (CARASI et al., 2014). Statistical data suggest that probiotic bacteria in the gut of kefir consumers are abundant and diverse, and microbial communities in the gut are closely correlated with health (ZHENG et al., 2013).

FIGURE 1.3 THE SCHEMATIC MODEL OF KEFIR GRAIN FORMATION



Font: Adapted from WANG et al. (2012)

The microorganisms in the kefir grains produce lactic acid, antibiotics and bactericide, which inhibit the development of degrading and pathogenic microorganisms in the kefir milk (LIU et al., 2002). Kefir is claimed to act against the pathogenic bacteria *Salmonella*, *Helocobacter*, *Shigella*, *Staphylococcus*, *Escherichia coli*, *Enterobacter aerogenes*, *Proteus vulgaris*, *Bacillus subtillis*, *Micrococcus luteus*, *Listeria monocytogenes*, *Streptococcus pyrogenes*, (LOPITZ-OTSOA et al., 2006), *Streptococcus faecalis* KR6, *Fusarium graminearum* CZ1 (ISMAIEL; GHALY; EL-NAGGAR, 2011) and the fungus *Candida albicans*. Besides, it has been tested that a mixture of kefir isolated bacteria and yeast was able to prevent diarrhea and enterocolitis triggered by *Clostridium difficile* (BOLLA et al., 2013). Besides Kefir showed a good efficacy inhibiting spore formation and aflatoxin B1 produced for the fungus *Aspergillus flavus* which is a potent toxic compound that is formed either in the field or during storage in foods (ISMAIEL; GHALY; EL-NAGGAR, 2011).

On the other hand, probiotic strains from kefir have the capacity to lower the cholesterol level. Lactic acid bacteria may alter serum cholesterol by three proposed mechanisms: a) Directly binding, absorbing cholesterol into the cell and assimilation before cholesterol can be absorbed into de body. b) deconjugating bile acids and produce free bile acids, which are more likely to be excreted from the body and drain the cholesterol pool as more bile acids are synthesized and c) inhibiting HMG – CoA reductase by some metabolites of the lactic acid bacteria like propionic acid (YANPING WANG et al., 2009). **Table 1.4** shows some microorganisms found in kefir and kefir grains with their bioactivities and origin.

On the other hand, it had been proved that many species of l*actobacilli* present in the kefir has S – layer proteins. Surface layers (S – layers) are planar arrays of proteinaceus or glycoproteinaceus subunits which can be aligned in unit cells of different symmetries on the outermost surface of many prokaryotic microorganisms (MOBILI et al., 2009). In concordance, it have been demonstrated that these S – layer proteins are involved in the interaction of bacterial cells with yeasts present in kefir grains. Also S – layer proteins are capable to exert a protective action inhibiting the invasion of *Salmonella enterica* Serovar enteritidis to Caco – 2 cells, besides has the ability to antagonize the effects of toxins from *Clostridium difficile* on eukaryotic cells *in vitro* (CARASI et al., 2012).

Biological activity of kefir grains, the cell – free fraction of kefir or lactic acid bacteria isolated from kefir such as antitumoral (GAO et al., 2013b), anti-inflammatory (DINIZ et al., 2003), antimicrobial (ANSELMO et al., 2010), immunoregulatory (HONG et al., 2009), antiallergic (WEI-SHENG HONG; YEN-PO CHEN; MING-JU CHEN, 2010), wound healing (HUSEINI et al., 2012), anti – diabetes (YOUNG-IN KWON; APOSTOLIDIS; SHETTY, 2006), antimutagenic (GUZEL-SEYDIM et al., 2006) and antigenotoxic (GRISHINA et al., 2011). In that way, it had been demonstrated that Kefir cell – free fraction has antiproliferative effects on the human gastric cancer SGC7901 cells (GAO et al., 2013b), colon adenocarcinoma cells (KHOURY et al., 2014), HuT – 102 malignant T lymphocytes, sarcoma 180 in mice, lewis lung carcinoma and human mammary cancer (RIZK; MAALOUF; BAYDOUN, 2009), reduce oxidative stress (PUNARO et al., 2014),

enhance the ability to digest lactose from lactose maldigestors, enhance of mucosal resistance to gastrointestinal pathogen infection (RIZK; MAALOUF; BAYDOUN, 2009). In addition to, KAKISU et al., (2007) demonstrated that kefir fermented milk is able to antagonize mechanisms involved in the growth of *Bacillus cereus*. Another study was shown that suspensions after 24 hours fermentation and Kefir grains mechanically disintegrated have a significant inhibition on the formation of granuloma tissues and an inhibition of 43% for the inflammatory process (DINIZ et al., 2003).

1.3.3 Kefir based products

Kefir can be made from any kind of milk; the milk can be pasteurized, unpasteurized, whole fat, skim and no fat. There are two ways to produce kefir, the traditional way and the commercial way. In the traditional way, kefir grains are added to fresh milk and incubated at 25°C during 24 h then the grains are separated with a sieve (ALTAY et al., 2013). Industrial kefir is mostly produced in Russia and other countries of the ex – Sovietic Union, Greece, Austria and Brazil. Nowadays the interest in developing functional foods is increasing because people want to improve their health and prevent diseases, consequently, the popularity of kefir increased in countries like the United States of America and Japan (POGACIC et al., 2013).

Commercial kefir is produced by two methods: The "Russian method" and the pure cultures. In the "Russian method" kefir is produced on a large scale, using a series fermentation process, beginning with the fermentation of the grains and using the percolate. The other method employs pure cultures isolated from kefir grains or commercial cultures (LEITE et al., 2013). Also, the industrial or commercial process uses direct – to – vat inoculation (DVI) or direct – to – vat set (DVS) kefir starter cultures. In addition, *Bifidobacterium* spp, *Lactobacillus* spp and probiotic yeast such as *Saccharomyces boulardii* may be used as adjunct cultures when blended with kefir grains or kefir DVI cultures (WSZOLEK et al., 2006).

TABLE 1.4 BIOACTIVITY OF BACTERIA ISOLATED FROM DIFFERENT TYPES OF KEFIR AND KEFIR GRAINS

Organism of interest	**Origin**	**Biological Activity**	**Source**
Lactobacillus plantarum MA2	Tibetan kefir	Hypocholesterolemic effect	YANPING WANG et al.(2009)
Lactobacillus plantarum Lp27	Tibetan kefir	Inhibited cholesterol absorption	YING HUANG et al. (2013)
Lactobacillus plantarum CIDCA 83114	Kefir grains - Argentina	Inhibit the growth of *Shigella sonnei in vitro* and also the cytotoxicity of *C. difficile* toxins on eukaryotic cells	BOLLA et al. (2013)
Lactobacillus kefir CIDCA 8348	Kefir grains - Argentina	Inhibit the growth of *Shigella sonnei in vitro* and also the cytotoxicity of *C. difficile* toxins on eukaryotic cells	BOLLA et al. (2013)
Lactobacillus plantarum ST8KF	Kefir grains – South Africa	Bactericida effect against: *Lactobacillus casei* *Lactobacillus salivarus* *Lactobacillus curvatus* *Listeria innocua*	POWELL et al. (2007)
Lactobacillus kefiranofaciens K1	Kefir grains - Taiwanese milk	Antiallergenic effect	CHEN et al. (2008); WEI-SHENG HONG et al. (2010)
Lactobacillus kefiranofaciens M1	Kefir grains – Taiwanese milk	Immunoregulatory effects – anticolitis effect	CHEN et al. (2012); HONG et al. (2009)
Lactobacillus lactis CIDCA 8221	Kefir grains - Argentina	Inhibit the growth of *Shigella sonnei in vitro* and also the cytotoxicity of *Clostridium difficile* toxins on eukaryotic cells	BOLLA et al. (2013)
Kluyveromyces marxianus CIDCA 8154	Kefir grains - Argentina	Inhibit the growth of *Shigella sonnei in vitro* and also the cytotoxicity of *Clostridium difficile* toxins on eukaryotic cells	BOLLA et al. (2013)
Saccharomyces cerevisiae CIDCA 8112	Kefir grains - Argentina	Inhibit the growth of *Shigella sonnei in vitro* and also the cytotoxicity of *Clostridium difficile* toxins on eukaryotic cells	BOLLA et al.(2013)
Lactobacillus lactis subsp. *cremoris*	Kefir grains – India	Activity against food spoilage bacteria	RAJA et al.(2009)

Font: The author

Keeping in mind that kefir is a beverage with high probiotic activity, and others biological activities, new companies are emerging around the world. One of the biggest kefir companies known is Lifeway, which started in 1986; their products can be obtained in the United States, Canada and Great Britain, all of them based in kefir beverages, frozen and cheese. Other companies are Evolve Kefir with its principal product, a smoothie; Wallaby Yogurt Company with Low Fat Kefir; and CocoKefir LLC, which provides drinks/beverages based mainly on coconut water cultured with a comprehensive blend of probiotics. **Table 1.5** summarizes the products provided these companies with some general information about each one. Others promising products are a new baker's yeast a simulated kefir drink and single cell protein which can be use as livestock feed or as a food additive and a starter culture for cheese ripening, it can be made using whey as the raw material (KOURKOUTAS et al., 2007).

1.3.4 Kefiran

In kefir grains the main polysaccharide is Kefiran a water – soluble glucogalactan mainly produced by *Lactobacillus kefiranofaciens* (ZAJŠEK; KOLAR; GORŠEK, 2011) in the center of the grain under anaerobic conditions (BADEL; BERNARDI; MICHAUD, 2011). This heteropolysaccharide composed by equal proportions of glucose and galactose is present in kefir grains and the fermented product (ISMAIEL; GHALY; EL-NAGGAR, 2011; PIERMARIA; DE LA CANAL; ABRAHAM, 2008) and its principal role in kefir grains is protect the microorganisms against desiccation (BADEL; BERNARDI; MICHAUD, 2011).

TABLE 1.5 MARKETED KEFIR – BASED PRODUCTS

Companies	Product	General Information
Lifeway • United States. • Canada • Great Britain	Low Fat Kefir	All-Natural 99% Lactose-Free Gluten-Free 12 Probiotic Cultures High in Protein and Calcium
	Non Fat Kefir	
	Veggie Kefir	
	Kefir Oats	All-Natural 99% Lactose-Free Gluten-Free 12 Probiotic Cultures Oat Fiber Enriched High in Protein and Calcium
	Perfect 12 Kefir	All-Natural 99% Lactose-Free Gluten-Free 12 Probiotic Cultures No Added Sugar High in Protein and Calcium
	Traditional Kefir	
	Greek Style Kefir	
	Low Fat Kefir (Organic)	USDA Certified Organic Oregon Tilth Certified Organic 99% Lactose-Free Gluten-Free 12 Probiotic Cultures High in Protein and Calcium
	Whole Milk Kefir (Organic)	USDA Certified Organic Oregon Tilth Certified Organic 99% Lactose-Free Gluten-Free 12 Probiotic Cultures No Added Sugar
	Helios Kefir (Organic)	USDA Certified Organic Oregon Tilth Certified Organic 99% Lactose-Free Gluten-Free 7 Probiotic Cultures Contains Inulin
	Green Kefir (Organic)	USDA Certified Organic Oregon Tilth Certified Organic 99% Lactose-Free Gluten-Free 12 Probiotic Cultures Phytoboost = 1 Serving of Vegetables
	ProBugs (organic)	USDA Certified Organic Oregon Tilth Certified Organic 99% Lactose-Free Gluten-Free 12 Probiotic Cultures No-Spill Pouch
	ProBugs Blast (Organic)	USDA Certified Organic Oregon Tilth Certified Organic 99% Lactose-Free Gluten-Free 12 Probiotic Cultures High in Protein and Calcium
	Frozen ProBugs (Organic)	All-Natural 99% Lactose-Free Gluten-Free 10 Probiotic Cultures High in Protein and Calcium

The first study about kefiran structure was published by KOOIMAN (1968) who proposed a structure composed by two units: Kefiran (Polysaccharide) and kefirose (Pentasaccharide). After that some authors had analyzed the polysaccharide structure with current techniques such as gas chromatography (GC), Fourier transform infrared spectroscopy (FTIR) (CHEN et al., 2015; WANG et al., 2008) and nuclear magnetic resonance (NMR) (GHASEMLOU et al., 2012a). Kefiran structure is composed by equal proportions of glucose and galactose and has a principal backbone of of (1→6) – linked Glc, (1→3) linked Gal, (1→4) linked Gal – linked Glc and (1→2,6) linked Gal (GHASEMLOU et al., 2012a; MAEDA et al., 2004b; WANG et al., 2008). **Figure 1.4** is showing the structure of kefiran, according to the most actual studies. On the other hand, dry Kefiran texture is brittle and rigid. **Figure 1.5** shows a SEM image of Kefiran which seems organized similar to a piece of wood or paper.

FIGURE 1.4 KEFIRAN STRUCTURE.

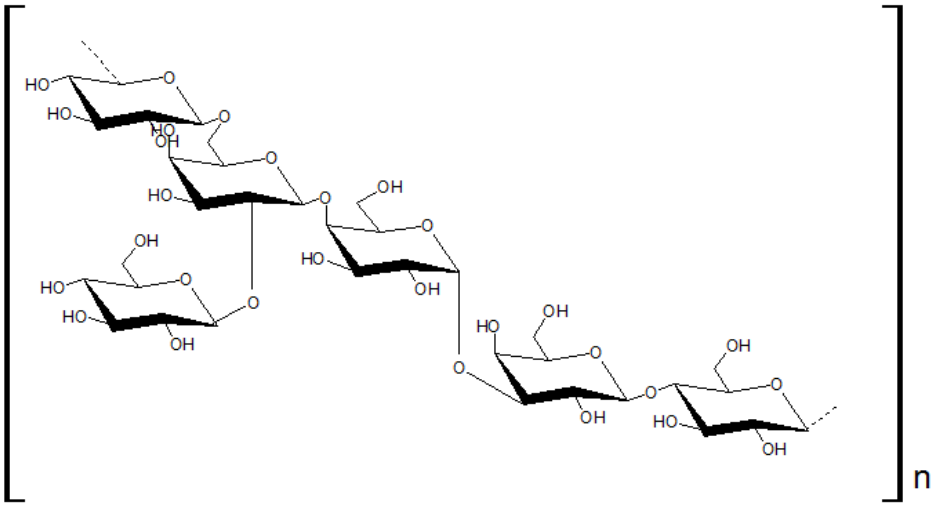


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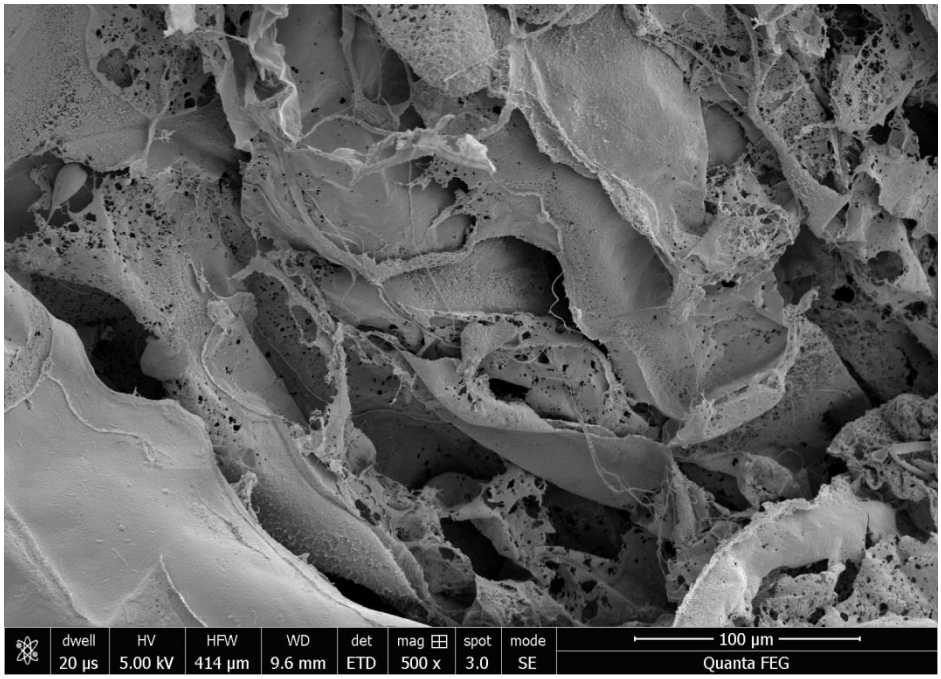
FIGURE 1.5 SEM IMAGE OF KEFIRAN



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In spite of, kefiran is produce by *Lactobacillus kefiranofaciens*, yeast role is important for kefiran production. CHEIRSILP; SHIMIZU; SHIOYA, (2007) made revealed the importance of yeast for kefiran production. The researcher realized that when lactic acid is produced by LAB in kefir grains the inhibition of *L. kefiranofaciens* caused by a low pH is softened by lactic acid – assimilating yeast like *Saccharomyces cerevisiae* avoiding the accumulation of lactic acid at high concentrations, and directly enhancing cell growth and kefiran production rate.

Kefiran has important mechanical, physical and biological properties, he has newtonian behavior in diluted solutions, becomes pseudoplastic at higher concentration and is able to form gels as a result of a cryogenic treatment, this cryogels are translucent and sufficiently cohesive to support their own weight (self – supporting) and have a high water – holing capacity (PIERMARIA; DE LA CANAL; ABRAHAM, 2008). Biologically, kefiran modulates the gut immune system (MEDRANO; PÉREZ; ABRAHAM, 2008a; SERAFINI et al., 2014; VINDEROLA et al., 2006a), protects epithelial cells against *Bacillus cereus 4exocelular factor* (MEDRANO; PÉREZ; ABRAHAM, 2008a), has antitumor, antibacterial (WANG et al., 2008), anti-inflammatory (RODRIGUES; CARVALHO; SCHNEEDORF, 2005),

healing (RODRIGUES et al., 2005), antioxidant activity (CHEN et al., 2015) and can reduce blood pressure induced by hypertension (MAEDA et al., 2004a).

Due to its biological activity, mechanical and physical properties kefiran will contribute to improve food and pharmaceutical industries. In food industry, Kefiran could be certified as a generally safe material (GRAS) a requirement of the governmental agencies such as the European Food Safety Authority (EFSA) or Food and Drug Administration (FDA) in the USA, kefiran can improve rheological properties of dairy products (ENIKEEV, 2012). There are future uses, like whey protein isolate nanocomposites, a great potential in the food packaging for long time storage (ZOLFI et al., 2014). Another interesting use is kefiran nanofibers through electrospinning process using distilled water as solvent as a good opportunity to use kefiran for encapsulation of food additives and in preparation of active and bioactive packaging (ESNAASHARI et al., 2014) and edible kefiran films as vehicle for probiotic organisms (PIERMARIA; DE LA CANAL; ABRAHAM, 2008).

In pharmaceutical industry kefiran could be used not only for his biological activities but also, because it is a water soluble glucalactan, and hydrophilic polymers are promising materials for use in clinical trials such as surface modification of biomaterials, as carriers of drugs, genes and oligonucleotides and for the modification of proteins and liposomes (KOPEČEK, 2013).

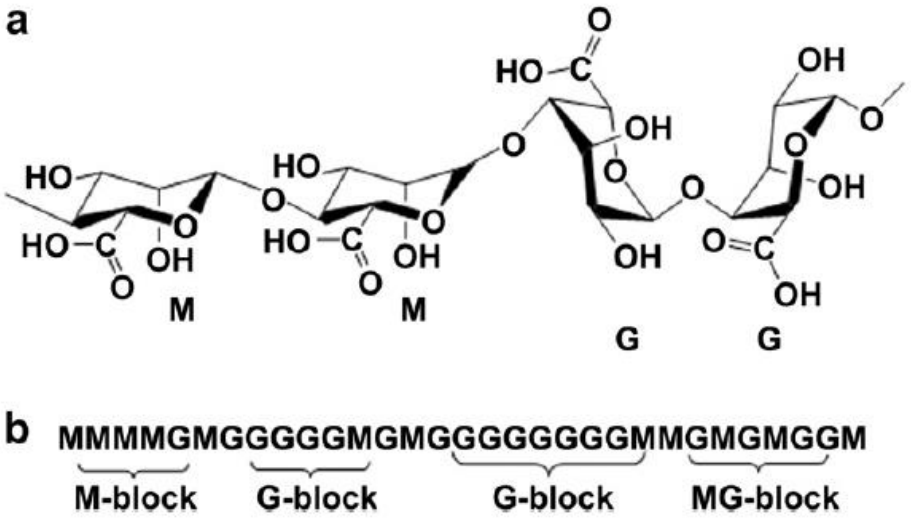
Figure 1.7 shows a diagram of kefiran extraction process from kefir grains, employed in the present work, according to the protocol developed by (RIMADA; ABRAHAM, 2003). Briefly, a quantity of kefir grains is suspended in distilled water boiling during 30 minutes with discontinuous stirring, the mixture obtained is centrifuged at 1000 g during 20 minutes at 20°C to separate the biomass, after that polymer precipitation is made with three volumes of cold absolute ethanol (Leave 12 hours at 0°C). Later, the mixture is centrifuged at 10000g during 20 minutes at 4°C and the precipitated obtained is suspended in hot water. Precipitation steps are repeated twice to remove biomass and residual proteins; finally, the last precipitate is dissolved in hot water, cooled and freeze-drying.

1.7 ALGINATE

Alginate is a biopolymer produced by brown algae (Ex: *Macrocystis pyrifera*) and some bacteria (Ex: *Pseudomonas aeruginosa*) (DONG; WANG; DU, 2006). Industrially, alginate is obtained from algae and its production is approximately of 30000 metric tons in the year, but its composition, sequence and molecular weight varies according to the specie (PAWAR; EDGAR, 2012). The first report of alginate structure was in 1966 (HAUG; LARSEN) and the structure proposed is composed of two uronic acids, with connections β – (1-4) linked with D - manuronic acid (M) and α (1-4) linked with L – guluronic acid (G), besides is composed with homopolymeric blocks M-M or G-G and blocks with alternating sequences M-G (LI et al., 2015). **Figure 1.6** shows alginate structure.

In brown algae alginate is found in the extracellular matrix as gels containing magnesium, calcium, sodium, strontium and barium ions. Figure 1.8 shows the alginate extraction process from algae. Short, the process is made in three parts. In the first part counterions are removed by the interchanged of protons using a mineral acid (0,1 – 0,2 M); in the second part the non – soluble alginic acid obtained is solubilized by neutralization with an alkali like sodium carbonate or sodium hydroxide to form sodium alginate, after that particulate materials are removed with sieving, floculation, centrifugation and filtration. Finally, sodium alginate is precipitated with alcohol, calcium chloride or an acid mineral, the product is dry and milled. On the other hand, for biomedical uses mitogens and cytotoxic impurities are removed in a more rigorous extraction process (PAWAR; EDGAR, 2012).

FIGURE 1.6 ALGINATE STRUCTURE



Font: Adapted from PAWAR; EDGAR, (2012)

Due to its gelling capacity in the presence of polyvalent cations such as Ca^{2+}, Ba^{2+}, Zn^{2+} and Cu^{2+}, its biocompatibility, low cost and toxicity, alginate has been used in various biomedical applications, like wound healing (THU; ZULFAKAR; NG, 2012), drug delivery (RUVINOV; COHEN, 2016) and tissue engineering (VENKATESAN et al., 2015). Besides alginate has an important role in cystic fibrosis where the bacterium biofilms are composed by alginate gels that are secreted by *Pseudomonas aeruginosa* (ISLAN; BOSIO; CASTRO, 2013). Nonetheless, alginate gels are instable in the presence of chelating cationic molecules present in biological fluids like phosphate, also, the tridimensional structure of the gel formed is lost after freeze-drying and rehydration in aqueous environments (CASTRO et al., 2007), a possible solution is the mixture of alginate with other polymers to improve its stability (BLANDÓN et al., 2016).

FIGURE 1.7 KEFIRAN EXTRACTION PROCESS FROM KEFIR GRAINS

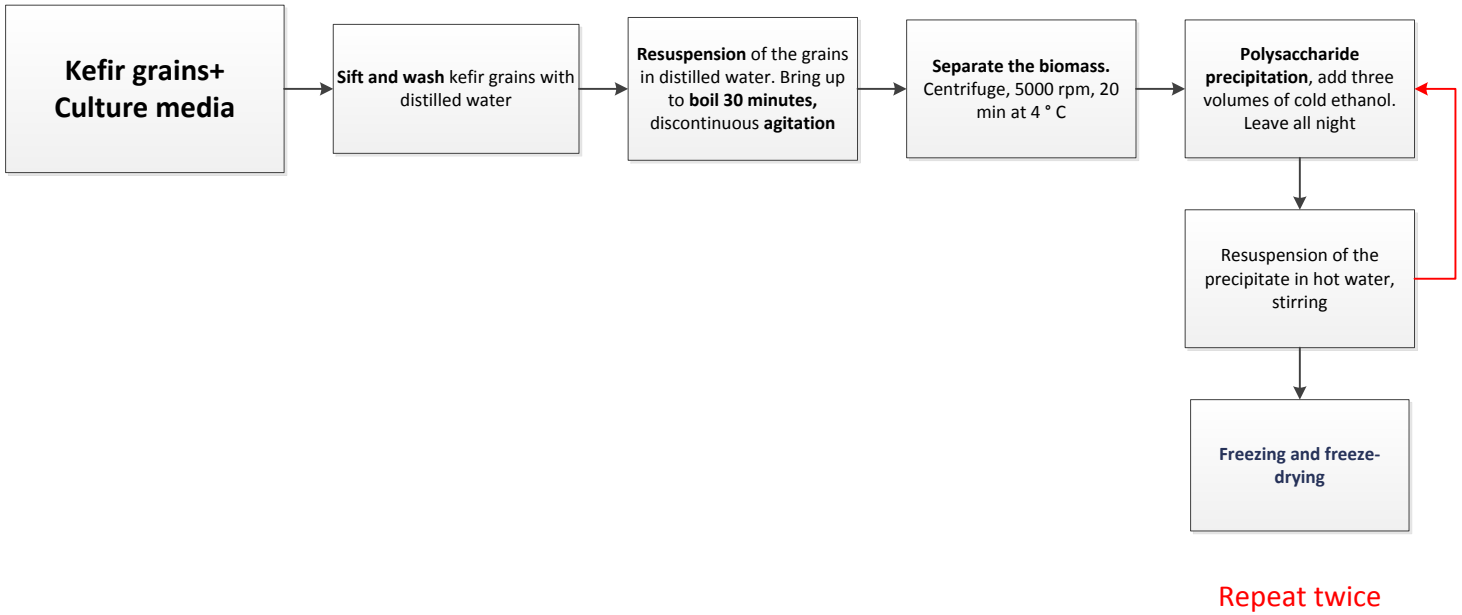


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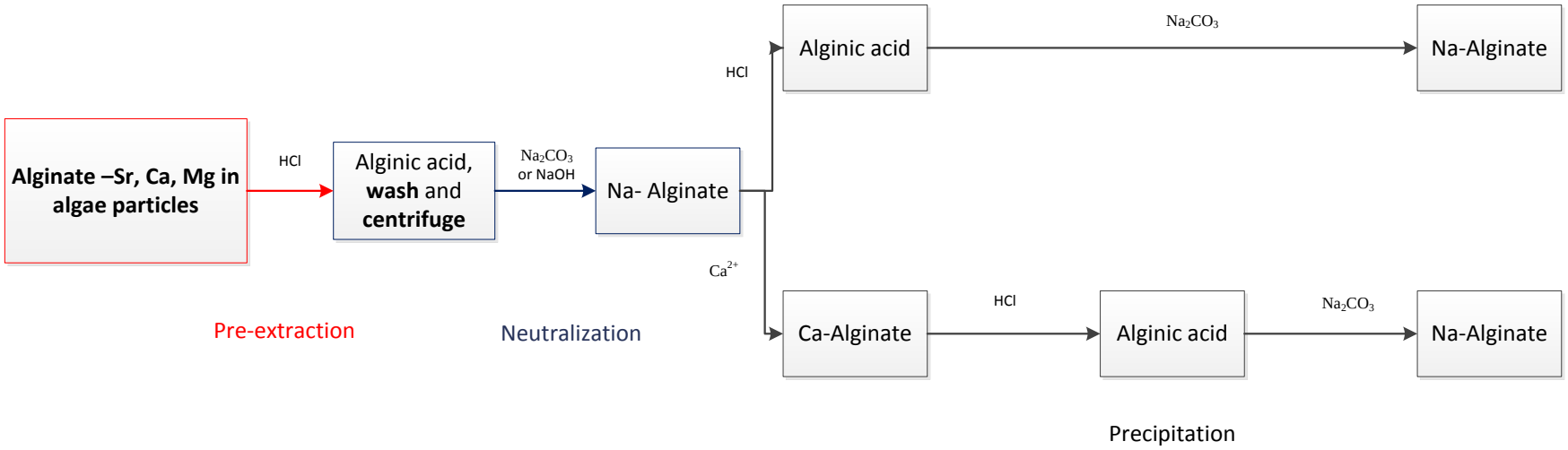
FIGURE 1.8 ALGINATE EXTRACTION PROCESS FROM ALGAE



Font: Adapted from PAWAR; EDGAR, (2012)

2. KEFIRAN PRODUCTION USING WHEY AS CULTURE MEDIUM

2.1 INTRODUCTION

Kefiran is a water-soluble glucogalactan exopolysaccharide produced by the bacterium *Lactobacillus kefiranofaciens* present in kefir grains (ZAJŠEK; KOLAR; GORŠEK, 2011). Kefir grains consist of a complex symbiotic microbial mixture of lactic acid bacteria (10^8 CFU/g), yeasts (10^6-10^7 CFU/g) and acetic acid bacteria (10^5 CFU/g) (CHEN et al., 2015; GARROTE; ABRAHAM; DE ANTONI, 2010). Recent studies demonstrated that kefiran could have biotechnological applications in food, and biopharmaceutical industries (BLANDÓN et al., 2016). Additionally, kefiran has beneficial biological activities, such as modulation of the gut immune system (MEDRANO et al., 2011; SERAFINI et al., 2014; VINDEROLA et al., 2006b), protection of epithelial cells against *Bacillus cereus* toxins (MEDRANO; PÉREZ; ABRAHAM, 2008b), antitumor, antibacterial and anti-inflammatory activities (RODRIGUES et al., 2005; RODRIGUES; CARVALHO; SCHNEEDORF, 2005; WANG; GHOSH, 2008), healing properties (RODRIGUES et al., 2005), antioxidant activity (CHEN et al., 2015) and reduction of blood pressure induced by hypertension (MAEDA et al., 2004a).

The production cost is crucial for the development of new products. The use of low-cost substrates is an important strategy to reduce production costs (FREITAS; ALVES; REIS, 2011). Whey is a liquid by-product obtained from casein precipitation during the production of cheese (BRANDELLI; DAROIT; CORRÊA, 2015). The whey volume is about 83% of the total milk used for cheese production, and it has 55% of the total milk nutrients (SEO et al., 2015). Nevertheless, the chemical composition of whey depends on many variables such as milk properties, processing, and cheese type. In addition, whey is primarily composed of lactose (main component), proteins (with all essential amino acids), salts (potassium, calcium, phosphorus, sodium, and magnesium), vitamins (thiamine, pantothenic acid, riboflavin, nicotinic acid, cobalamin) and fats (HUERTAS, 2009).

Consequently, whey is rich in many nutrients and for this reason it can be used as low-cost and environmentally friendly culture media for the development of biological processes. On the other hand, compared with other polysaccharides, bacterial polysaccharides have attracted less attention because of their low production levels (GHASEMLOU;

KHODAIYAN; OROMIEHIE, 2011), which justifies the need to produce these polymers in large quantities and with a low-cost process.

Kefiran production and optimization studies have been performed using different sources such as kefir grains, pure cultures of *Lactobacillus kefiranofaciens*, and some mixed cultures, which were tested under different culture media and physicochemical conditions, as shown in **Table 2.1**. The aim of this chapter was to evaluate the best physical conditions (temperature, agitation and time) and medium composition for kefiran production from kefir grains using cheese whey supplemented with a carbon source as substrate.

2.2 MATERIALS AND METHODS

2.2.1 Starter culture

Kefir grains were obtained from a culture of Tibetan kefir maintained in the bioprocesses engineering and biotechnology laboratory (Federal University of Parana, Curitiba – Brazil). The grains were kept at -20°C and reactivated by successive subcultures in 1L of fresh milk at room temperature without stirring. The medium was changed daily and the grains washed with cold running water.

2.2.2 Chemicals and culture media

Glucose, lactose, saccharose, galactose and ethanol were purchased from Sigma Aldrich (Sao Paulo, Brazil). Skim sweet cheese whey was a donation from Anila industries (Parana, Brazil).

2.2.3 Kefiran extraction

The biopolymer was extracted using a previously described protocol (RIMADA; ABRAHAM, 2003). A weighted amount of kefir grains maintained in whey supplemented with carbohydrates (based on the optimization design, see below) was suspended in boiling distilled water for 15 min with discontinuous stirring. After cooling, the mixture was centrifuged at 10,000xg for 20 min at 20°C to eliminate biomass residues. The soluble polysaccharide present in the supernatant was precipitated by the addition of three volumes of cold ethanol (at -20°C). The suspension was centrifuged at 10,000xg for 20 min at 4°C, and the precipitate

containing the biopolymer was dissolved in hot distilled water. The precipitation procedure was repeated twice to eliminate protein and biomass residues. Finally, the polysaccharide was freeze-dried (**Figure 1.7**)

2.2.4 Composition of the fermentation medium

Batch fermentations were carried out in 250 mL Erlenmeyer flasks at 30°C for 36 h. The fermentation volume was 100 mL, and the initial humid weight of kefir grains was 4.2 g. Under these conditions, culture medium composition was optimized by supplementing cheese whey with different carbon sources (*i.e.*, galactose, glucose, lactose, and saccharose) and tested at 5%, 10%, 15% and 20% (w/v). Whey medium without sugar supplement was used as control. Finally, kefir grains were separated from the fermentation product by filtration, washed with cold distilled water, dried carefully with filter paper, and weighed. Later, kefiran was extracted and two responses were determined: biomass increase (equation 2.1) (ZAJŠEK; GORŠEK; KOLAR, 2013) and kefiran productivity ($g.L^{-1}.h^{-1}$).

$$Biomass\ increase = \frac{Wf - Wi}{Wi} x100 \qquad (2.1)$$

Where W_i and W_f are the initial and final weights, respectively. Experiments were performed in triplicate. Statistic treatments were carried out by one way analysis of variance (ANOVA) with a significance level of ($\alpha = 0.05$) using R software (R version 3.2.3 (2015-12-10), R Foundation for Statistical Computing).

TABLE 2.1 KEFIRAN OPTIMIZATION STUDIES REPORTED IN THE LITERATURE

Source	Media Culture	Best Conditions	Productivity	Reference
Kefir grains CIDCA AGK1	Deproteinized whey	43°C.	57±2 and 103±4 mg/l, 5 days of fermentation, 10 and 100 g kefir grains.	RIMADA; ABRAHAM, (2001)
Lactobacillus kefiranofaciens JCM6985, *Torulaspora delbruekii* IFO1626, *Saccharomyces cerevisiae* IFO0216, *Saccharomyces unisporus* IFO0724, *Candida tenuis* IFO1303, and *Candida kefir* IFO10278	MRSL broth medium	Mixed culture of *Lactobacillus kefiranofaciens* and *Saccharomyces cerevisiae*	Anaerobic condition 36 mg $^{-1}$ h^{-1}, under aerobic condition 44 mg^{-1} h^{-1}, fedbatch culture 62 mg $^{-1}$ h^{-1}	CHEIRSILP; SHIMIZU; SHIOYA, (2003)
Lactobacillus kefiranofaciens WT-2BT (JCM = 6985 ATCC 43761)	Rice hydrolysate	7-day culture period, pH 5.0 and 33°C.	2.5 g/L	MAEDA et al., (2004c)
Lactobacillus kefiranofaciens JCM6985, *Saccharomyces cerevisiae* IFO0216	MRSL medium	Lactose-feeding batch operation with feedforward/feedback control during the coculture	In a fed-batch coculture 6.3 g in 102 h	TADA et al., (2007)
Lactobacillus kefiranofaciens	Low-cost sago starch	pH 5.5, temperature of 30°C, 4% starch and α-amylase to glucoamylase radio of 60:40	11.83 mg/l*h^{-1}	YEESANG; CHANTHACHUM; CHEIRSILP, (2007)
Lactobacillus kefiranofaciens JCM 6985, *Torulaspora delbruekii* IFO 1626, *Saccharomyces cerevisiae* IFO0216, *Debaryomyces hansenii* TISTR 5155, *Saccharomyces exiguus* TISTR 5081, *Zygosaccharomyces rouxii* TISTR5044, and *Saccharomyces carlsbergensis* TISTR 5018	MRS - Whey lactose 4%, yeast extract 4%	pH 5.5, initial amount of *Lactobacilllus kefiranofaciens* and *Sacharomyces cereviciae* of 2.1x10^7 and 4.0x10^6 CFU/mL respectively. Scale up of the mixed culture in a 2L bioreactor with dissolved oxygen control at 5% and pH control at 5.5	2580 mg/mL in batch culture and 3250 mg/mL in fed batch culture.	CHEIRSILP; RADCHABUT, (2011)
Kefir grains	Cheese whey	67 g/l Lactose, 13 g/l yeast extract, pH 5.7 and 24°C	Approx. 678 mg/L (Surface response analysis)	GHASEMLOU et al., (2012b)
Kefir grains company, Kele & Kele d.o.o., Slovenia	Customized fresh milk	25°C, 80 rpm, customized milk supplemented with 5% (w/v) lactose, 0.1% thiamine 0.1% and $FeCl_3$, 24 hours		ZAJŠEK; GORŠEK; KOLAR, (2013).
Lactobacillus kefiranofaciens ATCC 8007	Sucrose, yeast extract and K_2HPO_4 at 20.0, 6.0, 0.25 g L^{-1}	Batch culture under un-controlled pH conditions in 16-L scale bioreactor	1.91 g/L^{-1}	DAILIN et al. (2015)

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2.2.5 Process parameters

Kefir grains from the batch cultures were set up in 4.2 g, and the selected carbon source was the best one found (glucose) in the first part of the optimization experiment (see Results and discussion, **Figure 2.1**). Three physicochemical parameters were optimized: temperature, time and agitation. A central composite design was developed with six axial points and four central points. The variables coded levels used for the study are shown in **Table 2.2**. The three independent parameters were studied at five different levels (-1.68, -1, 0, +1, and +1.68) and a set of 29 experiments was performed (**Table 2.3)**. ANOVA and response surfaces were carried out using the "rsm"package of the R program (R version 3.2.3 (2015-12-10), R Foundation for Statistical Computing) (LENTH, 2009). At the end of each experiment, kefir grains were separated and processed as mentioned above and responses of biomass increase and productivity ($g.L^{-1}.h^{-1}$) were determined.

TABLE 2.2 - EXPERIMENTAL DESIGN:INDEPENDENT VARIABLES AND THEIR CODED LEVELS CHOSEN FOR COMPLETE FACTORIAL DESIGN

Independent Variables		**-1,68**	**-1**	**0**	**1**	**1,68**
Temperature (°C)	X1	25	27	30	33	35
Time (h)	X2	12	16	24	32	36
Agitation (rpm)	X3	50	60	70	80	90

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TABLE 2.3 - CODED LEVELS AND REAL VALUES (IN THE PARENTHESIS) FOR THE EXPERIMENTAL DESIGN

	Independent Variables			Y1 Biomass Increase (%)		Y2 Productivity ($g.L^{-1}.h^{-1}$)	
	X1	**X2**	**X3**	**Predicted**	**Observed**	**Predicted**	**Observed**
1	-1 (27)	-1 (16)	-1 (60)	9.358	7.937	0,166	0,180
2	+1 (33)	-1 (16)	-1 (60)	14.914	15.873	0,177	0,186
3	-1 (27)	+1 (32)	-1 (60)	11.012	10.317	0,121	0,117
4	+1 (33)	+1 (32)	-1 (60)	19.744	21.429	0,107	0,102
5	-1 (27)	-1 (16)	+1 (80)	5.548	4.388	0,146	0,156
6	+1 (33)	-1 (16)	+1 (80)	2.748	3.968	0,186	0,190
7	-1 (27)	+1 (32)	+1 (80)	3.196	2.762	0,091	0,086
8	+1 (33)	+1 (32)	+1 (80)	1.229	3.175	0,106	0,090
9	-1.68 (25)	0 (24)	0 (70)	20.670	20.635	0,094	0,092
10	+1.68 (35)	0 (24)	0 (70)	3.072	2.381	0,067	0,067
11	0 (30)	-1.68 (12)	0 (70)	3.629	4.130	0,282	0,251
12	0 (30)	+1.68 (36)	0 (70)	4,609	3,175	0,120	0,132
13	0 (30)	0 (24)	-1.68 (50)	11,374	13,492	0,123	0,120
14	0 (30)	0 (24)	+1.68 (90)	16,134	13,492	0,133	0,135
15	0 (30)	0 (24)	0 (70)	19,825	19,048	0,129	0.128
16	0 (30)	0 (24)	0 (70)	19,825	19,048	0,129	0,128
17	0 (30)	0 (24)	0 (70)	19,825	19,048	0,129	0,123
18	0 (30)	0 (24)	0 (70)	19,825	23,050	3,103	3,330
19	0 (30)	0 (24)	0 (70)	19.825	19,048	3,103	2,970

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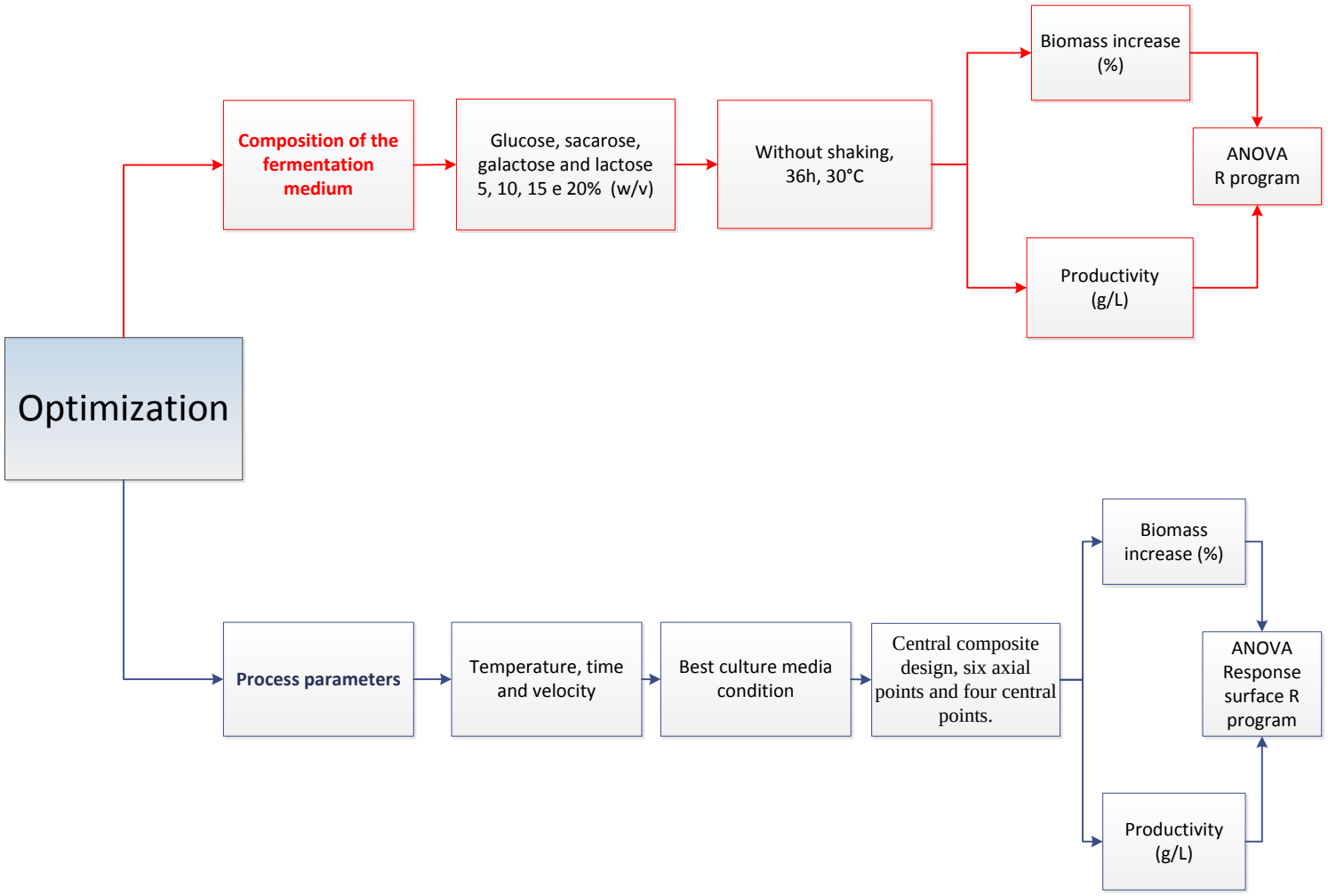
FIGURE 2.1 DIAGRAM OF THE OPTIMIZATION METHODS



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2.3. RESULTS AND DISCUSION

2.3.1 Composition of the fermentation media

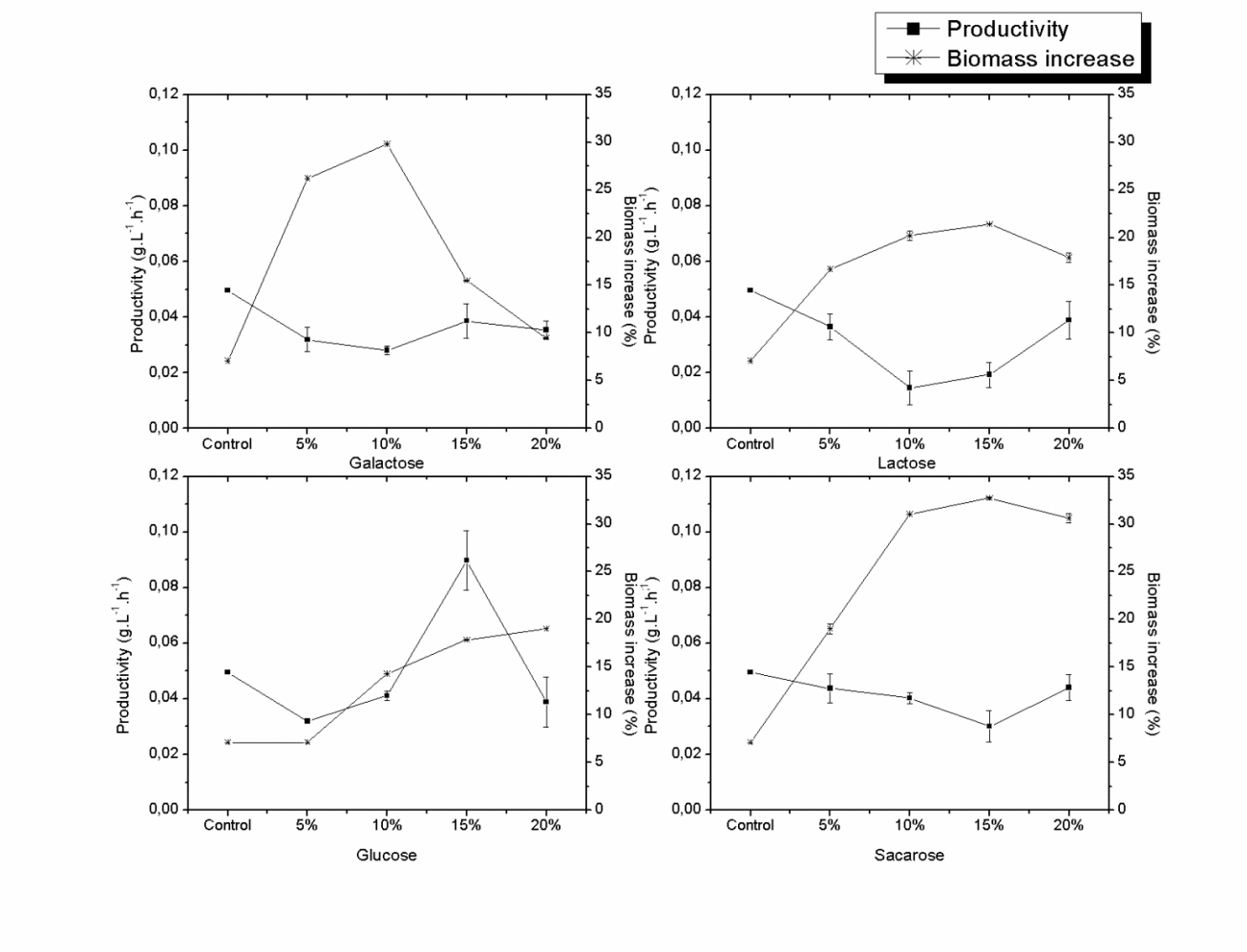
Whey is an interesting source for biological processes due not only to its high amount and diversity of nutrients but also to the environmental concerns related to the possibility to diminish whey wastes. Besides, the production process could be more cost - effective, reducing one of the main problems in the commercialization of products: production costs, mainly related to the substrate costs. EPS synthesis is high in conditions of carbon excess and limitation of other nutrients (FREITAS; ALVES; REIS, 2011); for this reason, the whey extract was supplemented separately with four carbon sources: galactose, glucose, lactose, and sucrose at four different concentrations (5%, 10%, 15%, and 20%, w/v). Fructose was not tested because previous studies showed little productivity with this carbon source (ZAJŠEK; GORŠEK; KOLAR, 2013).

The whey pH was set at 5.0 based on a mathematical model used to determine the optimal pH in batch culture for kefiran production previously reported (CHEIRSILP; SHIMIZU; SHIOYA, 2001). On the other hand, low pH in the media culture can be softened by the presence of non-lactose assimilating yeasts present in kefir grains because they use lactic acid as a carbon and energy source avoiding the accumulation of lactic acid and consequently enhancing kefiran production (CHEIRSILP; RADCHABUT, 2011; CHEIRSILP; SHIMIZU; SHIOYA, 2007).

The effects of carbon sources on biomass increase and kefiran productivity are shown in **Figure 2.2**. In general, the highest values of biomass values were found using saccharose and galactose as carbon sources, but a high biomass increase does not necessarily correlates with high kefiran content into the grain, probably because the weight increase could be related to high cell division.

Previous studies using UHT milk supplemented with the same carbon sources found the best kefiran production was that with 5% (w/v) lactose, giving a yield of 4.2% (w/w) kefiran/kefir grains ratio (ZAJŠEK; GORŠEK; KOLAR, 2013). Our best result was obtained with 15% glucose as carbon source, giving a yield of 6.5% (w/w) kefiran/kefir grains ratio that corresponds to 0.090 ± 0.07 $g.L^{-1}.h^{-1}$ productivity. This discrepancy could be attributed to the differences in medium composition.

FIGURE 2.2 - EFFECT OF SUPPLEMENTING WHEY WITH DIFFERENT CARBON SOURCES ON THE BIOMASS INCREASE AND THE KEFIRAN PRODUCTION (ERRORS SD ≤ 10%, n = 3)



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2.3.2 Process parameters

A central composite design was developed in order to evaluate the interaction among the three independent process variables, i.e., Temperature, time and agitation, and to determine their potential interaction. Aeration was not taken into account because kefiran is produced by *Lactobacillus kefiranofaciens*, which is positioned in the center of the grains under anaerobic conditions, as previously reported (BADEL; BERNARDI; MICHAUD,

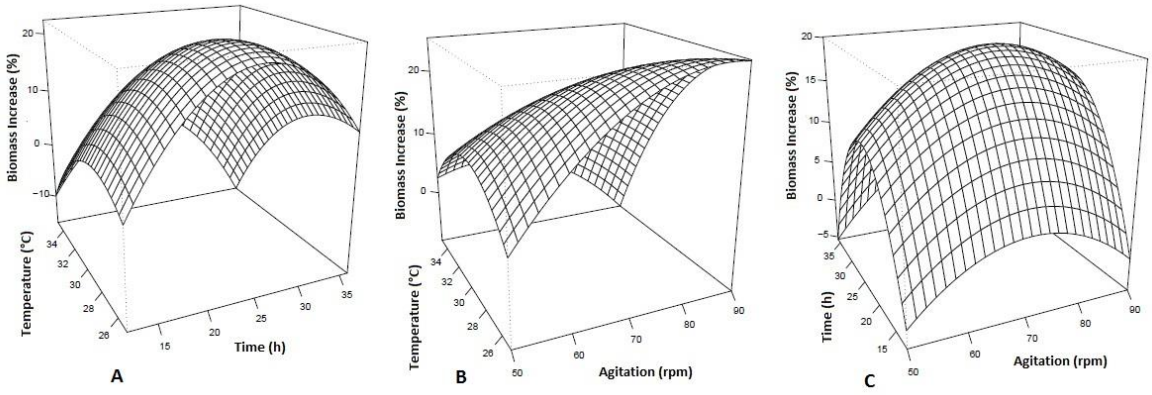
2011). **Table 2.3** lists the matrix results of biomass increase and kefiran productivity obtained under different conditions (statistical data are included in the supplementary material)

$$Y_1 = -554.670^{***} + 24.145{X_1}^{***} + 6.456X_2^{***} + 4.476X_3^{**} - 0.079X_1X_3^{*} - 0.317{X_1^2}^{***} - 0.109{X_2^2}^{***} - 0.015{X_3^2}^{**} \quad (2.2)$$

Where Y_1 is the biomass increase; X_1, temperature; X_2, time; and X_3, agitation speed.

The ANOVA quadratic regression verified the suitability of the model at P= $5.40*10^{-5}$ with an R^2 of 0.96 and an adjusted R^2 of 0.92. **Figure 2.3** displays the resulting surface response plots**. Equation 2.2** shows the great significance of the three studied parameters for biomass production. Particularly, agitation has a strong negative effect on biomass production, as previously reported (KOURKOUTAS et al., 2007).

FIGURE 2.3 - RESPONSE SURFACE PLOTS FOR THE BIOMASS INCREASE A. TEMPERATURA AND TIME, B. TEMPERATURE AND AGITATION, C. TIME AND AGITACION



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The effect of temperature and time on biomass increase at the fixed center value for agitation is shown in **Figure 2.3 A**. The biomass increases with the increase in temperature and time until 25°C for 24 h, when the highest value was reached (20%); from that point on, biomass starts to decrease. In concordance with **Figures 2,3B and 2.3C**, the highest value of biomass increase is observed at 25°C and 75 rpm, approximately.

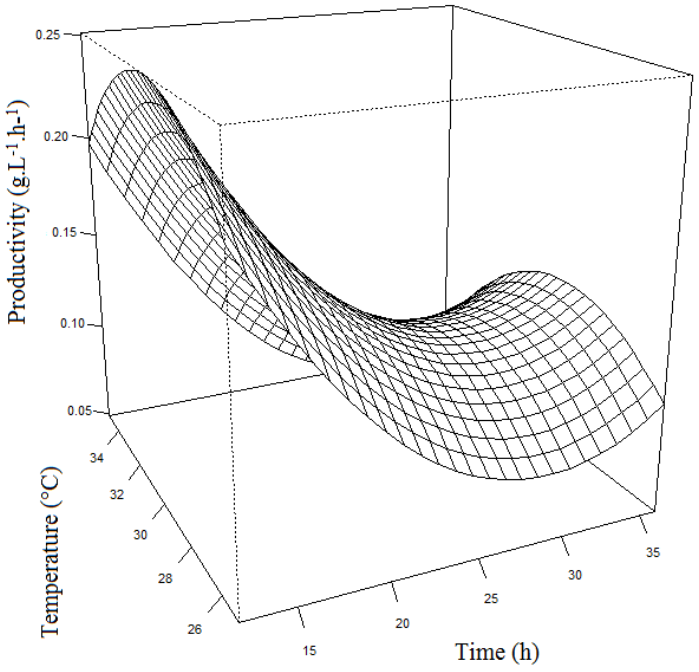
Unlike biomass increase, kefiran production does not depends on the agitation. Equation 2.3 shows the equation model for kefiran productivity.

$$Y_2 = 0.103X_1^{*} - 0.002X_1^{2^{**}} + 4.393 * 10^{-4}X_2^{2^{*}} \qquad (2.3)$$

Where Y_2 is the productivity; X_1, temperature; and X_2, time.

The ANOVA quadratic regression model verified that the model was suitable (P= 0.0002) with an R^2 of 0.96 and an adjusted R^2 of 0.90. **Figure 2.4** shows the resulting response surface plot.

FIGURE 2.4- .RESPONSE SURFACE PLOT FOR PRODUCTIVITY



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The best temperature and time values for kefiran production are 30°C and 10 h, respectively. In that way, 30°C is the limiting temperature for biomass increase (the best model was run at 25°C). Under 30°C, kefiran production is stimulated during the first 10 h because the major physicochemical role of kefiran in kefir grains is to create a physical barrier protecting the microorganisms against desiccation (BADEL; BERNARDI; MICHAUD, 2011), enhancing the cell-protection mechanism. On the other hand, kefiran will become more soluble and could diffuse from the grains to the culture medium when the time and temperature increase.

In a previous report, the effects of temperature and agitation on kefir growth and kefiran production were studied independently, and it was found that the physical parameters were not related to each other (ZAJŠEK; GORŠEK; KOLAR, 2013). However, this approach is very limited from the conceptual point of view of microorganisms' growth.

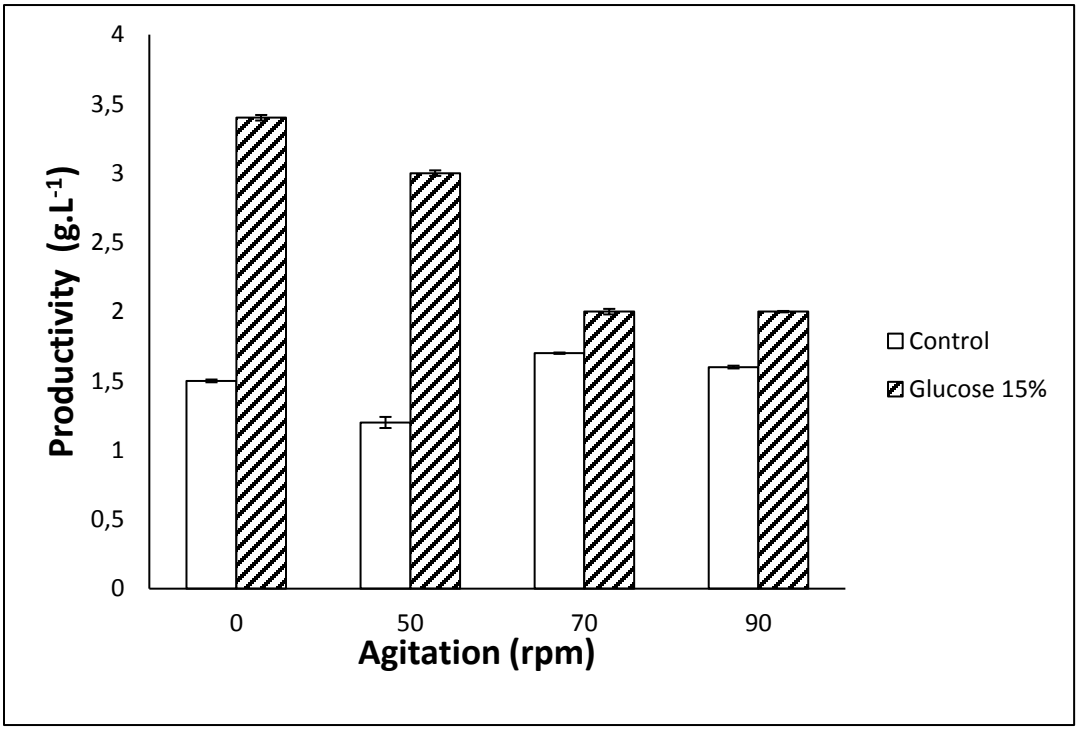
There are previous studies on kefiran optimization from different sources: kefir grains, mixed cultures of bacteria and yeast, and pure *Lactobacillus kefiranofaciens* cultures (**Table 2.1**). From the practical point of view, an inexpensive and less complicated process is to obtain kefiran directly from kefir grains, because additional purification steps are expensive and labor and time consuming. In comparison with other studies, RIMADA; ABRAHAM (2001) reported a best temperature condition of 43°C with a productivity of 57±2 and 103±4 mg/L in 5 days of fermentation using 10 and 100 g kefir grains, respectively, with deproteinized whey. The high temperature value could be because it is directly extracted from the culture medium rather than from kefir grains, which corroborates the fact that a higher temperature involves kefiran dissolution in the culture media. On the other hand, ZAJŠEK; GORŠEK; KOLAR, (2013) used customized fresh milk supplemented with lactose, thiamine and $FeCl_3$, a more expensive culture medium.

In order to validate the model and results, three different experiments were performed under the optimal conditions for growth (25°C, 75 rpm, 24 h) and kefiran production (30°C, 0 rpm, 10 h) showing the mean biomass increase value of 19.84±0.35% and the mean kefiran production value of 0.20±0.04 $g.L^{-1}.h^{-1}$.

To see in more detail the effect of agitation on kefiran production, an independent study was done to compare kefiran production in a culture medium composed of whey supplemented with 15% glucose and whey alone (control) at the velocities studied (**Figure**

2.5). The results corroborated the model, showing that the best conditions for kefiran production do not need agitation and that kefiran production decreases when shaking increases.

FIGURE 2.5 -KEFIRAN PRODUCTION UNDER DIFFERENT VELOCITIES



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2.4 CONCLUSION

The present study demonstrated that kefiran can be produced with high productivity from low-cost culture media under minimal control conditions by a simple purification method. The optimization of culture conditions using three parameters in a central composite design showed that the best kefiran production medium is the one that contains cheese whey supplemented with 15% (w/v) of glucose at 30°C for 10 h without agitation. Under these conditions, 0.20 $g.L^{-1}.h^{-1}$ of kefiran can be obtained from 42 g/L starter kefir grains. Our study also showed that good growing conditions for kefir grains not necessarily imply that the best kefiran productivity will be obtained.

3 KEFIRAN STRUCTURE ANALYSIS AND BIOCIDAL PROPERTIES

3.1 INTRODUCTION

Previous studies have demonstrated that kefiran is a water-soluble branched heteropolysaccharide composed of equal proportions of glucose and galactose and has a backbone consisting of [(→6)-β-D-Glc*p*-(1→6)-β-D-Gal*p*-(1→4)-α-D-Gal*p*-(1→3)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→] (KOOIMAN, 1967; CHEN et al., 2015; GHASEMLOU et al., 2012b; MAEDA et al., 2004c; WANG et al., 2008).

In the previous chapter (chapter 2), kefiran production optimization from kefir grains was study using as media culture cheese whey, nonetheless, it has been demonstrated that EPS produced by different strains alone or in a mixed culture could differ in properties and monosaccharide composition, and that the composition of culture media can influence too. Also, the EPS produced by *L. kefiranofaciens* ZW3 is mainly composed of glucose and galactose (AHMED et al., 2013a), but when the same bacteria are cultured with strains that are not EPS producers such as *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, the product obtained presents different thermodynamic properties, surface morphology and is composed of glucose, galactose, arabinose and xylose (AHMED et al., 2013b). Furthermore, BOTELHO et al.,(2014) obtained a polysaccharide composed only of glucose fermenting soy milk with kefir grains.

The aim of present study was to analyze the resulting polysaccharide produced in chapter 2, using High Pressure Size Exclusion Chromatography (HPSEC), gas chromatography - mass spectrometry (GC-MS), nuclear magnetic resonance (NMR), Fourier transform infrared (FTIR), Raman spectroscopy. The antimicrobial activity of the polysaccharide was determined using live and dead test to confirm the persistency of polymer biocidal activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and four strains of *Salmonella typhimurium.*

3.2 MATERIALS AND METHODS

3.2.1 HPSEC

HPSEC was carried out at 25°C with 1.0 mg.mL^{-1} of kefiran, dissolved in 0.1 M $NaNO_2$ containing NaN_3 (0.2 g.L^{-1}); the sample was filtered using 0.2 mm cellulose acetate membranes. The biopolymer analysis was performed using a differential

refractive index detector (RID, Waters 2410) coupled with a multi-angle laser light scattering detector (MALLS, Wyatt Technology model Dawn DSP). The products were separated isocratically at 0.6 $mL.min^{-1}$ (Waters peristaltic pump 515) and four size exclusion columns were placed in series, with exclusion limits of 7.10^6, 4.10^5, 8.10^4, and 5.10^3 $g.mol^{-1}$ (Waters, Ma., USA). The HPSEC data were collected and analyzed with ASTRA program (ASTRA, Wyatt Technology, Ma., USA).

3.2.2 Monosaccharide composition

To determine monosaccharide composition, the polysaccharide was totally hydrolyzed. Briefly, 2.0 mg of the polysaccharide was solubilized in 0.5 mL of TFA 2 M and maintained at 100°C for 2 h, and then TFA was evaporated with nitrogen. The hydrolyzed sample was solubilized in 0.5 mL of distilled water, and the monosaccharides were reduced with $NaBH_4$ for 12 h. Then the solution was acidified with acetic acid (up to pH 5), dried with nitrogen, and the residual boric acid was codistilled with methanol (3x). The resulting alditols were acetylated with 0.5 mL of acetic anhydride for 1 h at 120°C, and the corresponding alditol acetates were analyzed by (GC-MS).

3.2.3 NMR

For NMR analyses, kefiran samples were dissolved in D_2O 40 $mg.mL^{-1}$ for ^{13}C and in 15 $mg.mL^{-1}$ for ^{1}H, and were recorded at 50°C using a Bruker Avance DRX400 spectrometer. The base frequency was 400.13 and 100.61 MHz for ^{1}H and ^{13}C nuclei, respectively. Chemical shifts are expressed relative to acetone (internal standard) at 30.59 for ^{13}C.

3.2.4 FTIR

The FTIR spectrum was obtained using KBr pellets. The kefiran sample was pressed into KBr (0.1% w/w), and the FTIR spectrum was recorded (Bomen-Hartmann & Braun, MB-series) with a resolution of 4 cm^{-1} and 32 scans per minute, using transmittance technique. The range of wavenumber scanned was from 400 cm^{-1} to 4,000 cm^{-1}. The data obtained were analyzed using the ACD/NMR processor academic version.

3.2.5 RAMAN spectroscopy

Raman spectroscopy was performed on an upgraded WITec UHTS300 (Witec GmbH, Ulm, Germany) confocal microscope system with a 532 nm frequency-doubled Nd:YAG laser and a 100 Nikon objective. Raman spectra were measured with a 600 nm grating in the 350 to 3980 cm^{-1} range. An area of 50 x 50 mm^2 was scanned in 0.39 mm steps, giving a map with 1024 x 127 pixels. A survey scan with 100 s integration times was run to determine the quality of the sample in the chosen area, and then a full scan was measured with 1 s/pixel to obtain reasonable spectra.

3.2.6 Biocidal properties

The antimicrobial activity of kefiran against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium* LT2 (the type strain of *S. typhimurium*, resistant to streptomycin, rifampicin, and nalidixic acid), *S. typhimurium* CQ27 (resistant to amikacin and gentamicin), *S. typhimurium* CQ28 (resistant to amikacin, gentamicin and nitrofurantoin) and *S. typhimurium* CQ29 (resistant to nitrofurantoin) was followed with Live/Dead BacLight® kit. Bacteria were grown at late exponential phase and inoculated in a soft nutrient agar (1/10 dilution), then a 20 µL drop was placed on the surface of a glass slide, followed by incubation for 24 h to allow biofilm formation. Subsequently, the biofilm was covered with 1.0% (wt) kefiran for 30 and 60 min. After treatment, the biofilms were carefully washed with deionized water.

Biofilm staining was prepared by mixing both dyes in equal proportions (0.75 µL of each one in 0.5 mL of sterile deionized water). The resulting dye solution was applied onto the entire biofilm and held in darkness for 20 min. Then, the samples were washed using deionized water and observed in an epifluorescence microscope (Leica DM 2500, Germany) equipped with UV filters (495–505 nm) to determine the bacteria viability.

The detection was set at excitation wavelengths between 510 and 550 nm, and emission wavelength at 590 nm (U-MWG2 filter) for alive bacteria (green), and at 460 nm excitation and 490-520 nm emission wavelengths (U-MWB2 filter) for dead bacteria (red).

3.3 RESULTS AND DISCUSSION

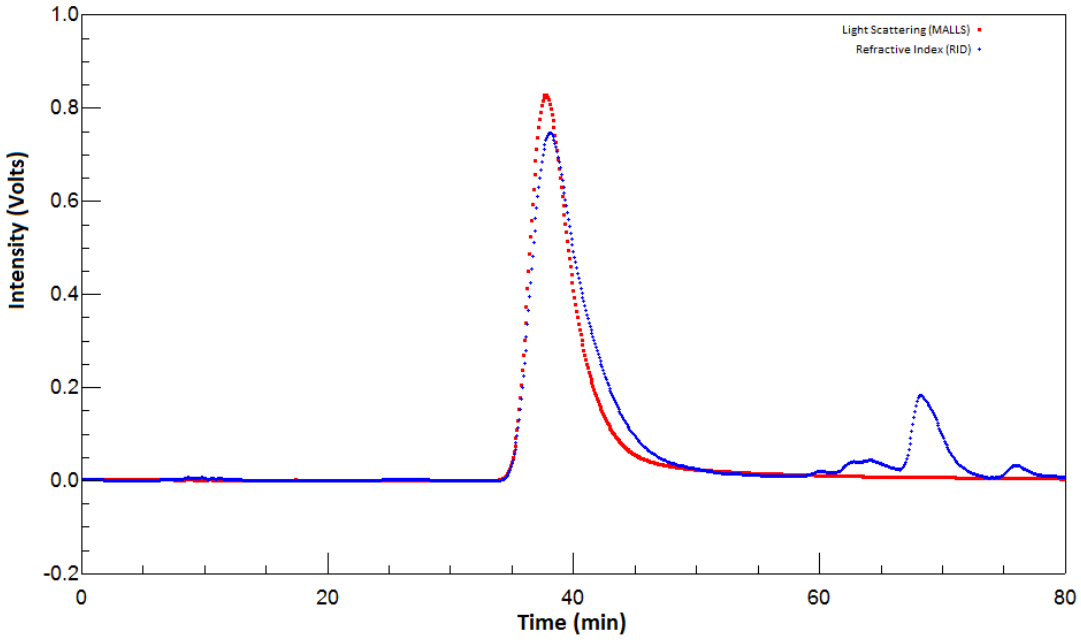
3.3.1. Structure analysis

The biopolymer obtained using the optimized conditions was analyzed by chromatographic and spectroscopic techniques. HPSEC analysis (**Figure 3.1**) was used to determine the homogeneity of the biopolymer sample and the spectroscopic techniques (*i.e.*, Raman and FTIR, **Figure 3.4,** and NMR, **Figures 3.2** and **3.3**) were used to characterize the polysaccharidic nature of the biopolymer produced under the optimized conditions.

The HPSEC elution profile of the biopolymer shows a unique and symmetric peak, detected by the light scattering (MALLS) and refractive index (RID) detectors, indicating a homogeneous molecular mass distribution of the polysaccharide.

The monosaccharide composition, as determined by GC-MS, shows that the biopolymer is composed of galactose and glucose.

FIGURE 3.1 HPSEC-MALLS-RID ELUTION PROFILE OF THE BIOPOLYMER OBTAINED USING THE OPTIMIZED CONDITIONS



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^{1}H- and ^{13}C-NMR analyses (**Figures 3.2** and **3.3**) of the polysaccharide obtained using the optimized conditions are in accordance with previously reported NMR spectra of kefiran (MAEDA et al., 2004c).

FIGURE 3.2 ^{1}H NMR SPECTRUM OF THE BIOPOLYMER OBTAINED USING THE OPTIMIZED CONDITIONS

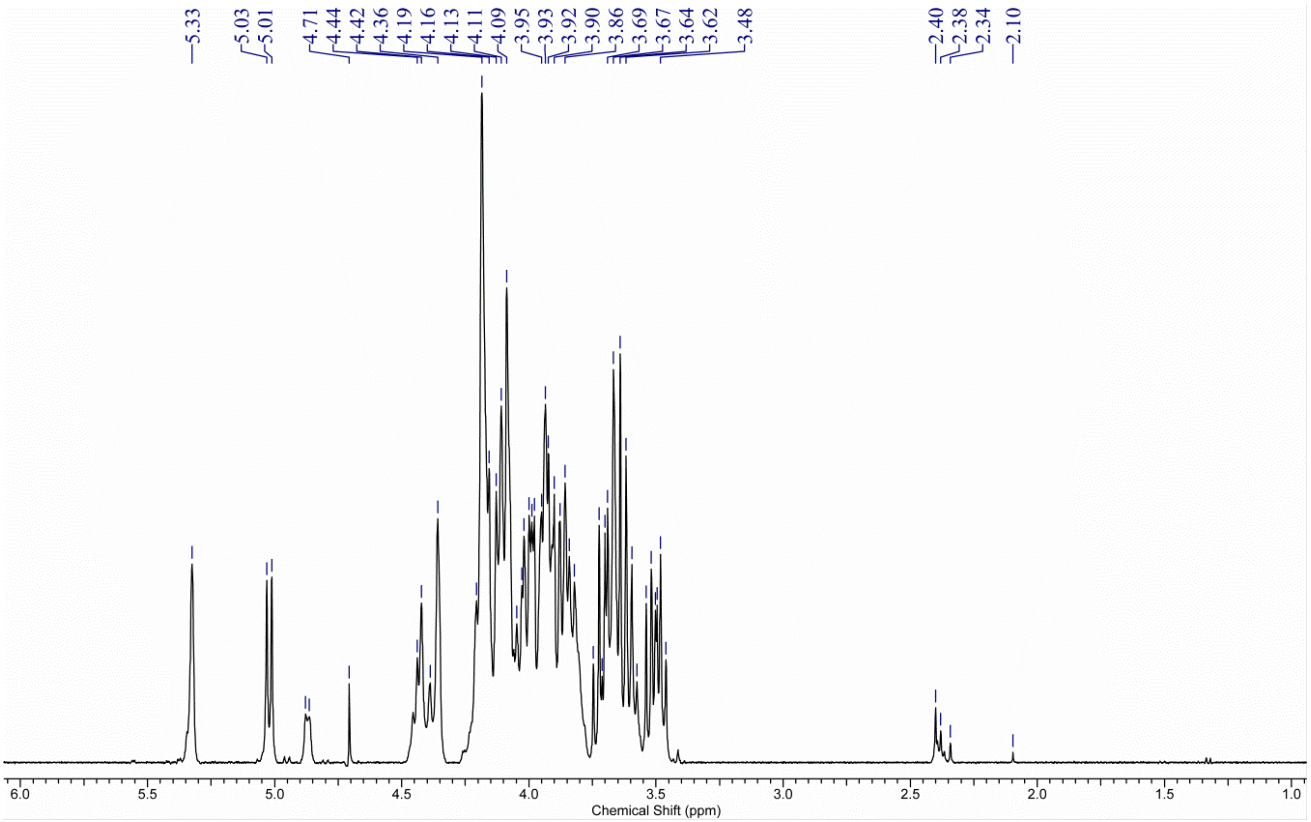

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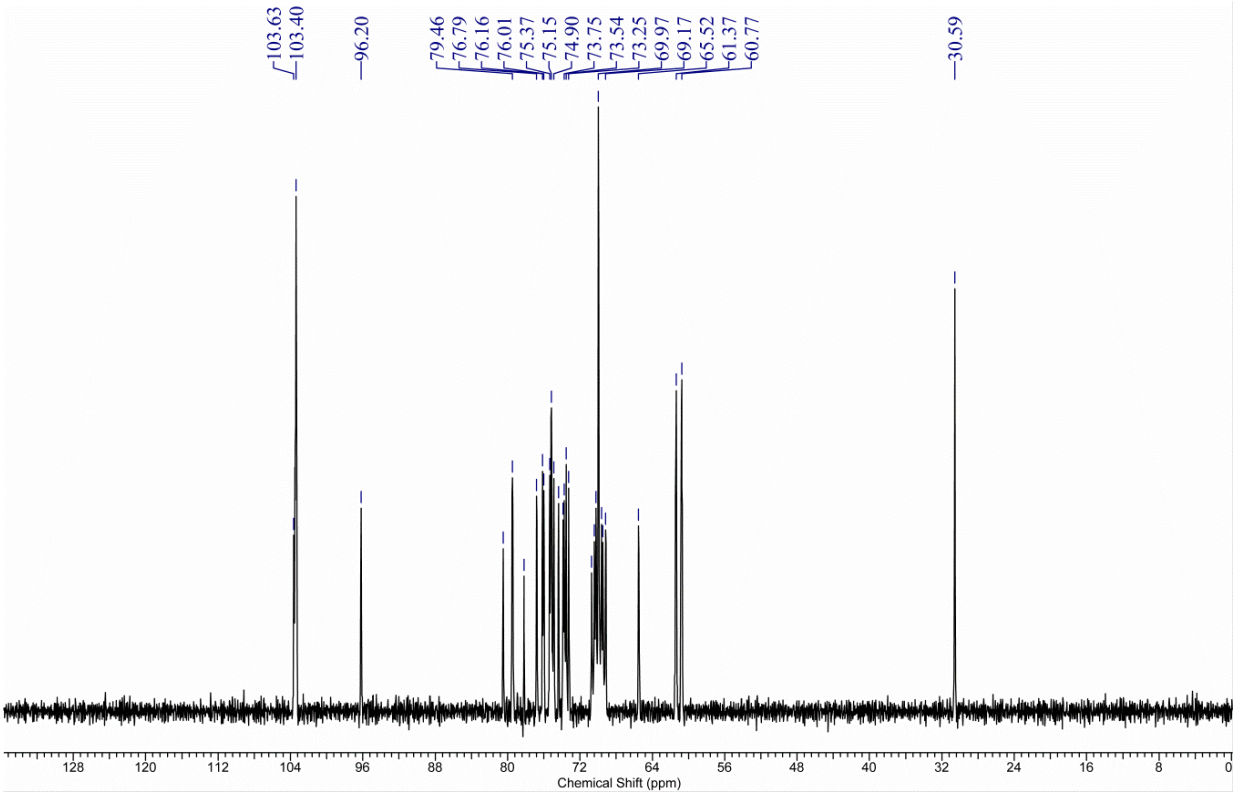
FIGURE 3.3. ^{13}C NMR SPECTRUM OF THE BIOPOLYMER OBTAINED USING THE OPTIMIZED CONDITIONS



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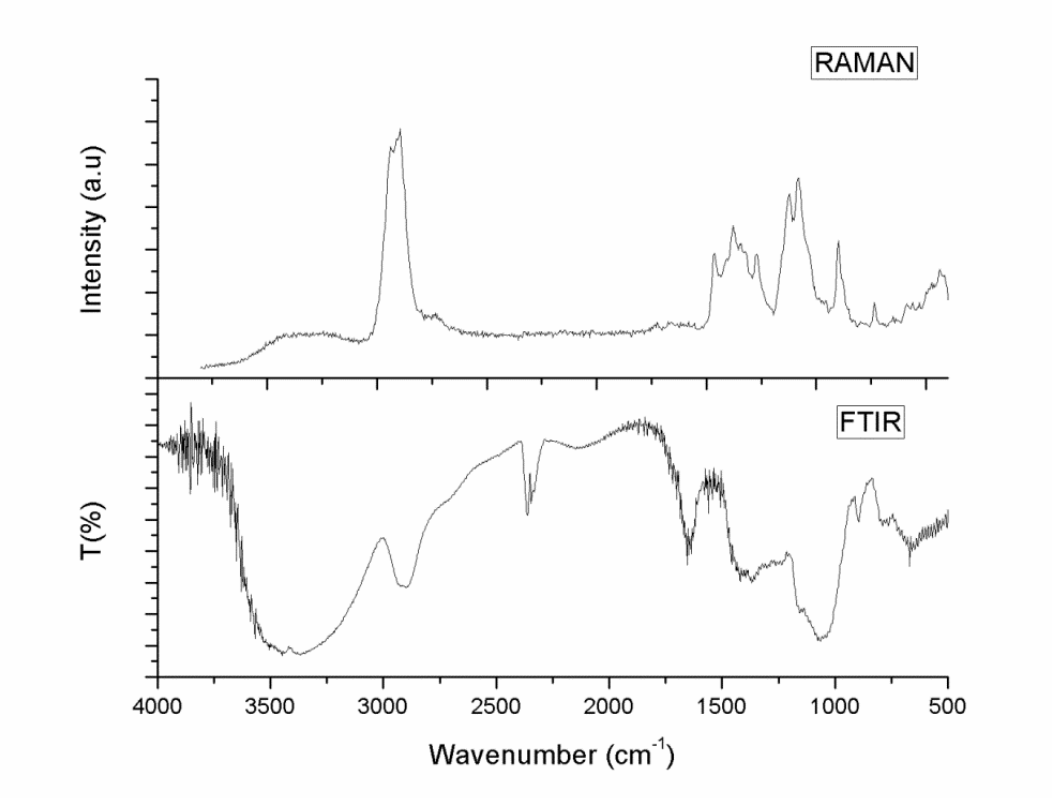
FTIR and Raman spectra of the biopolymer show evidence of a polysaccharidic structure (**Figure 3.4**), as previously reported (BLANDÓN et al., 2016). The broad band around 3480 cm^{-1} in the FTIR spectrum was assigned to the O-H stretching vibrations of the hydroxyl groups typically associated with polysaccharide structures (PIERMARIA et al., 2011; WANG et al., 2008). The peaks around 1400 cm^{-1} and 1270 cm^{-1} in the Raman spectrum indicates CH_2 or CH_3 bending vibrations, and CH_2OH related mode and CH_2 deformation (XING et al., 2016). The peaks around 1038 cm^{-1} in the FTIR spectrum and 1080 in the Raman spectrum could also 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 (CAEL; KOENIG; BLACKWELL, 1973;

SEMENIUC, 2013). Finally, the vibration bands around 895 cm^{-1} indicate the presence of β-glycosidic linkages (CHEN et al., 2015) and around 736 cm^{-1} in the Raman spectrum, indicated C-H bending (AMBROSE et al., 2016).

FIGURE 3.4 FTIR AND RAMAN SPECTRA OF THE BIOPOLYMER OBTAINED USING THE OPTIMIZED CONDITIONS



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3.3.2 Biocidal properties

Kefiran antibacterial activity was reported previously and confirmed by our group (BLANDÓN et al., 2016; RODRIGUES et al., 2005). However, the antimicrobial effect of kefiran was previously determined by indirect techniques such as inhibitory plate haloes or by following optical density. In the present work and for the first time, the antimicrobial activity of kefiran was observed in real time by

Live/Dead BacLight® kit allowing us to establish the effectivity of the polymer biocidal activity. In this sense, seven pathogenic bacteria were tested (**Figure 3.5**).

Among Gram-negative strains, *P. aeruginosa* seems to be more sensitive to kefiran (1.0% for 30 min) treatment than other tested microbes, because of the considerable red stain observed under fluorescence microscopy. Red images indicate a compromised bacterial membrane resulting from cell damage or membrane integrity loss due to contact of the biopolymer with the cell surface. In the case of *E. coli*, a fifty percent of the population was affected just after 30-min incubation with kefiran, but most of the cells were found dead after one-hour polymer treatment. On the other hand, Gram-positive *S. aereus* exhibited a lower kefiran bactericidal effect after 30-min incubation compared to Gram-negative bacteria (observed as green spots). This fact could be attributed to the presence of a bacterial cell wall composed of peptidoglycan reducing kefiran diffusion to the cell membrane. However, the presence of deteriorated bacteria was noticed in the images after 1 h of polymer incubation under the same experimental conditions.

Considering the potential aplication of kefiran as a biocontrol in the food industry, the bioactivity was tested against several *S. typhimurium* strains with different antiobiotic resistant markers. **Figure 3.6** shows that the type strain of *Salmonella typhimurium LT2* was less susceptible to kefiran treatment and only after one hour a small population of dead bacteria began to appear, probably because of the reported antibiotic multiresistance to streptomycin, rifampicin, and nalidixic acid of this strain. Among the clinical isolates, *S typhimurium* CQ27 (resistant to amikacin and gentamicin) was the most suceptible, while *S. typhimurium* CQ28 (resistant to amikacin, gentamicin and nitrofurantoin) seemed to be resistant to the kefiran treatment. Finally, the clinical isolate *S. typhimurium* CQ29 (resistant suspect to nitrofurantoin) showed an intermediate behavior, being resistant after a 30 min exposure to kefiran, but red spots in the images showed compromissed bacteria after one - hour incubation.

Although more antimicrobiological assays are needed to establish the mechanism of kefiran action on different strains, the results clearly showed the tendency observed in previous reports, and the time dependence was elucidated

acording to the membrane composition and the presence of resistant genes (BARBOSA et al., 2011; BLANDÓN et al., 2016; RODRIGUES; CARVALHO; SCHNEEDORF, 2005).

FIGURE 3.5 KEFIRAN (1% wt) EFFECT ON BACTERIAL ON VIABILITY AFTER 30 AND 60 MINUTES TREATMENT, TESTED BY LIVE/DEAD BACLIGHT® KIT.GREEN COLOR INDICATES LIVE BACTERIA WHILE RED ARE DEAD ONES.

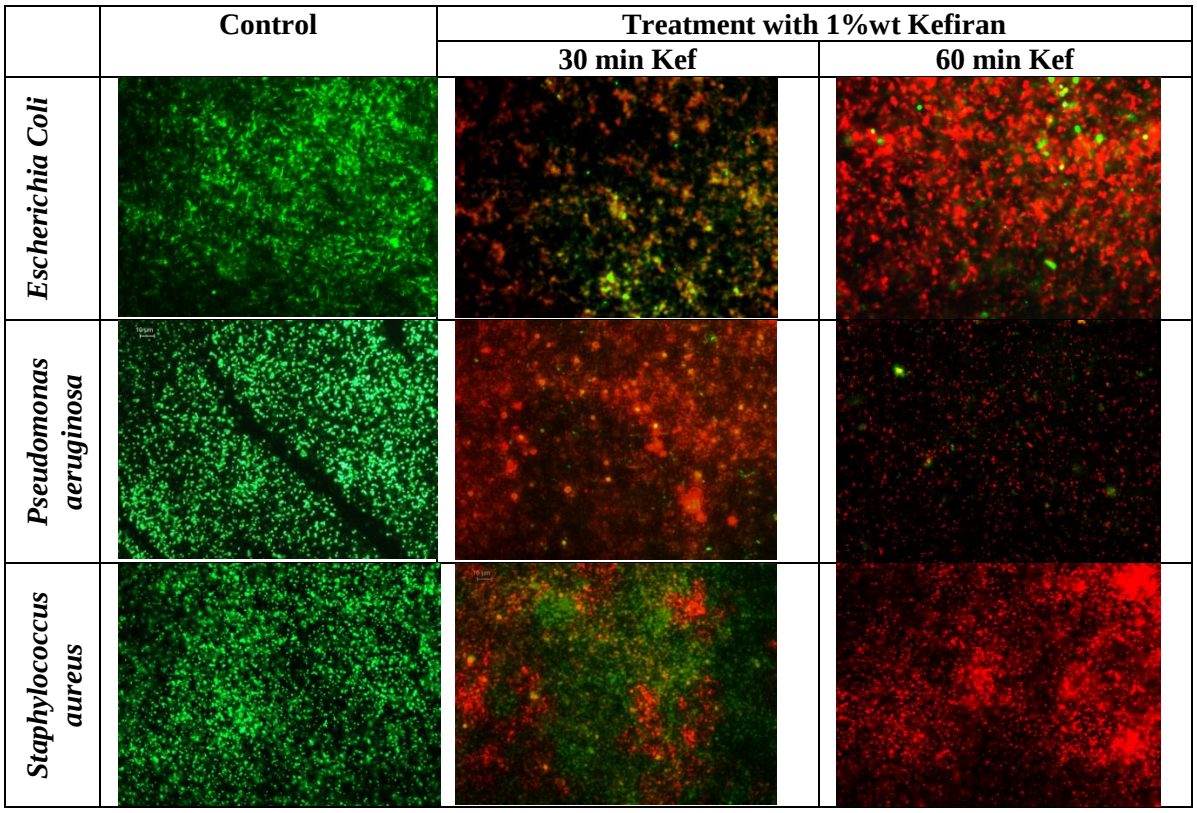

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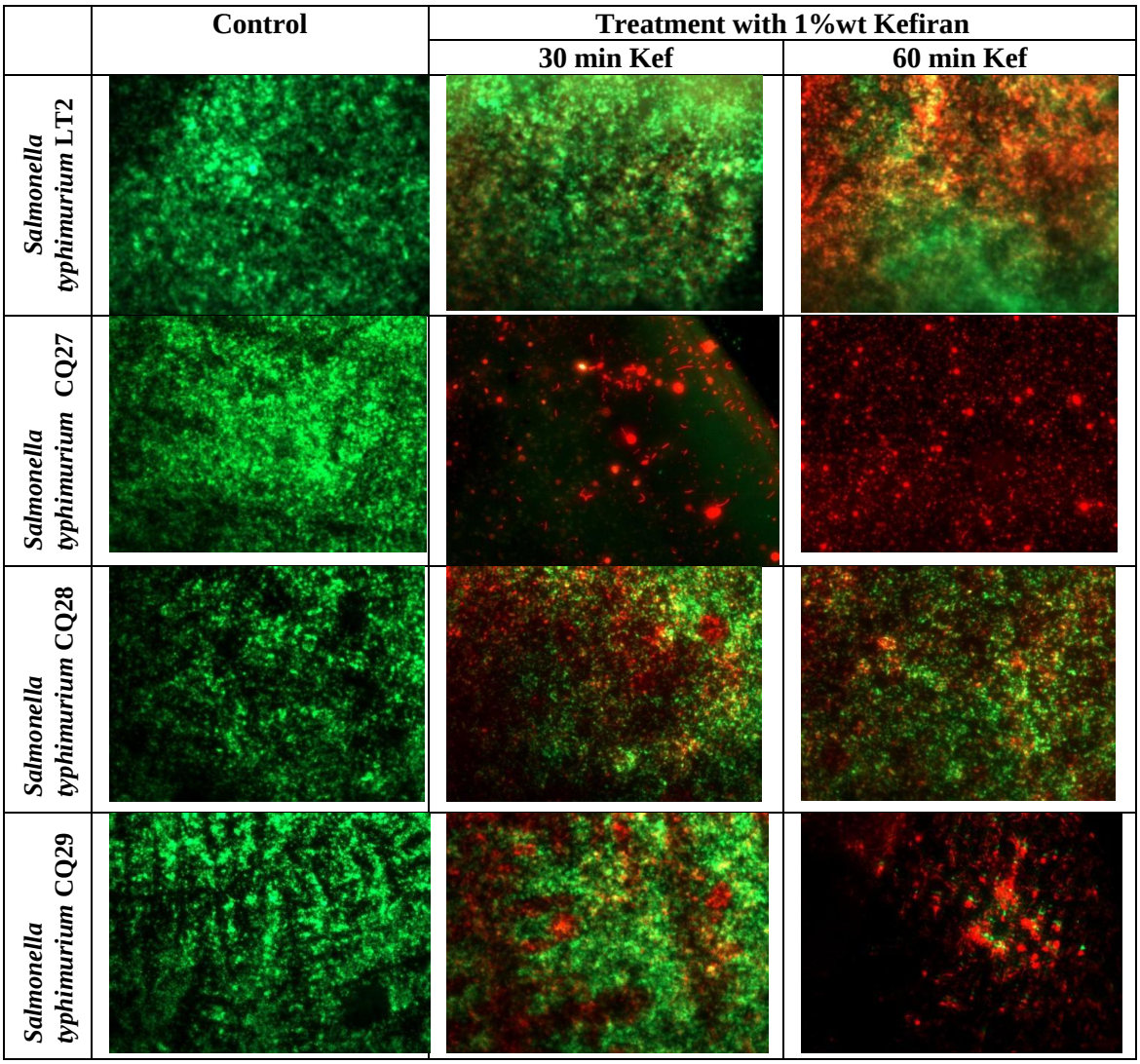
FIGURE 3.6 KEFIRAN (1% wt) EFFECT ON BACTERIAL VIABILITY AFTER 30 AND 60 MINUTES TREATMENT AGAINST DIFFERENT *Salmonella typhimurim* STRAINS, TESTED BY LIVE / DEAD BACLIGHT® KIT. GREEN COLOR INDICATES LIVE BACTERIA WHILE RED ARE DEAD ONES.



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3.4. CONCLUSION

The kinetic analysis of kefiran against selected microbes using fluorescent dyes showed high antimicrobial activity against *P. aeruginosa* > *E. coli* > *S. aureus*, which could suggest high microbiocide activity against Gram-negative microorganisms compared to Gram-positive bacteria. However, the antimicrobial activity of kefiran in different *S. typhimurium* strains showed diverse biocide results probably associated with the presence of antibiotic multiresistance markers. Also, the biocidal properties of the polymer were not altered by the optimization process, and kefiran antimicrobial activity was strain and time dependent. However, more studies on kefiran antimicrobial activity are required to establish the potential mechanisms of the biopolymer action on bacteria and the metabolic role of the antibiotic microbial resistance in kefiran.

4. KEFIRAN – ALGINATE MICROSPHERES FOR ORAL DELIVERY OF CIPROFLOXACIN

4.1 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 (ISLAN; MUKHERJEE; CASTRO, 2015). On the other hand, Cip is associated with gastric and intestinal disorders in humans when the antibiotic is orally administered during long periods (KELESIDIS; FLEISHER; TSIODRAS, 2010). 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 is a linear polysaccharide produced by some brown algae (i.e., *Macrocystis pyrifera*, among others) and some bacteria (i.e., *Pseudomonas aeruginosa*). Alginate 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. Alginate 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 (PAWAR; EDGAR, 2012; RUVINOV; COHEN, 2016). Also, Alginate is biocompatible (THU; ZULFAKAR; NG, 2012), of non thrombogenic nature (RUVINOV; COHEN, 2016), and low cost (LI et al., 2015). Nevertheless, Alginate 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 Alginate gel swelling and to produce matrix structure stabilization, one feasible strategy is to combine Alginate with other polymers (CASTRO et al., 2007).

Kefiran is a water-soluble glucogalactan produced by *Lactobacillus kefiranofaciens* and is present in kefir grains (ZAJŠEK; KOLAR; GORŠEK, 2011). Kefiran 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 (PIERMARIA; DE LA CANAL; ABRAHAM, 2008). On the other hand, it has been reported that Kefiran modulates the gut immune system (VINDEROLA et al., 2006b), protecting epithelial cells against *Bacillus cereus* toxin (MEDRANO; PÉREZ; ABRAHAM, 2008a), and has many beneficial activities, such as antitumor, antibacterial (WANG et al., 2008), anti-inflammatory (RODRIGUES; CARVALHO; SCHNEEDORF, 2005), healing (RODRIGUES et al., 2005) and antioxidant (CHEN et al., 2015). Also, some studies reported the antibiotic activity of kefiran against Gram-positive, Gram-negative bacteria and the yeast *Candida albicans* (ISMAIEL; GHALY; EL-NAGGAR, 2011; PIERMARIA; DE LA CANAL; ABRAHAM, 2008).

Some of the polymers most commonly used in drug delivery systems are collagen, chitosan, cellulose esters and silk fibroin. Specifically, collagen sponges have been used to increase gastric residence time for the delivery of active agents to the stomach, cellulose esters are a good matrix for drug delivery systems due to its little toxicity, stability, high water permeability, film – forming capability and compatibility with most bioactive agents (PAL; PAULSON; ROUSSEAU, 2013). Besides its applications in tissue engineering, silk fibroin has potent applications in drug delivery because its processability in aqueous media and at ambient conditions offers an efficient physical barrier against external proteases and peptidases. On the other hand, promising chitosan based vehicles for drug delivery have been developed, but there are some concerns about the use of chitosan because may cause blood cell aggregation and can activate macrophage and stimulate cytokine production (MERKLE, 2015).

A disadvantage of the use of biopolymers is their susceptibility to microbial contamination (PAL; PAULSON; ROUSSEAU, 2013), a risk that can be diminished employing kefiran. In that way, the aims of the present work were to develop hybrid Kefiran-Alginate 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 Kefiran and Cip against pathogenic bacteria. Analyses of the gel microspheres were performed by infrared spectroscopy (FTIR), scanning electron microscopy (SEM), thermogravimetry, and antimicrobial tests in agar and liquid media.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Ciprofloxacino (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).

4.2.2 Kefiran extraction

The extraction procedure was based on the protocol previously described by PIERMARIA et al., (2009) with some modifications. 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. 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.

4.2.3 Interaction analysis between Ciprofloxacin and Kefiran

4.2.3.1 Binding assay

Based on the previously described protocol with some modifications (ISLAN et al., 2012), solutions of 100 µg/mL Cip and 1.0% (w/v) Kefiran

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 Kefiran was obtained according to the following equation:

$$Binding\ percentage = \frac{Drug\ concentration\ in\ the\ control - Drug\ concentration\ in\ the\ essay}{Drug\ concentration\ in\ the\ control} * 100 \quad (3.1)$$

4.2.3.2 Viscosity determinations

Kinematic viscosities were measured at 2.0% (w/v) Alginate-Kefiran blends as follows: 0.0% Alginate – 2.0% Kefiran, 1.5% Alginate – 0.5% Kefiran, 1.0% Alginate – 1.0% Kefiran, 0.5%Alginate –1.5% Kefiran 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.

4.2.3.3 FTIR

The mixture Kefiran-Ciprofloxacin (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.

4.2.4. Thermal properties

The thermal analyses of Kefiran, Ciprofloxacin, alginate, the mixture of Cip-Kef (1:2) and the mixture of Alg – Kef (1:1) 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.

4.2.5 Encapsulation of ciprofloxacin in microspheres and scanning electron microscopy analysis

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 (Islan et al., 2012). 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:

$$Percentage\ of\ encapsulation = \frac{Drug\ concentration\ in\ the\ calcium\ solution - Drug\ initial\ concentration}{Drug\ concentration\ in\ the\ calcium\ solution} \quad (3.2)$$

4.2.5.1 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.

4.2.5.2 *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.

4.2.6 Antimicrobial assay

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.

For Antimicrobial activity of Cip, Kef and Cip-Kef in liquid medium, 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.

4.3 RESULTS AND DISCUSSION

4.3.1 Interaction analyses

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 4.2**, and the relevant assignation bands are displayed in **Table 4.1.**

TABLE 4.1 FTIR BAND POSITIONS (cm^{-1}) AND ASSIGNMENTS FOR CIP MOLECULE, KEF AND FORMULATION CIP/KEF (2:4) AT pH = 5.

Wavenumbers (cm^{-1})			**Assignments**
Cip	**Kef**	**Cip/Kef**	
1732	-	1718	stretching vibrations of –CO.
1628	-	1629	vibration of phenyl framework conjugated to –COH.
1395	-	1385	Protonation of amine group in the piperazine moiety.
1265	-	1273	Stretching vibration of C-F bond.
-	3417	3380	The O-H stretching vibrations (from water and hydroxyl groups in the kefiran) give rise to absorption bands with maximum intensity.
-	2924	2910	Weak C–H stretching peak of methyl group at 2924 cm^{-1}.
-	1645	1629	The bands in the region 1630 - 1655 cm^{-1} were assigned to the OH bending mode of water molecules.
-	1067	1058	1200 - 800 cm^{-1} is typical for each polysaccharide; this region is dominated by ring vibrations overlapped with stretching vibrations of (C–OH) side groups and (C–O–C) glycosidic band vibration.
-	896	894	890 cm^{-1} indicated the of β-glycosidic linkage in the EPS.
1732	-	1718	stretching vibrations of –COOH.

FIGURE 4.1 METODOLOGY USED FOR KEFIRAN – ALGINATE GELS FOR ORAL DELIVERY OF CIPROFLOXACIN

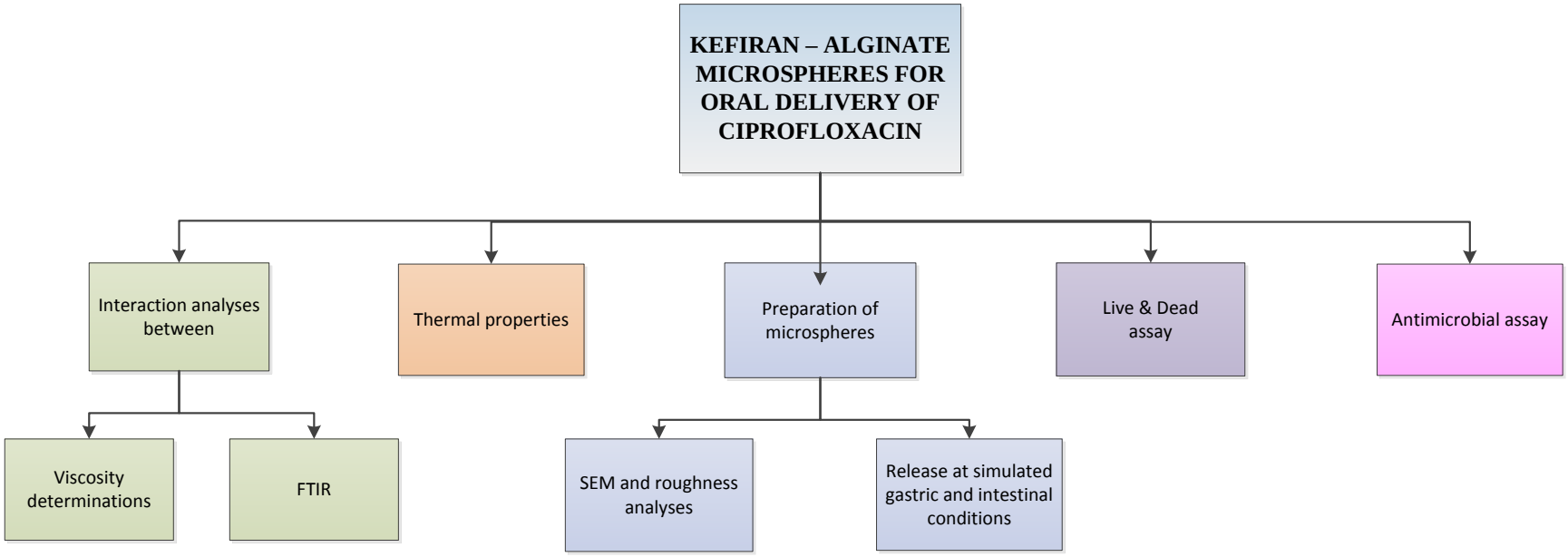

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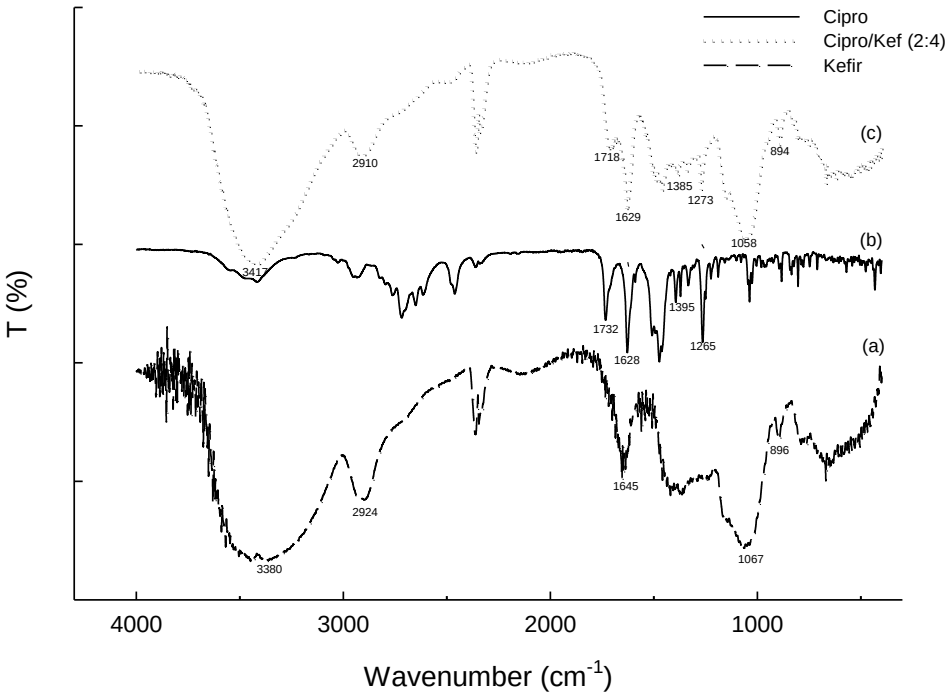
FIGURE 4.2 FTIR SPECTRA OF CIP, KEF, AND THE CIP/KEF FORMULATION (2:4 RATIO) AT pH = 5.0



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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 (DONG; WANG; DU, 2006). 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 (ISLAN et al., 2012). 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 (WANG et al., 2008). 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 (SEMENIUC, 2013). 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 (CHEN et al., 2015). In **Figure 4.1**, 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.

FTIR spectra of Alg and Alg-Kef microspheres (**figure 4.3**) 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 (ISLAN et al., 2012). 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 4.1**) 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.

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 4.2**). 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.

FIGURE 4.3 FTIR SPECTRA OF ALGINATE AND KEFIRAN

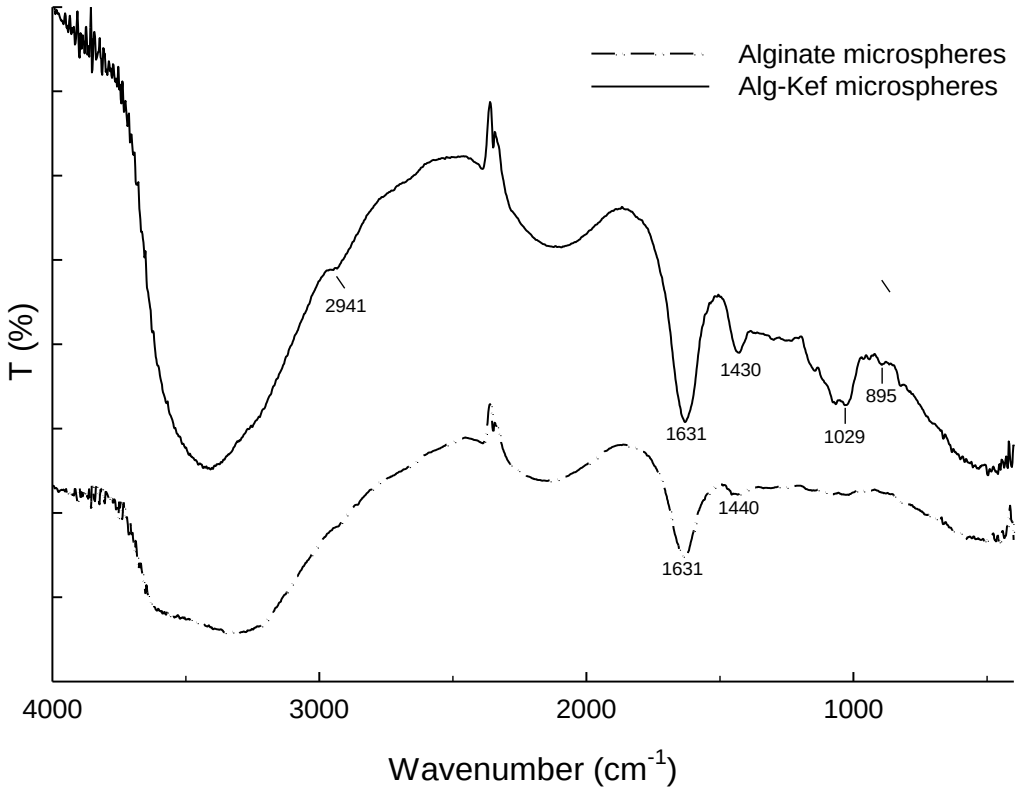

TABLE 4.2 GEL STRENGHT AND VISCOSIMETRIC ANALYSIS OF ALGINATE – KEFIRAN FORMULATIONS OF DIFFERENT

Polymer composition (w/v, %)		**Viscosity (mm^2/S)**	**Gel strength**
Alginate	**Kefiran**		
0.0	2.0	21.3 ± 1.1	ND
0.5	1.5	156.9 ± 1.2	Weak
1.0	1.0	422.1 ± 11.5	Stable
1.5	0.5	550.3 ± 2.4	Strong
2.0	0.0	ND	Strong

Obs.: ND, no detectable.

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4.3.2 Thermal analysis

Thermal properties of Cip, Kef and Cip-Kef (1:2) complex were determined (**Figure 4.4**). 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 (SUROV et al., 2015). In Figure 3.4B, 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 4.4 A**, 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.

FIGURE 4.4 THERMOGRAVIMETRIC CURVES OF CIPROFLOXACIN (…..), KEFIRAN (------) AND CIP – KEF (___). TGA (A) AND DTGA (B)

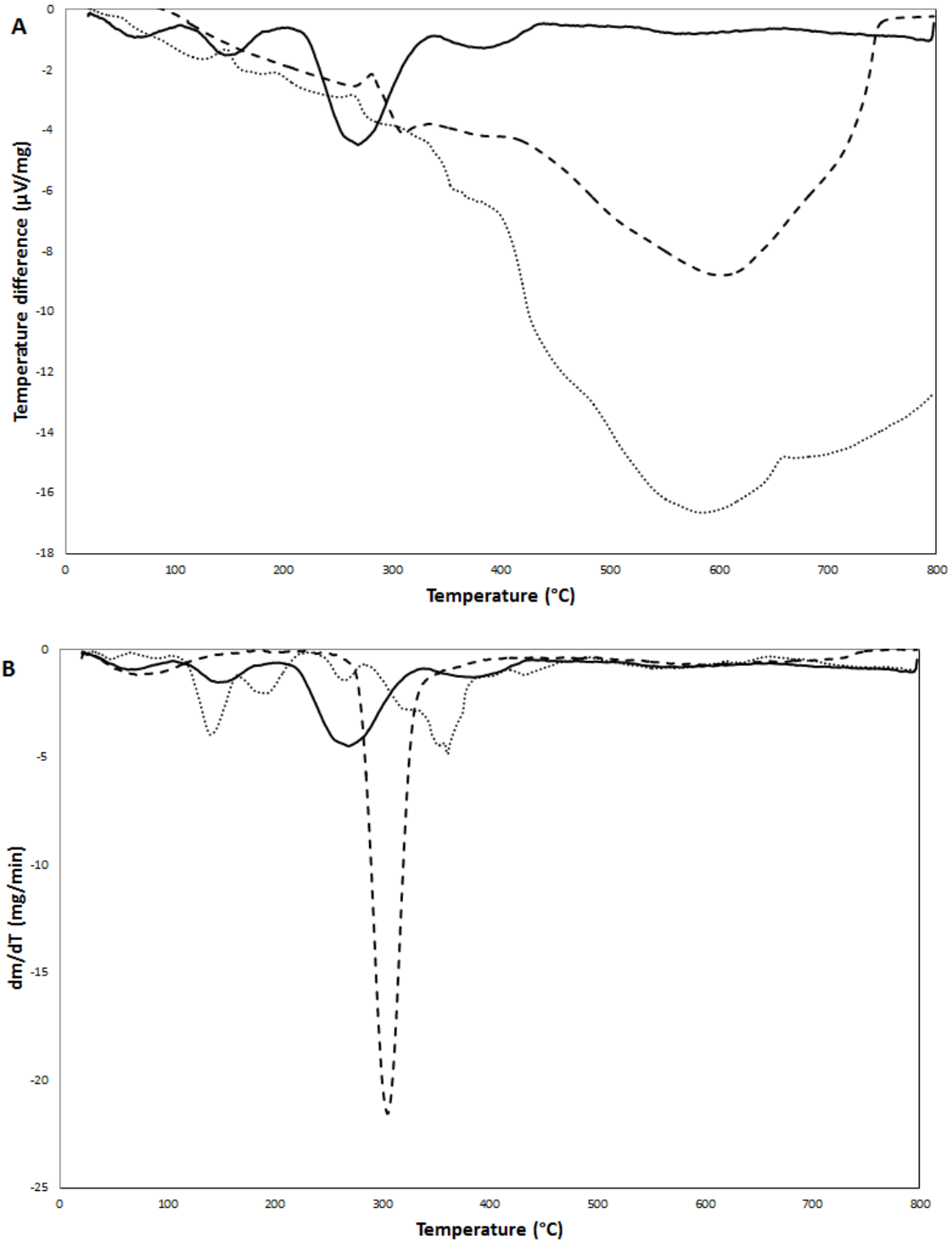

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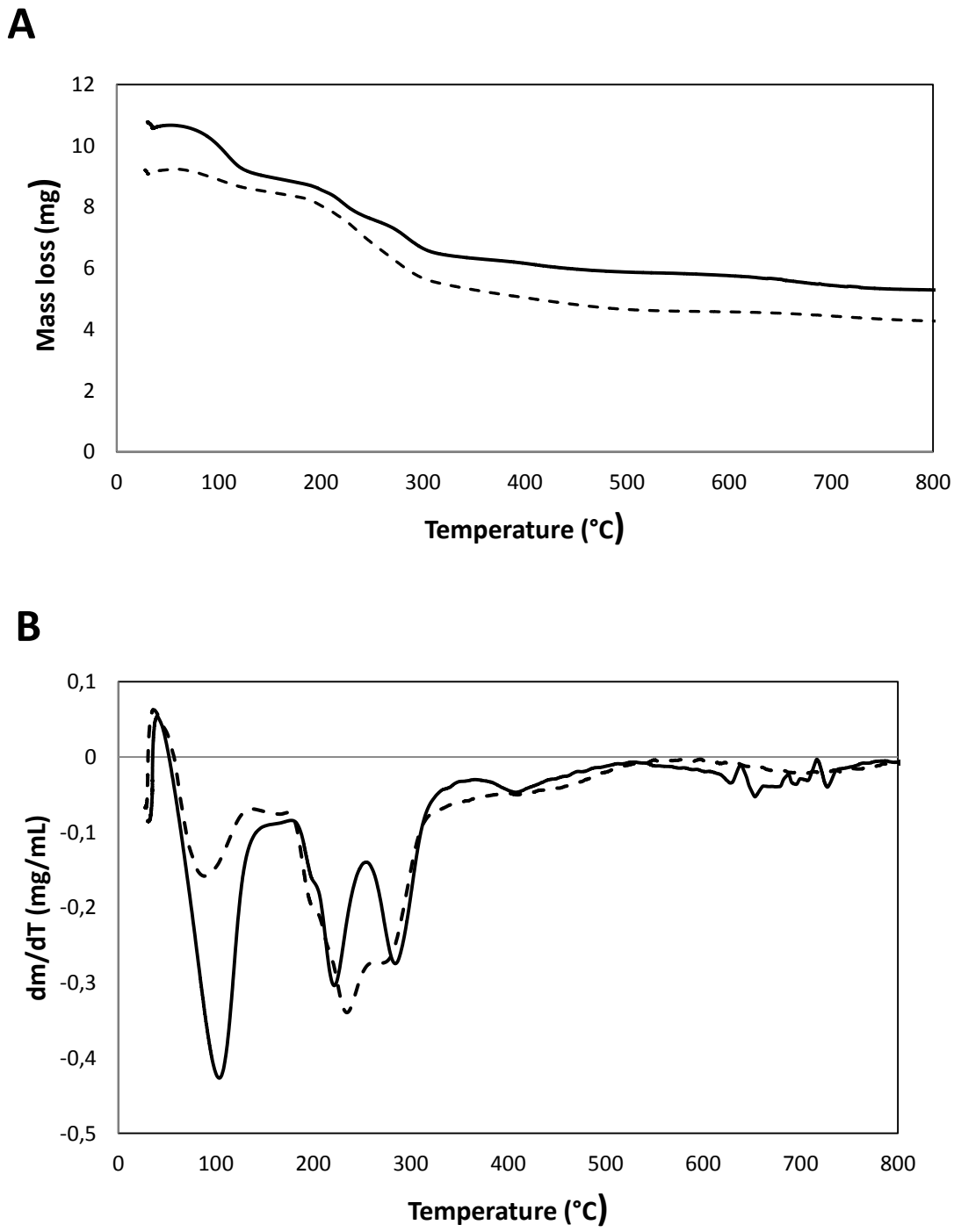
FIGURE 4.5 THERMOGRAVIMETRIC CURVES OF ALGINATE (—) AND ALGINATE – KEFIRAN MIXTURE (---). TGA (A), AND DTGA (B).



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The thermal analysis of Alg and Alg-Kef (1:1) microspheres showed similar weight loss profiles (**Figure 4.5 A**). However, the DTGA graph showed three distinctive endothermic peaks (**Figure 4.5 B**). 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 4.5 B**).

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 4.5 B**).

4.3.3 Encapsulation of ciprofloxacin in microspheres and scanning electron microscopy analysis

Nowadays, drug delivery using microcapsules is an evolving field, with the aim to avoid adverse side effects related to drug concentration in oral delivery (DESAI et al., 2010). 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 4.6A and 4.6B**). 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 4.6C and 4.6D**).

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.

FIGURE 4.6 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).

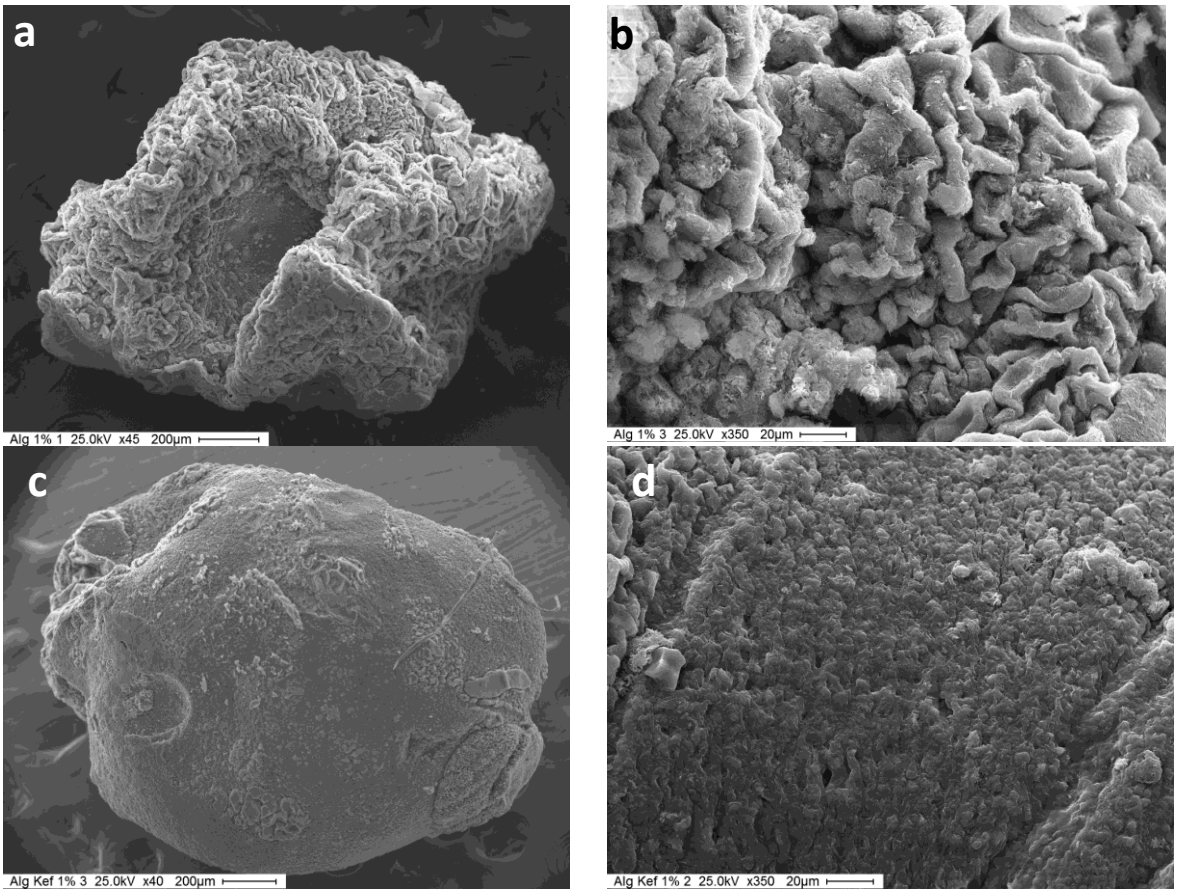

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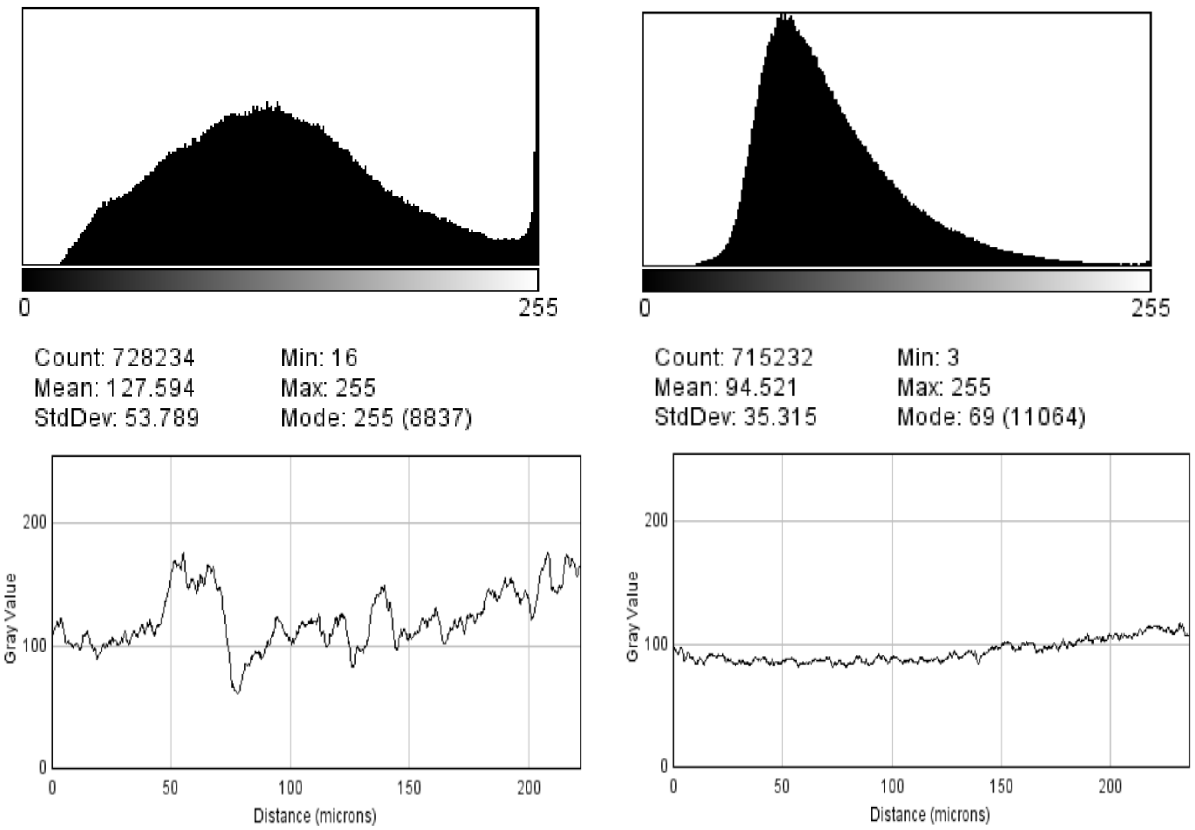
FIGURA 4.7 ROUGHNESS ANALYSIS OF SEM IMAGES OF MICROSPHERES COMPOSED OF: 1.0% ALG AT 45 (LEFT) AND 1.0% ALG – 1.0%KEF (RIGHT). AT THE TOP, THE HISTOGRAMS OF THE BEADS SURFACE ARE OBTAINED AND AT THE BOTTOM THE PLOT PROFILE BY IMAGEJ SOFTWARE



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The results observed by the SEM images were correlated with the ImageJ profiles **(Figure 4.7**). 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.

4.3.4 Release at Simulated Gastric and Intestinal Conditions

In nature release mechanisms can be chemical or physical, always involving a form of diffusion, some of the main factors that affect diffusion are the properties of the polymeric network and the solvent – polymer interaction (PAL; PAULSON; ROUSSEAU, 2013). Drug release kinetics from a microcapsule depends on microcapsule size, calcium chloride and biopolymer concentration (DESAI et al., 2010). Previously, biopolymer concentration was evaluated finding the best combination for kef-alg matrix (1:1). **Figure 4.8** 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 4.7**, indicating that microspheres made with Alg-Kef matrix with a diameter of 800 μm approximately, showed better stability and their spherical morphology was preserved. Additionally, microspheres diameter

4.3.5 Antimicrobial assay

A disadvantage of the use of biopolymers is their susceptibility to microbial contamination (PAL; PAULSON; ROUSSEAU, 2013). In that way, the presence of kef in the matrix help to reduce contamination risk and in this case, could improve Cip action.

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 3.9 and 3.10. 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 (ISMAIEL; GHALY; EL-NAGGAR, 2011). In addition to live &dead test corroborated kef bactericidal effect (figure 3.11) showing the bactericidal effect at 30 and 60 minutes exposition to kef, including the antibiotic resistant bacteria *Salmonella typhimurium* CQ24 (resistant to amikacin and gentamicin), *Salmonella typhimurium* CQ27 (resistant to amikacin, gentamicin and nitrofurantoin) and *Salmonella typhimurium* CQ28 (possible resistant nitrofurantoin).

FIGURE 4.8 KINETIC OF CIPROFLOXACIN RELEASE FROM ALG AND ALG – KEF GEL MICROSPHERES pH 1.2 (A), AND pH 7.4 (B)

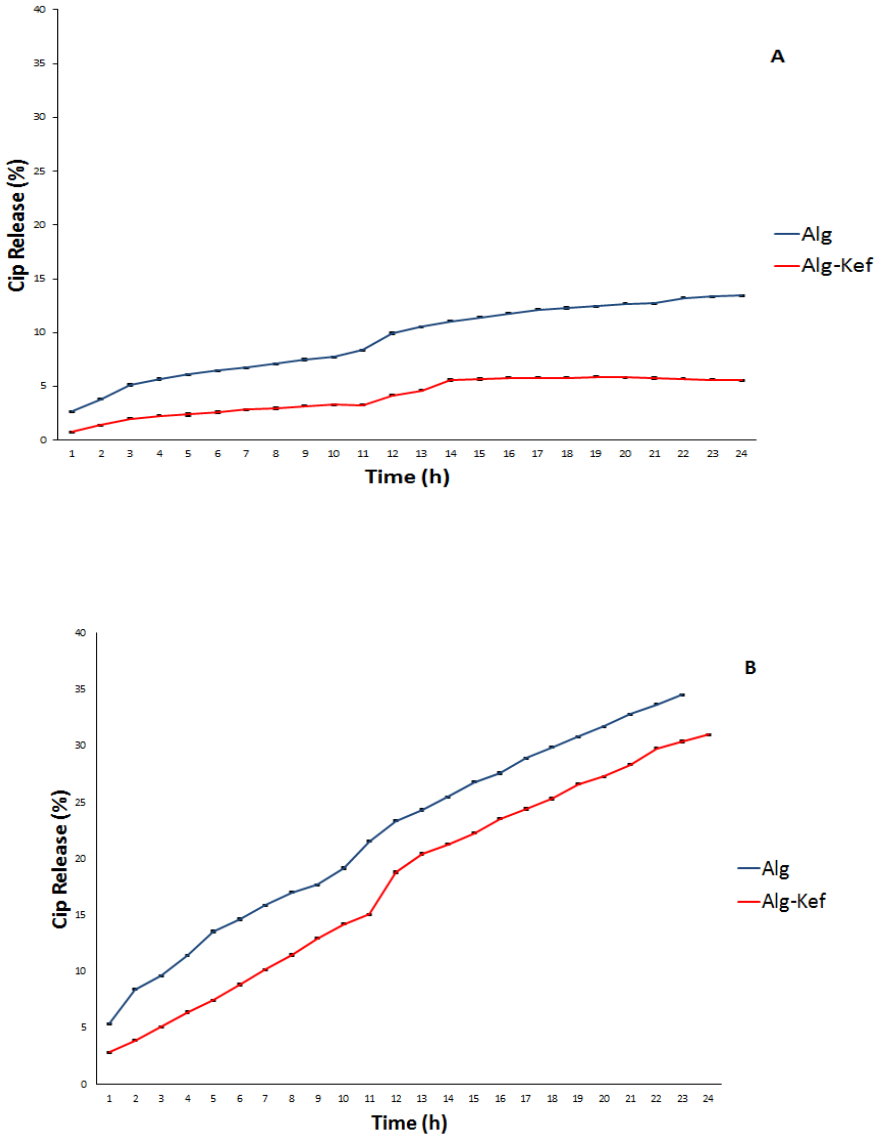

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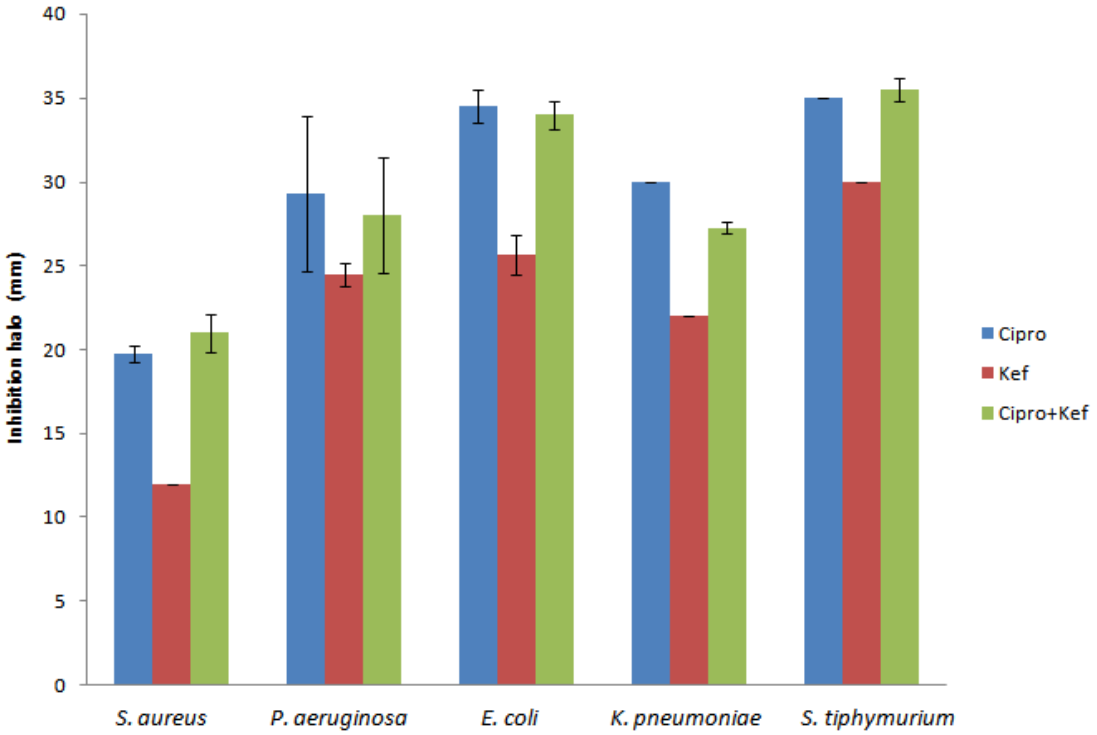
FIGURE 4.9 ANTIBACTERIAL SUSCEPTIBILITY ASSAY OF CIPROFLOXACIN (Cip), KEFIRAN (Kef), AND A MIXTURE OF CIPROFLOXACIN WITH KEFIRAN (Cip – Kef) AGAINST PATHOGENIC BACTERIA S. AUREUS, P. AERUGINOSA, E. COLI, K. PNEUMONIAE, S. TIPHYMURIUM



On the other hand, 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 (**Figure 4.12**). 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 4.9**)

FIGURE 4.10 ANTIBACTERIAL SUSCEPTIBILITY ASSAY OF CIPROFLOXACIN (Cip), KEFIRAN (Kef), AND A MIXTURE OF CIPROFLOXACIN WITH KEFIRAN (Cip – Kef) AGAINST THE PATHOGENIC BACTERIA BACILUS CEREUS

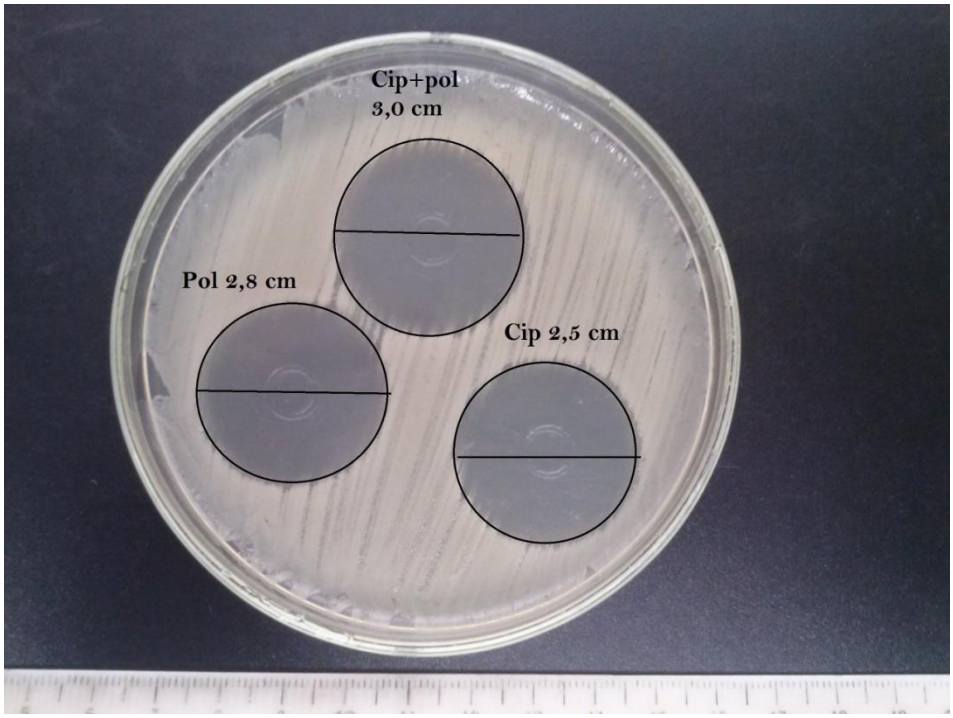


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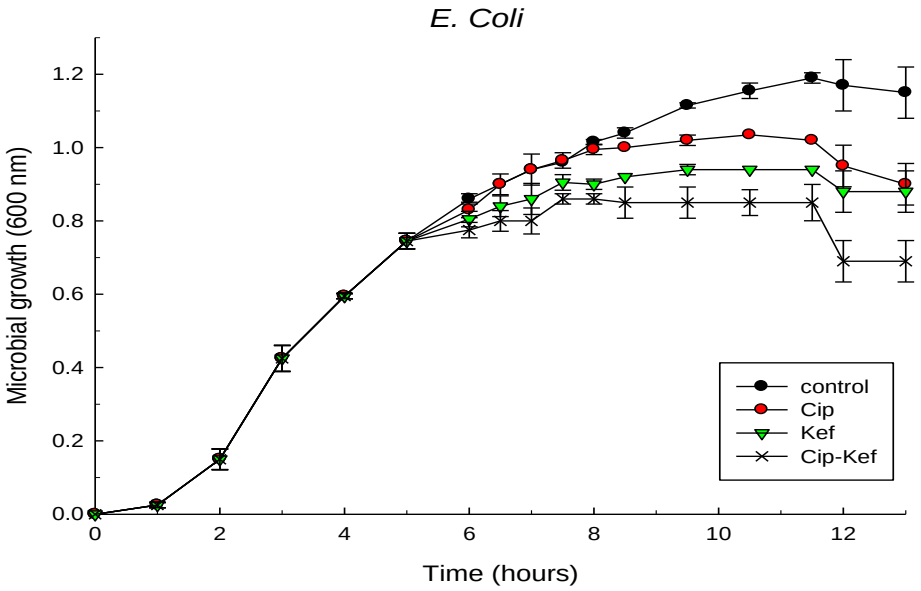
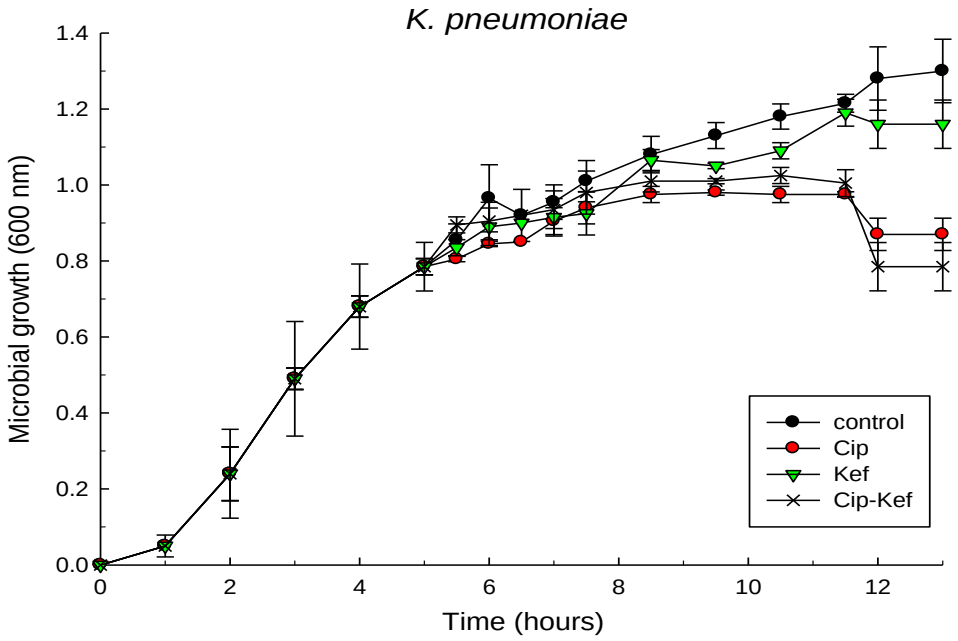
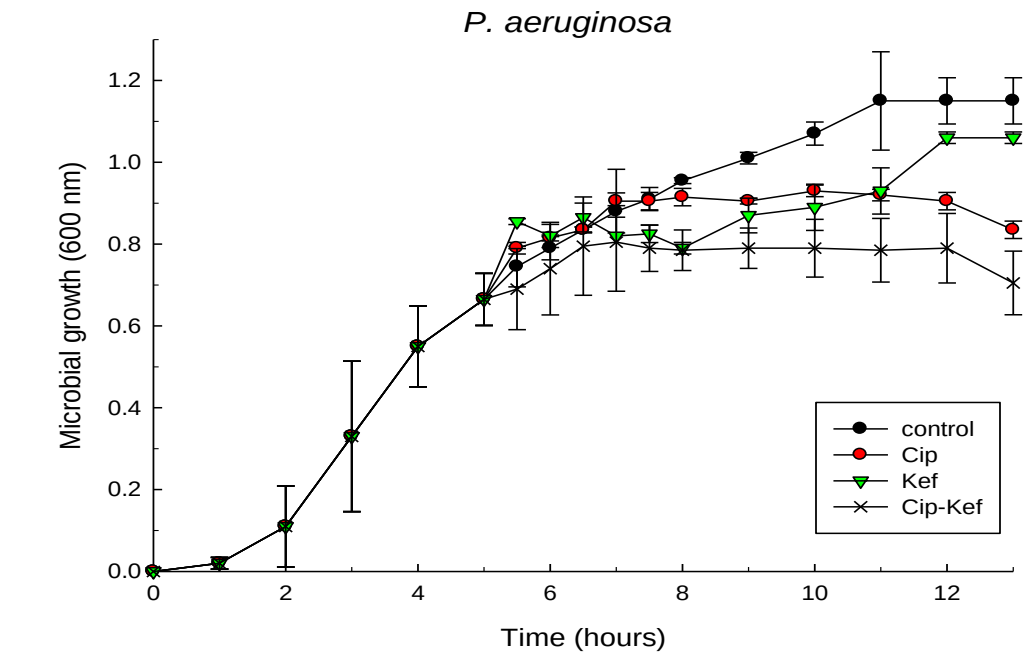
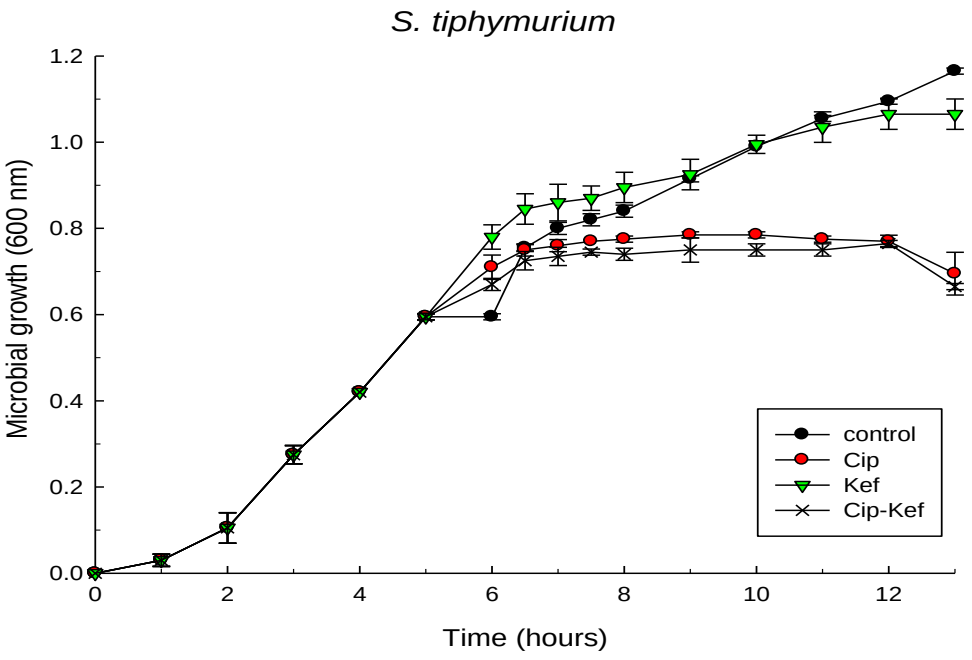
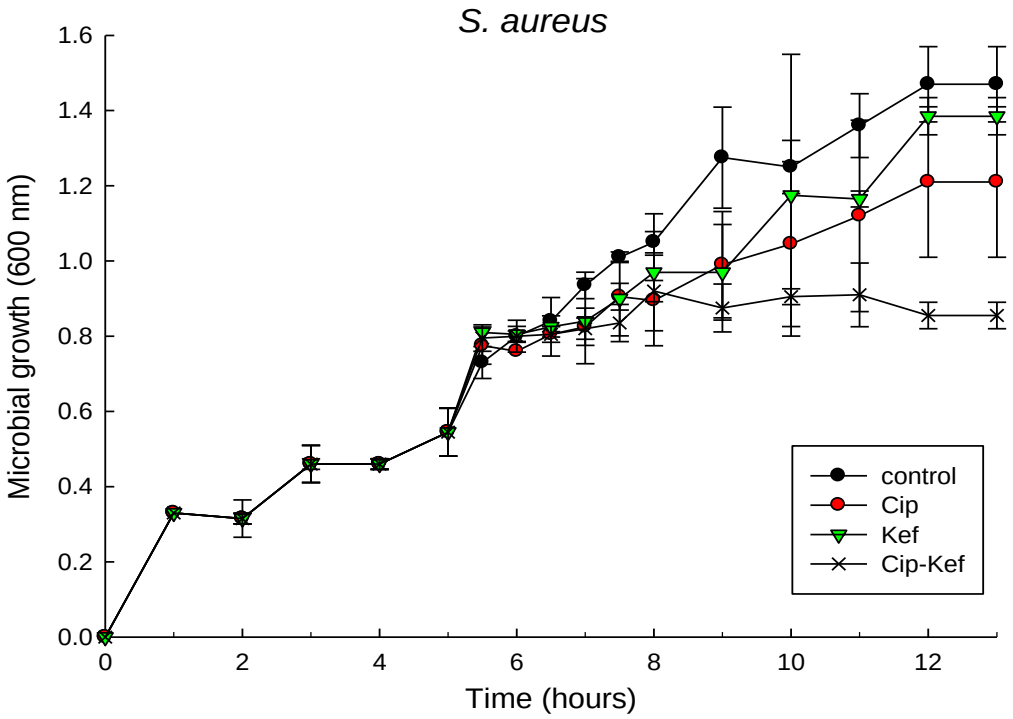
FIGURE 4.11 ANTIMICROBIAL ASSAY OF CIPROFLOXACIN (Cip) KEFIRAN (Kef), AND THE FORMULATION OF KEFIRAN CONTAINING CIPROFLOXACIN (Cip-kef) AGAINST PATHOGENIC BACTERIA: E. COLI, K. PNEUMONIAE, P. AERUGINOSA, S. TYPHIMURIUM, S, AUREUS (Errors: SD≤ 10%, n = 3).






P. aeruginosa
Microbial growth (600 nm)
Time (hours)
control
Cip
Kef
Cip-Kef


S. tiphymurium
Microbial growth (600 nm)
Time (hours)
control
Cip
Kef
Cip-Kef



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4.4 CONCLUSION

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 bonds, 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 (PRADO et al., 2015).

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4. SUPPLEMENTARY MATERIAL

TABLE 6.1 ANOVA ANALYSIS OF THE GROWN MODEL

Analysis of variance (ANOVA)					
Source	**Df**	**Sum Square**	**Mean square**	***F*-value**	***P*-value**
FO (X_1, X_2, X_3)	3	0.02	134.36	29.62	$5.40*10^{-5}$
TWI (X_1, X_2, X_3)	3	$1.00*10^{-3}$	20.27	4.47	0.04
PQ (X_1, X_2, X_3)	3	0.01	178.74	39.40	$1.67*10^{-5}$
Residuals	9	$1.00*10^{-3}$	4.54		
Lack of fit	5	$1.00*10^{-3}$	5.60	1.75	0.30
Pure error	4	0.00	3.20		

FO: First order, TWI: Two way interaction, PQ: Pure quadratic, SO: Second order

5.

TABLE 6.2 ANOVA ANALYSIS OF THE STATISTICAL PARAMETERS OF THE GROWN MODEL

Analysis of variance (ANOVA)				
Source	**Estimate**	**Std. Error**	**T value**	**Pr (>|t|)**
Intercept	-0.06	89.57	-6.19	$1.60*10^{-4}$ ***
X_1	24.14	4.36	5.54	$3.62*10^{-4}$ ***
X_2	64.56	1.26	5.11	$6.32*10^{-4}$ ***
X_3	4.47	0.99	4.53	$1.43*10^{-3}$ **
X_1:X_2	-0.05	0.03	-1.72	0.12
X_1:X_3	-0.08	0.02	-3.16	0.01*
X_2:X_3	$6.26*10^{-3}$	$9.41*10^{-3}$	0.66	0.52
X_1^2	-0.32	0.06	-4.87	$8.85*10^{-4}$ ***
X_2^2	-0.11	0.01	-10.13	$3.22*10^{-6}$ ***
X_3^2	-0.01	0.04	-3.56	$6.15*10^{-3}$ **

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Multiple R-squared: 0.96, Adjusted R-squared: 0.92. F-statistic: 24.5 on 9 and 9 DF, p-value: $2.77*10^{-5}$

TABLE 6.3 ANOVA ANALYSIS OF THE PRODUCTIVITY MODEL

Analysis of variance (ANOVA)					
Source	**Df**	**Sum Square**	**Mean square**	***F*-value**	***P*-value**
FO (X_1, X_2, X_3)	3	0.02	0.01	32.69	$2.00*10^{-4}$
TWI (X_1, X_2, X_3)	3	$1.00*10^{-3}$	0.00	1.10	0.43
PQ (X_1, X_2, X_3)	3	0.01	0.04	20.32	$1.00*10^{-4}$
Residuals	9	$1.00*10^{-3}$	0.00		
Lack of fit	5	$1.00*10^{-3}$	0.00	3.49	0.24
Pure error	4	0.00	0.00		

FO: First order, TWI: Two way interaction, PQ: Pure quadratic, SO: Second order

TABLE 6.4 ANOVA ANALYSIS OF THE STATISTICAL PARAMENTERS PF THE PRODUCTIVITY MODEL

Analysis of variance (ANOVA)				
Source	**Estimate**	**Std. Error**	**T value**	**Pr (>|t|)**
Intercept	-1.01	0.66	-1.52	0.17
X_1	0.10	0.03	3.21	0.02*
X_2	-0.02	$8.77*10^{-3}$	-1.92	0.10
X_3	$-3.38*10^{-3}$	$6.98*10^{-3}$	-0.49	0.64
$X_1:X_2$	$-1.20*10^{-4}$	$2.14*10^{-4}$	-0.56	0.59
$X_1:X_3$	$1.93*10^{-4}$	$1.71*10^{-4}$	1.14	0.29
$X_2:X_3$	$-7.97*10^{-5}$	$6.42*10^{-5}$	-1.24	0.25
X_1^2	$-1.94*10^{-4}$	$4.90*10^{-4}$	-3.96	$5.00*10^{-3}$ **
X_2^2	4.3910^{-4}	$8.15*10^{-5}$	5.39	$1.00*10^{-3}$ **
X_3^2	$-1.51*10^{6}$	$3.19*10^{-5}$	-0.05	0.92

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Multiple R-squared: 0.96, Adjusted R-squared: 0.90. F-statistic: 18.02 on 9 and 7 DF, p-value: $4.79*10^{-4}$