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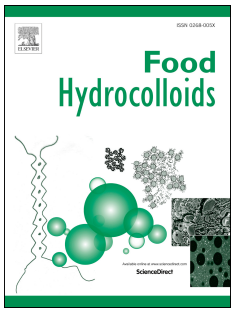

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PII: S0268-005X(18)31084-1

DOI: 10.1016/j.foodhyd.2018.08.057

Reference: FOOHYD 4662

To appear in: *Food Hydrocolloids*

Received Date: 13 June 2018

Revised Date: 31 July 2018

Accepted Date: 4 August 2018

Please cite this article as: Singh, P., Magalhães, S., Alves, L., Antunes, F., Miguel, M., Lindman, Bjö., Medronho, B., Cellulose-based edible films for probiotic entrapment, *Food Hydrocolloids* (2018), doi: https://doi.org/10.1016/j.foodhyd.2018.08.057.

Graphical abstract

“Celullose-Based Edible Films for Probiotic Entrapment”

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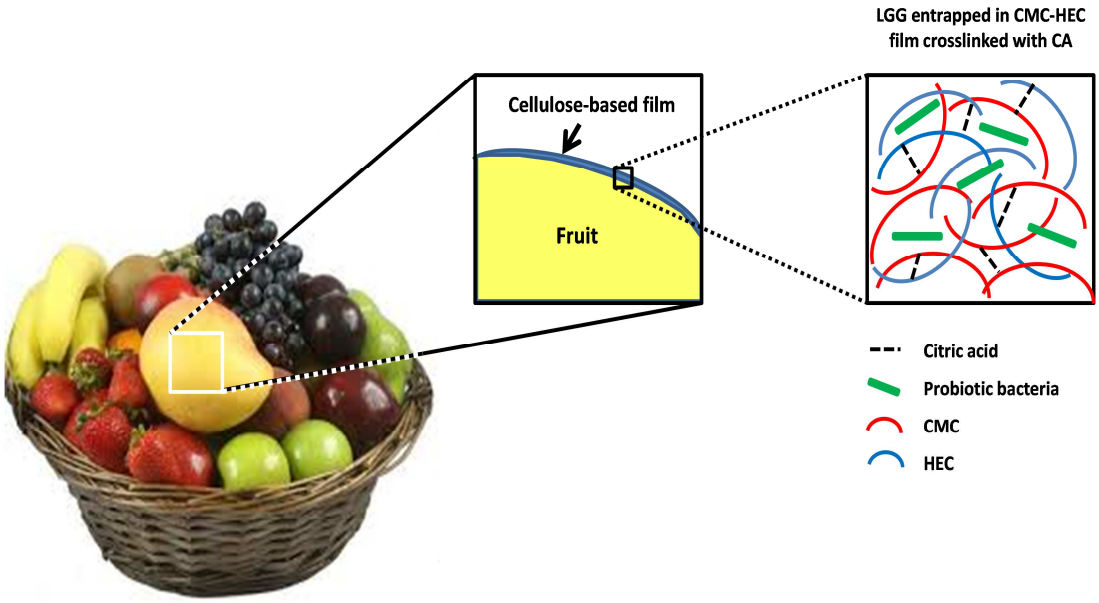

Cellulose-Based Edible Films for Probiotic Entrapment

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Abstract:

Encapsulation with edible films is a promising approach that may solve the disadvantages associated with the use of bioactive compounds as food additives. This is particularly relevant in the case of probiotics, since their stability in food matrices and in the gastrointestinal tract may be rather poor. Therefore, new cellulose-based edible films have been successfully developed and characterized. Sodium carboxymethyl cellulose (CMC) and hydroxyethyl cellulose (HEC) were used for the film preparation and cross-linked with citric acid (CA) under reasonably mild conditions. Model probiotic bacteria (*Lactobacillus rhamnosus* GG) were incorporated in the films either during the film formation and casting or after the film synthesis, via bacteria diffusion and adsorption. The later approach could efficiently entrap and preserve viable bacteria. The mechanical properties and swelling ability could be tuned by varying the HEC/CMC ratio and the amount of CA. Moreover, the surface area and total pore volume of the films considerably decreased after cross-linking. Overall, these novel films are regarded as promising inexpensive and friendly matrices for food protection and packaging applications.

Keywords: Edible films; citric acid; carboxymethyl cellulose; hydroxyethyl cellulose; probiotic bacteria

1. Introduction

Nowadays, the use of edible coatings to improve the quality of food products is an interesting approach employed routinely. Typically, these systems work as physical barriers for gases, moisture and other compounds such as aromas and lipids (Biquet & Labuza, 1988; Cuq, Gontard, & Guilbert, 1995; Kester & Fennema, 1986; Quiros-Sauceda, Ayala-Zavala, Olivas, & Gonzalez-Aguilar, 2014). Apart from the barrier functionality, some innovative applications consider their use to entrap bioactive compounds of interest combined with later controlled release of the cargo at a specific target. These functionalized systems are interesting not only to extend shelf life and reduce the risk of pathogen growth on food products, but also to provide a functional product with health benefits to the consumer (Dhall, 2013; Pothakamury & BarbosaCanovas, 1995). Antioxidants, nutraceuticals, antimicrobials, flavors and probiotics are among the most used bioactive agents (Espitia, Batista, Azeredo, & Otoni, 2016; Muranyi, 2013). Among them, probiotic bacteria are particularly relevant since these living microorganisms are believed to provide beneficial health effects to the host by replenishing the natural gastrointestinal microbiota (Espitia, et al., 2016; Holzapfel, Haberer, Snel, Schillinger, & Huis in't Veld, 1998; Pamer, 2016; Salminen, Ouwehand, Benno, & Lee, 1999). Entrapment of probiotics into polymeric films can protect them from premature degradation and enhance their controlled release (Corona-Hernandez, et al., 2013; Haffner, Diab, & Pasc, 2016; P. Singh, et al., 2017). The practicability of coatings based on edible polysaccharides (e.g. alginate or gellan films) to carry and support viable bifidobacteria in food products was firstly demonstrated by Tapia et al. in fresh-cut fruits (Tapia, et al., 2007). Later, in a related study, *L. acidophilus* probiotics have been immobilized in an alginate-coated surface for strawberries (Moayednia, et al., 2010). Such immobilization of *L. acidophilus* in the alginate-based film effectively protected the bacteria against the low temperature storage, with no significant change in the viability count (Moayednia, et al., 2010).

Among the systems developed so far, a high focus has been given to biopolymers such as chitosan, gums, cellulose, pectins and seaweed extracts (Baldwin, Hagenmaier, & Bai, 2012; Bourtoom, 2008; Espitia, Du, Avena-Bustillos, Soares, & McHugh, 2014). Cellulose is particularly special since it is a highly available, renewable and biocompatible raw material and therefore it emerges as a future key resource for a sustainable planet (Klemm, Heublein, Fink, & Bohn, 2005; P.; Singh, et al., 2015).

The film production often requires the use of harsh chemicals and cross-linking agents, such as formaldehyde-based compounds, that may limit their potential use in food applications. An interesting alternative considers the use of citric acid, CA, as a natural cross-linker. Thus, the resulting materials are acceptable in the food and medical fields, due to their excellent biocompatibility and hydrophilicity (Christian Demitri, et al., 2008; Raucci, et al., 2015). Even if not well documented in the available literature, cellulose systems chemically cross-linked by CA are expected to form three-dimensional networks poorly soluble in water or in biological fluids (Coma, Sebti, Pardon, Pichavant, & Deschamps, 2003; Glusker, 1980; Wang & Chen, 2005; Xie, Liu, & Cui, 2006; Yang & Wang, 1998). These hydrogels have also been suggested as new superabsorbent systems potentially interesting in personal care (i.e. diapers and napkins) or in agriculture and horticulture for strategical water management (C. Demitri, Scalera, Madaghiele, Sannino, & Maffezzoli, 2013; Sannino, Demitri, & Madaghiele, 2009). To the best of our knowledge, the potential use of such systems to entrap probiotic bacteria has never been explored before. Therefore, in the present work, two cellulose derivatives, sodium carboxymethylcellulose (CMC) and hydroxyethylcellulose (HEC) were used for film preparation, cross-linked with CA and loaded with probiotic bacteria. The film formation, structure and mechanical properties were characterized, as well as the viability of the entrapped bacteria under different conditions.

2. Experimental

2.1. Materials and Methods

The cellulose derivatives, CMC (Mw of ca. 250 kDa with a degree of substitution ca. 0.80-0.85) and HEC (Mw of ca. 720 kDa with a molecular substitution of 2.5 mol per mol of cellulose), were purchased from VWR international (Belgium) and Sigma Aldrich (USA), respectively, and used as received. The CA and HCl were obtained from Sigma Aldrich (USA). The chemical structures of CA, CMC and HEC can be found in supporting material section, Figure S1. *Lactobacillus rhamnosus* GG LMG 18243 (LGG) was bought from the Belgian Coordinated Collection of Microorganisms. The MRS broth pH 6.4 and MRS agar pH 5.7 were obtained from VWR International. The PBS buffer was prepared in the laboratory using disodium hydrogen phosphate, sodium chloride, potassium chloride and potassium dihydrogen phosphate, all with analytical grade from Sigma Aldrich (USA). The Live/Dead® BacLight™ Bacterial Viability Kit

L7012 was purchased from Thermofisher Scientific, USA. Milli-Q water (18.2 MΩ.cm-1 at 25 °C, MQ) was used for the preparation of all samples.

2.2. Film formation

The films were obtained by mixing different amounts of CMC, HEC and CA in Milli-Q water until full dissolution was achieved. The total polymer concentration was set to 2 wt% while the ratio between the cellulose derivatives was systematically varied. The CA (cross-linking agent) amount ranged from 5 to 10 wt% based on the total polymer concentration. Lower amounts of CA were also tested but the obtained films were too fragile for handling. After obtaining a clear solution, 15 g of it were poured into Petri dishes and allowed to cure in an oven at 50 °C for 15 h. in such conditions, the cross-linking reaction between the cellulose derivatives and CA occurs. The film thickness depends on the amount of CA and presents an average value of ca. 0.12 mm. It should be noted that this is a fairly mild procedure in comparison with the standard cross-linking approach reported elsewhere (i.e. 80 °C for 24 h) (Christian Demitri, et al., 2008).

2.3. Lactobacillus rhamnosus GG culture and entrapment in the films

All culture media and buffers were autoclaved for sterilization at 121 °C for 15 min. Freeze-dried cells of *Lactobacillus rhamnosus* GG were rehydrated in 5 mL MRS broth and incubated at 37 °C for 40 h in a CO_2 incubator. Cell growth was followed for two days and, in order to evaluate the purity of the bacteria, one inoculation loop with bacteria was streaked on a MRS agar plate while a plate with pure sterilized culture was treated as control. The optical density was measured with a UV/vis spectrophotometer.

The incorporation of bacteria into the films was performed in two ways: 1) soaking the cross-linked films in a bacteria medium for 30 min at room temperature and removing the excess of medium to allow a moderate drying at 37 °C for 30 min. 2) mixing the bacteria inoculum in the CMC/HEC/CA mixture before casting and curing. While in the former approach the bacteria are expected to diffuse and adsorb into the film during its swelling and solvent uptake, in the latter the bacteria are expected to be entrapped in the cellulosic polymer matrix already before casting and curing. The results from both methodologies will be discussed later.

2.4. Evaluation of the Viability of Lactobacillus rhamnosus GG.

To estimate the viable counts and matrix effect, the encapsulated bacteria were released by re-suspending ca. 0.01 g of film either in a PBS buffer solution (pH 7.4) or in HCl aqueous solution (pH 2.4) at 37 °C for 30 min, under stirring. Sequential dilutions were performed following the Miles and Misra approach to count the number of viable bacteria (Hedges, 2002; Miles, Misra, & Irwin, 1938). All different dilutions were plated in triplicates and kept in a CO_2 incubator for 48 h before the bacterial colony was counted using a colony reader. The experiments were performed in Faster BH-EN and BHG Class II Microbiological Safety Cabinets.

2.5. Rheology

The rheological measurements on the gel-like films were carried out on a HAAKE MARS III rheometer (Thermo Fisher Scientific, Germany) set with a plate-plate geometry (35 mm, 0.2 mm gap). A Peltier unit was used to ensure strict temperature control, which was set at 20.0 ± 0.1 °C. The storage modulus, G', was accessed by performing dynamic oscillatory experiments from 10 to 0.01 Hz, at a constant stress of 5 Pa.

2.6. Fourier transform infrared (FTIR) spectroscopy

The cross-linking between the cellulose derivatives and CA was investigated by FTIR at 25 °C with an ATR-FTIR spectrophotometer Thermo Nicolet IR300 (USA), using a universal ATR sampling accessory. The FTIR spectral analysis was performed between 400–4000 cm^{-1}. A total of 256 scans were performed in the transmission mode for each spectrum, with a resolution of 1 cm^{-1}.

2.7. Thermal gravimetric analysis (TGA)

TGA was performed on a TG 209 F3Tarsus thermogravimetric analyzer (Netzsch Instruments). The films (ca. 8-10 mg) were weighed in alumina pans and heated from 30 to 650 °C with a constant heating rate of 10 °C min^{-1} under N_2 atmosphere.

2.8. Water uptake

The equilibrium swelling measurements for all chemical films were carried out in Milli-Q water or in aqueous media at different pHs. The percentage of swelling (S) was estimated by weighing a ca. 1 g square-shaped specimen before and after its immersion

in the aqueous media for about 24 h. The films were observed to reach their swelling equilibrium within this period. S is defined as follows:

$$S = \frac{(Ws - Wd)}{Wd} \times 100$$

where W_s is the weight of the swollen film and W_d is the weight of the dried sample.

2.9. Scanning Electron Microscopy (SEM)

A VEGA3 SBH from TESCAN scanning electron microscope, equipped with a selected energy dispersive X-ray microanalyser, was used to observe the superficial morphology of the cross-linked dried films. Briefly, the freeze-dried samples were deposited directly over the carbon tape on the support and sputtered with an approximately 6 nm thin Au/Pd film by cathodic pulverization using a SPI Module Sputter Coater before SEM analysis. The accelerating voltage ranged from 5 to 15 kV.

2.10. Mechanical analysis

A Texture Analyzer TA.XT Plus (Stable Micro Systems Ltd., Surrey, UK) was used to access the mechanical properties of the films following the ASTM D882-12 standard. Tensile grips with 35 mm were used to hold the specimens which consisted of 40 mm x 40 mm films. Up to four repetitions were made for each film formulation using a grip speed of 1.0 mm/s.

2.11. Surface area and porosimetry

The surface area and the total pore volume were determined by N_2 gas adsorption isotherms using an ASAP 2000, from Micromeritics and considering the BET (Brunauer, Emmettand Teller) model for evaluation (Brunauer, Emmett, & Teller, 1938; Sing, 2001).

2.12. Statistical analysis

Most of the experiments were performed in triplicate and data was subjected to one-way analysis of variance (ANOVA). Multiple comparisons were performed by LSD test. Statistical significance was set at $p < 0.05$ using SPSS (SPSS Inc, USA).

3. Results and discussion

3.1. Characterization of the cellulose-based films

Photographs of the films formed following the procedure described in the experimental section are presented in Figure 1. The films made from HEC (#1) and CMC (#2) alone are reasonably flexible and transparent. The same is observed for the physical mixture of HEC and CMC (#3). On the other hand, the addition of CA followed by cross-linking resulted in less flexible and brittle films. The transparency is also observed to decrease with the amount of CA. While the HEC (#1), CMC (#2) and their physical mixture (#3) dissolve in water, the cross-linked films (#4 and #5) do not.

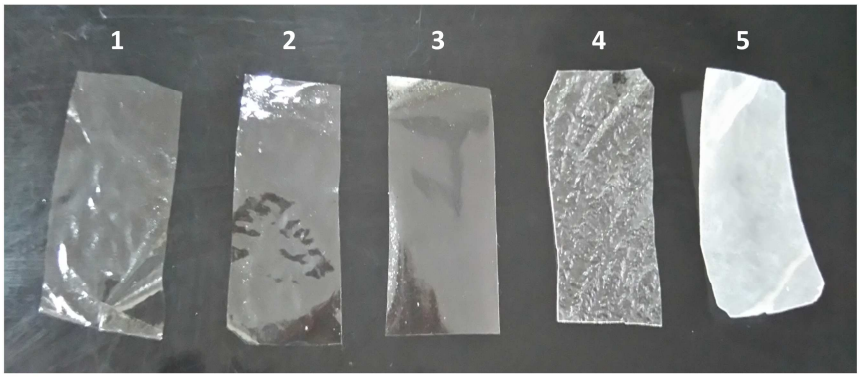

Figure 1. Photograph of cellulose-based film strips after being casted and dried in Petri dishes. 1) 2 wt% HEC; 2) 2 wt% CMC; 3) 1 wt% HEC + 1 wt% CMC; 4) 1 wt% HEC + 1 wt% CMC + 5 wt% CA; 5) 1 wt% HEC+ 1 wt% CMC + 10 wt% CA.

The synthesized films were further characterized by FTIR spectroscopy (Figure S2 in supporting material section). The spectra of the films were compared to those of the individual compounds, highlighting the region of interest between 1100 and 1900cm^{-1}. The cross-linking mechanism between polycarboxylic acids and cellulose has been proposed to occur firstly by molecular dehydration of the acid followed by the reaction of esterification (Figure S3 in the supporting material section). Indeed, for the cross-linked films, a new band is observed at 1722 cm^{-1} (dashed line in Figure S2) which is assigned to the stretching mode of the C=O ester bond, expected to appear at higher frequencies than for carboxylic acids. All the remaining bands of CMC and HEC are essentially kept after reaction. Overall, the FTIR data supports the success of the esterification reaction between the cellulose derivatives and CA.

The obtained films (cross-linked or not) were subjected to tensile tests in order to study their mechanical properties. In Figure 2 it is possible to observe that the CMC film presents the higher tensile strength (maximum load at break) while the mixture of CMC and HEC cross-linked with 5% CA is the weaker one. On the other hand, a significant difference in the tensile strength ($p < 0.05$) is observed when increasing the CA content to 10 wt%. These results should not be overrated since the film thickness slightly changes with the CA amount and this is expected to influence the mechanical properties. It is also interesting to note that the tensile strength and elongation to break follow an opposite trend, as reported in related systems (Borges, et al., 2004). Increasing the CA content improves the tensile strength but decreases the elongation to break (compare films #4 and #5). Overall, the mechanical properties obtained are promising even without the use of any plasticizer.

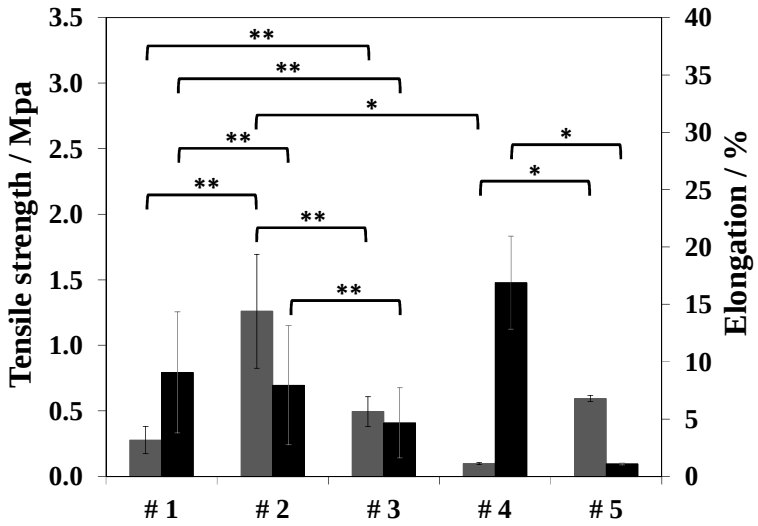

Figure 2. Elongation to break (black bars) and tensile strength (grey bars) of the cellulose-based films. 1) 2 wt% HEC; 2) 2 wt% CMC; 3) 1 wt% HEC + 1 wt% CMC; 4) 1 wt% HEC + 1 wt% CMC + 5 wt% CA; 5) 1 wt% HEC+ 1 wt% CMC + 10 wt% CA. The data is presented as means ± SD. *Significance at $p \leq 0.05$, ** not significant.

The BET surface area was also assessed and it is summarized in Table 1. The first observation is that the non cross-linked films present a substantial higher BET surface area when compared with the cross-linked films. Moreover, increasing the CA amount decreases the BET surface area. This decrease is certainly related with the denser matrix formation due to the cross-linking reaction. The total pore volume decreased ca. 50% when the films were cross-linked with 5 wt% CA. For the films with 10 wt% CA, the

pore volume drop is even more remarkable, ca. 95% less in comparison to the non cross-linked film.

Table 1. N_2 surface area and total pore volume of the novel cellulose-based films.

Film composition	BET surface area ($m^2.g^{-1}$)	Total pore volume (cm^3/g)
2 wt% HEC	4.079	0.022069
2 wt% CMC	3.142	0.002636
1 wt% HEC + 1 wt% CMC	5.934	0.005838
1 wt% HEC + 1 wt% CMC + 5 wt% CA	0.782	0.003025
1 wt% HEC + 1 wt% CMC + 10 wt% CA	0.196	0.000311

The amount of water adsorbed by the different films was measured after 24 h of equilibration in aqueous media at different pHs (Table 2). Note that all the physical films, i.e. without chemical cross-linking, dissolved in water after a couple of minutes and thus their average swelling is not possible to report. It was observed that all the tested systems, except the cross-linked HEC film, present a rather similar trend with a low swelling capacity at low pH (ca. pH 2) and two to three-fold swelling increment at high pH, ca. pH 10. The integrity of the cross-linked films was essentially preserved regardless of the pH used. All results are statisticaly different ($p < 0.05$) whitin the same pH.

Table 2. Average equilibrium swelling of the cross-linked films in water, at 25 °C at different pHs. Mean values with the same letter are not significantly different ($p < 0.05$).

Sample	Average swelling / %		
	pH 2	**pH 6**	**pH 10**
CMC + 5% CA	83 ± 6.2^a	236 ± 10.8	273 ± 7.5^c
HEC + 5% CA	2578 ± 13.7	2630 ± 8.4^b	2650 ± 10.0^b
CMC + HEC + 5% CA	171 ± 8.5	280 ± 15.0^c	312 ± 11.8
CMC + HEC + 10% CA	59 ± 4.0	86 ± 9.0^a	93 ± 2.5^a

The cross-linked HEC film presents the highest swellability, ca. 20 times more than cellophane (Stamm, 1956). The drawback of such a huge water uptake is that the highly

swollen film behaves as a weak gel (rheology data in Figure 3) and thus its handling is rather dificult in contrast to other formulations. Comparing the films containing only CMC and CMC/HEC, both cross-linked with 5% CA, it is interesting to observe an increase in swelling when HEC is present. The data suggests that CMC is more reactive under the present conditions, leading to a better cross-linking when compared with the HEC film. Previous work reported a different trend where the higher HEC reactivity, in comparison with CMC, was attributed to lower sterical hindrance (Christian Demitri, et al., 2008). On the other hand, since the film forming solution has a pH ca. 4, CMC is charged and swelling may induce some structural changes/rearrangements thus allowing a more efficient cross-linking. Another interesting observation is related to the lower swelling of the films when the CA concentration increases. Most likely, the increment in CA concentration leads to an increase in the cross-linking density and netowork regidity, thus reducing the swelling ability. This observation is also in agreeemtent with the observed low BET surface area and total pore size of the films cross-linked with 10 wt% CA.

If not protected, the growth and survival of probiotics during their transit in the gastrointestinal tract may be seriously compromised. Moreover, the inherent physical and chemical properties of food carriers, such as the low pH found in, for instance, fruit juices, salads and condiments, may represent an additional problem for probiotic survival (Rodgers, 2007). The data suggests that these cellulose-based films, cross-linked with CA, may be a viable alternative for entrapping viable probiotics and protect them from any unfavorable food matrix properties or even from the harsh gastrointestinal conditions.

As previously mentioned, after soaking and swelling the films for 24 h in aqueous media they behave as jelly-like materials. The rheological mechanical spectra are represented in Figure 4.

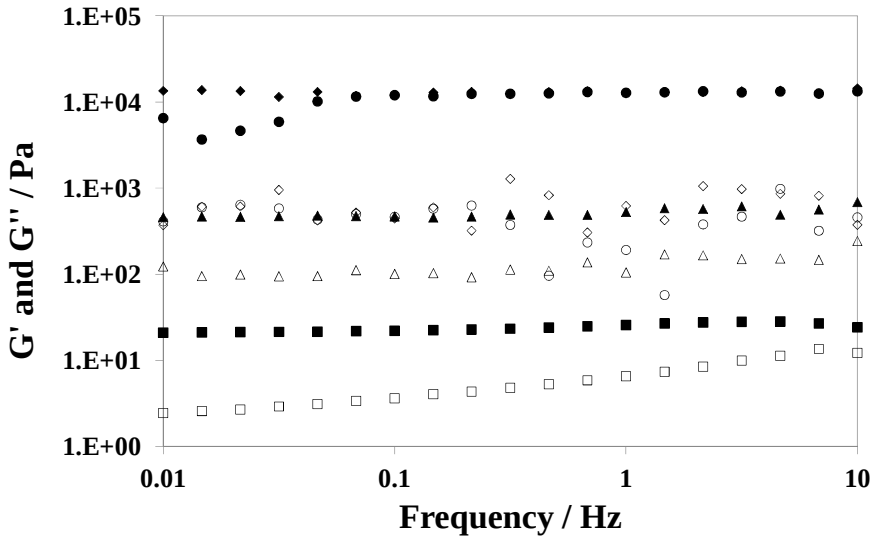

Figure 3. Mechanical spectra (frequency sweep) of the films after 24h swelling in Milli-Q water (neutral pH) at 25 °C. G' (filled symbols) and G'' (empty symbols) of 2 wt% CMC + 5 wt% CA (diamond); 2 wt% HEC + 5 wt% CA (square); 1 wt% HEC + 1 wt% CMC + 5 wt% CA (triangle) and1 wt% HEC + 1 wt% CMC + 10 wt% CA (circles).

The cross-linked CMC films show a reasonably high storage modulus, G', almost three orders of magnitude higher than the cross-linked HEC films, which behave as a weak gel-like material. Nevertheless, as observed in Table 2, such high elasticity compromises the extension of swelling. HEC films have a lower cross-linking density and thus swell up to one order magnitude more than the CMC films. The combination of the two polymers results in films with interesting rheological properties, where G' stands in between the values of the films composed by the individual polymers and the swelling degree is higher than the film of CMC alone. The role of CA cannot be neglected; an increase in CA concentration from 5 to 10 wt% leads to a significant increase in rigidity of the polymer network which results in the highest G' observed and very poor swelling ability. Therefore, the mechanical, rheological and swelling properties can be optimized by properly tuning the CMC/HEC/CA ratios.

Figure 4 shows the thermal decomposition of the different films as well as the corresponding derivative thermogravimetric curves (DTG).

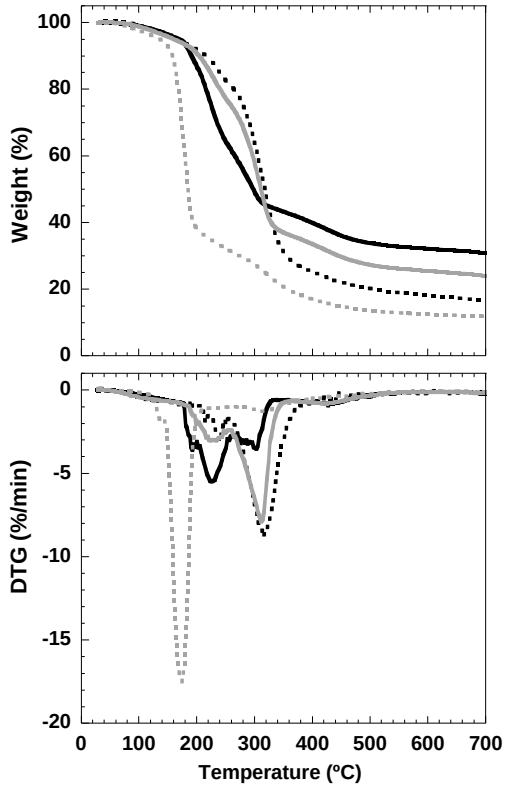

Figure 4. TGA (top) and corresponding DTG (bottom) of cellulose-based cross-linked films. 2 wt% CMC + 5 wt% CA (full black line); 2 wt% HEC + 5 wt% CA (dashed black line); 1 wt% HEC + 1 wt% CMC + 5 wt% CA (full grey line); 1 wt% HEC + 1 wt% CMC + 10 wt% CA (dashed grey line).

The CMC-based films generally present a lower thermal stability when compared with the formulations using HEC. The thermal degradation, T_{dm}, of the native polymers HEC and CMC occurs at ca. 310 °C and 280 °C, respectively (data not shown) but after the cross-linking reaction with 5 wt% CA, T_{dm} is shifted to ca. 315 °C and 230 °C, respectively. That is, while the HEC films slightly improve their stability, the CMC based films display a substantial reduction. Moreover, it is interesting to note that the film containing HEC, CMC and 5 wt% CA presents a T_{dm} similar to HEC cross-linked with CA but with an intermediate final mass loss of the individual cross-linked polymers. The formulation containing 10 wt% CA results in films with decreased thermal stability (i.e. T_{dm} is ca. 175 °C). This reduction in T_{dm} can be related to a higher disorder in the structure due to the high concentration of CA in the reaction. Such behaviour has been observed in other systems where a too high cross-linking density may lead to a less organized material and, consequently, to a lower thermal stability (Capanema, et al., 2018). Nevertheless, the TGA curves indicate that the obtained

chemically cross-linked cellulose-based films are thermally stable in the temperature range suitable for entrapment and delivery of probiotic bacteria.

The surface morphology of the cross-linked films was characterized using SEM (Figure 5).

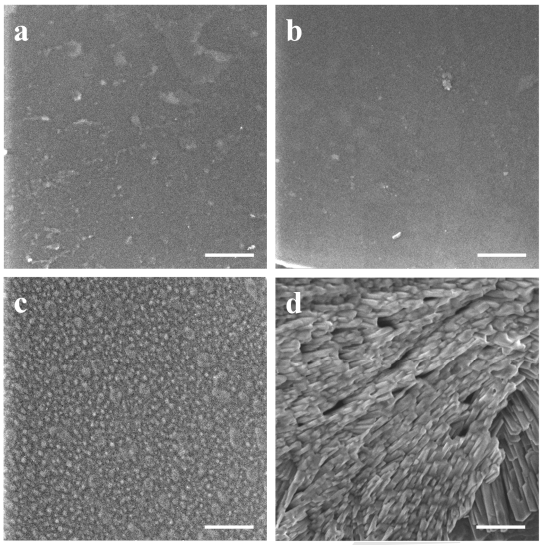

Figure 5. SEM images of different cross-linked films: a) 2 wt% CMC + 5 wt% CA; b) 2 wt% HEC + 5 wt% CA; c) 1 wt% HEC + 1 wt% CMC + 5 wt% CA; d) 1 wt% HEC + 1 wt% CMC + 10 wt% CA. The scale bar represents 5 µm.

The cross-linked films of HEC or CMC alone (Figures 5a and 5b) present a rather similar and homogeneous morphology with an apparent low porosity, which contrasts to the much less dense structure and higher porosity of the non-cross-linked homologues (data not shown). When CMC and HEC are mixed and cross-linked together, the porosity and heterogeneity increases (Figure 5c) and a “fibrillar”-like morphology is obtained for the highest CA concentration used (Figure 5d). Such an aggregated fibrillar structure is expected to account for the high elasticity of the wet films (Figure 3) and poor elongation to break (Figure 2). The increased heterogeneity in the film, with visible fracture points, may be responsible for the observed brittleness and decrease in thermal stability (Figure 4).

3.2. Probiotic encapsulation and viability

In order to evaluate the ability of the different cellulose-based films to work as matrices for probiotic entrapment, LGG was selected as model bacteria. As described in the experimental section, the bacteria were entrapped in the films following two distinct

methods. Briefly, in a first approach, LGG was mixed directly with all compounds and casted in the Petri dishes. A typical SEM example of LGG embedded in the film matrix is shown in Figure 6a with a zoomed area presented in Figure 6b. However, even if the curing conditions for the cross-linking reaction are far milder than the standard procedure reported in literature, the entrapped bacteria are found to have a quite low viability, as qualitatively evaluated by fluorescence microscopy using a Dead/Alive kit (Figure 6d). In this test, the viable bacteria appear as green rods while the dead bacteria are red. As it is clearly visible, the curing conditions (i.e. 50 °C for 24h) are still too harsh with the majority of the bacteria being found dead; 3 Log CFU/mL (CFU: colony-forming units) is estimated from the plate counting technique. Although lower temperatures are beneficial for probiotic viability, the films do not form below 50 °C.

It has been speculated that some mechanisms behind the probiotic health effects may not entirely dependent on the viability of the cells but on other factors such as probiotic adhesion. It has been argued that probiotic adhesion to host tissues facilitates the host-microbial interactions, such as the effects of microbes on the immune system of the host. In fact, some reports suggest that viable and non-viable probiotics are equally adherent to intestinal mucus (Lahtinen, 2012). Apart from the hypothetic effect of non-viable probiotics, further studies are planned to improve the synthesis conditions in order to guarantee a higher probiotic viability in the films.

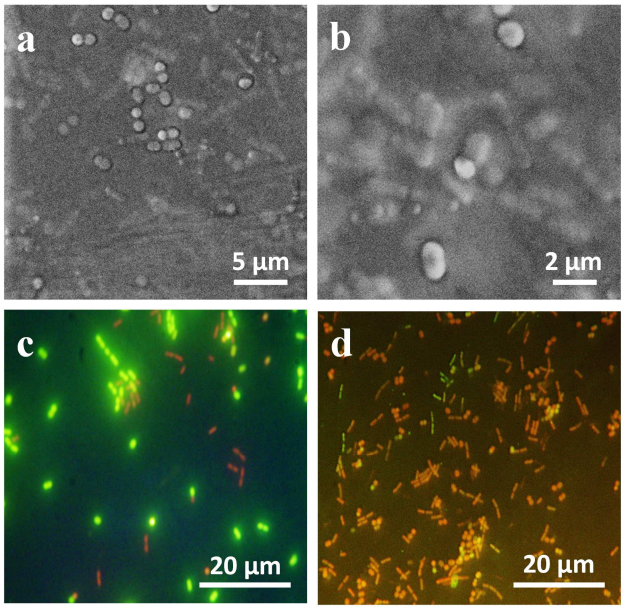

Figure 6. SEM of probiotic bacteria entrapped in CMC-based films (a and b) and the corresponding fluorescent microscopy image using the Dead/Alive kit (d). The control is LGG in the polymer matrix before curing (c).

An alternative approach was tested to endow the films with viable cells. The method is based on probiotic adsorption onto the films after soaking them in a LGG solution for 30 min at room temperature. The films were subsequently rinsed to remove any adsorbed bacteria at the film surface. The film ability to release the trapped bacteria into an aqueous media of different pH was evaluated. As can be seen in Figures 7a and 8b, the majority of the bacteria appear as green, which is a strong qualitative indication of high viability even after exposure to the different pH conditions. Note that the images in Figure 7 are not as clear as those in Figure 6 since the cross-linked mixture of CMC and HEC results in films with poorer transparency, which contributes to blur the fluorescence images. Additionally, during the cell diffusion into the film matrix, it is expected that bacteria accumulate at the larger pores and structural defects in the films thus resulting in a less homogeneous distribution of the bacteria. This can be inferred from Figure 7b where a rather large green area is observed. The viability of LGG after entrapment in the films was also quantitatively checked by plate counting (Figure 8).

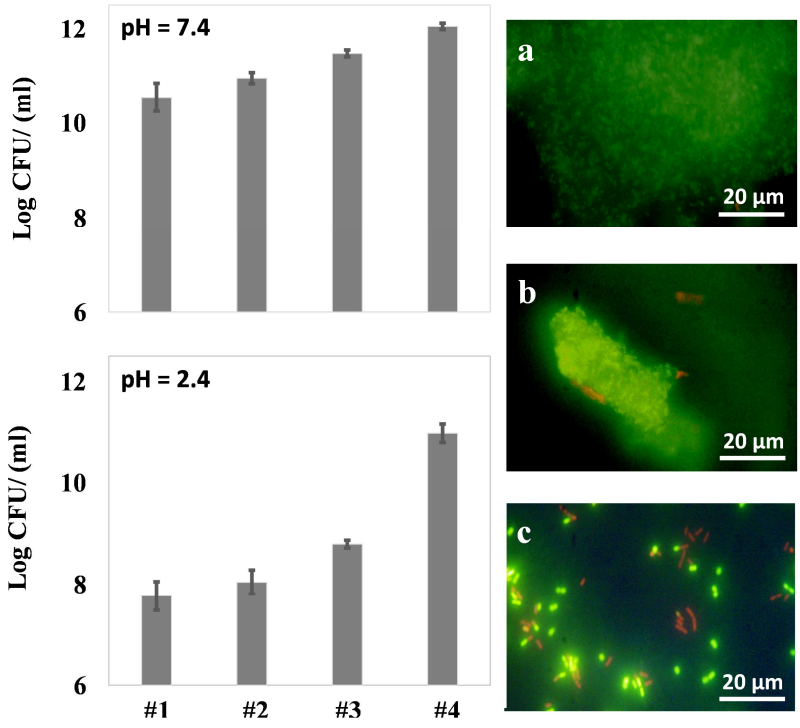

Figure 7. Left: Viability counts in Log CFU/mL of the different systems developed after the bacteria have been released in PBS, pH 7.4 (top) and in an aqueous solution of HCl, pH 2.4 (bottom). #1: 2 wt% CMC + 5 wt% CA; #2: 2 wt% HEC + 1 wt% CMC + 5 wt% CA; #3: 1 wt% HEC + 1 wt% CMC + 10 wt% CA; #4: naked bacteria.

Fluorescence microscopy images of the 1 wt% HEC + 1 wt% CMC + 5 wt% CA film after bacteria soaking and later exposure to entrapped bacteria of the CMC + HEC + 10% CA films after their exposure to a) pH 7.4 and b) pH 2.4. Image c) refers to naked bacteria at pH 7.4. The films were stained with the Live and Dead viability kit. The error bars represent the standard deviation.

There is a significant difference ($p < 0.05$) in the viability when the CMC+HEC+10 wt% CA films are compared to all other samples at pH 7.4. However, no significant difference is observed at pH 2.4 among the films. The larger the content of CA, the higher is the cell counting. This observation is most likely related to the fact that the film defects increase with CA (see Figure 5). Note that the HEC film was not selected for these tests since the mechanical properties of the wet film are rather poor. While at pH 7.4 the viability for all films tested is above ca. 10 log CFU/mL, it is noticeable that at 2.4 the viability count drops significantly to ca. 7-8 log CFU/mL. This viability counting is expected to be underestimated due to the presence of remaining entrapped bacteria in the film matrix. The fluorescence data suggests that not all the bacteria is released, which might be due to the poor swelling of the film. On the other hand, at high pH, film swelling is favoured and the bacteria are more efficiently released thus resulting in a higher viability counting.

In general, viable LGG bacteria can be effectively entrapped in the cross-linked cellulose based films and their release is strongly dependent on the film composition and pH of the media.

4. Conclusions

Both CMC and HEC can be successfully cross-linked using CA and the reaction can be followed by FTIR. The film properties can be controlled by the cross-linker amount. An increase in CA concentration leads to a decrease in swelling ratio of the films, increased rigidity and substantial decrease in the surface area. However, higher CA content also induces the formation of fibrillar-like aggregates and structural defects in the films. Viable LGG could be effectively entrapped in the films after soaking them in bacteria medium. The direct mixing of bacteria with the film components followed by casting and curing was found to be very harsh for LGG. Overall, the films developed proved to be promising matrices for bacteria entrapment in, for instance, food applications where the mechanical, swelling and release properties can be tuned by

HEC/CMC ratio and amount of cross-linker. The systems developed represent promising advances in the search for new applications of edible cellulose based films and coatings as carriers of diverse probiotics and open new possibilities for the development of novel food probiotic products. Additionally, these films may inspire the formation of related systems, such as micro-beads as carriers for probiotic bacteria and delivery in the gastrointestinal tract. The use of plasticizers is also expected to improve the mechanical performance of the films and this will be tried in the future.

Acknowledgements

This work was supported by funding from the People Program (Marie Curie Actions) of the European Union's Seventh Framework Program FP7/2007-2013/ under REA grant agreement n° 606713 BIBAFOODS. The Portuguese Foundation for Science and Technology (FCT) is acknowledged through the project PTDC/AGR-TEC/4814/2014 and researcher grant IF/01005/2014. The Coimbra Chemistry Centre, CQC, supported by FCT, through the project PEstOE/QUI/UI0313/2013 is also acknowledged.

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Research Highlights

- Cellulose-based films cross-linked with citric acid were developed.
- Novel edible coatings for probiotic bacteria entrapment and delivery are proposed.
- Suitable thermal, swelling and mechanical properties identified.
- Probiotic bacteria successfully entrapped into the films with acceptable viability.