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RELATIONSHIP BETWEEN CASEINATE/CARRAGEENAN EDIBLE FILM AS LACTIC ACID BACTERIA CARRIER AND ITS ANTIMICROBIAL ACTIVITY AGAINST PATHOGENS IN VITRO: EFFECT OF CARRAGEENAN TYPE

M. Lourdes Pérez-Chabela[1], Reyna Gutierrez[2], Alfonso Totosaus[2*]

[1]Universidad Autonoma Metropolitana Iztapapala. Av. San Rafael Atlixo 186, Mexico City 09340, Mexico.

[2]Food Science Lab & Pilot Plant. Tecnologico Estudios Superiores Ecatepec. Av. Tecnológico esq. Av. Central s/n, Ecatepec 55210, México.

** alfonso.totosaus@gmail.com*

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ABSTRACT

Edible films can be elaborated with proteins and/or polysaccharides. Composite edible films combine the functionality of each component, enhancing physical properties. In this research edible films were elaborated with sodium caseinate and different types of carrageenan (iota, kappa or lambda), to manipulate the interaction among them as carrier of lactic acid bacteria. Lambda carrageenan resulted in less soluble and tougher edible films, due to their higher sulfate group content and higher interaction with proteins. Higher solubility and hence a less ductile film structure enhanced P. pentosaceus bacteria viability and its antimicrobial activity, at least for Listeria and E. coli. The solubility and structural characteristics of caseinate edible films can be manipulated depending on carrageenan type employed, to enhance their capacity to active packaging for probiotic bacteria.

1.Introduction

Edible films are elaborated with any GRAS material employed to enrobing foods to extend shelf-life and that may be eaten together with the food, providing replacement and/or fortification of natural layers to avoid moisture losses and control gases exchange, to prevent loss of important components, with a thickness lower than 0.3 mm (Pavlath and Orts, 2009). The functionality of edible films depends on the nature of the different components and on their final composition and structure (proteins and polysaccharides). In general, polysaccharide based films absorbs more water and are more readily disintegrated with poor barrier properties than protein based films (Cuq et al., 1995). Polysaccharides impart structural cohesion serving as structural matrix, and proteins give rise to a very firm structure by both inter- or intramolecular folding and interaction (Wu et al., 2002).

The polyelectrolyte character of milk proteins in the interaction with hydrocolloids like carrageenan plays an important role in determining mixed biopolymer behavior (Dickinson, 98). Caseins and caseinates can readily form edible films from aqueous solutions since caseins are quite soluble in water due to the structure and amino acid composition of caseins, it is likely that hydrogen bonds, electrostatic interactions

and most probably hydrophobic forces are involved in the formation of casein-based edible films (Schou et al., 2005; Frinault et al., 2006). The number and position of sulfate groups entails a negative charge that affects the functionality of the different carrageenan types (Langendorff et al 2000). Carrageenans, which are film-formers, are used mainly in the food industry as texturizing agents with potential use as coating agents that control transfer of moisture, gases, flavors, and lipids in diverse food systems (Soliva-Fortuny et al., 2012).

The incorporation of antimicrobial agents to packaging materials slows down their release and helps keeping high concentrations of the active compounds on the product surface for extended periods of time (Kristo et al., 2008). Many antimicrobials are proposed to be used in the formulation of edible films and coatings to inhibit the spoilage flora and to decrease the risk of pathogens. There is a trend to select the antimicrobials from natural sources and to use generally recognized as safe (GRAS) compounds to satisfy consumer demands for healthy foods, free of chemical additives (Devlieghere et al. 2004). The advantage in having a film material carrying a biocide is that continued inhibition can occur during storage or distribution of the food product. Application of package-based biocides to reduce post process growth of food pathogens has shown promise, since biocides incorporated into the matrix of films will release their antimicrobial activity to the surrounding environment. To place an antimicrobial hurdle during storage would reduce the chance of cell numbers increasing to a dangerous level (Dawson et al, 2002).

The objective of this work was to evaluate physical and mechanical properties of sodium caseinate edible films with different types of carrageenans (iota, kappa and lambda) to employed as carrier for thermotolerant lactic acid bacteria evaluating their effect on viability and antimicrobial capacity in vitro.

2. Materials and methods

2.1. Edible films elaboration

Edible films were elaborated with sodium caseinate (DVA Mexicana, Naucalpan) and glycerol as plasticizer and carrageenan. In order to establish the effect of carrageenan type, Viscarin SD389 iota carrageenan, Viscarin GP209 lambda carrageenan, or Gelcarin GP8612 kappa carrageenan (FMC Biopolymers, Philadelphia) were employed. Edible films were prepared according to the casting technique, dehydrating the filmogenic protein-carrageenan-plasticizer solution. Sodium caseinate (8%, w/v) was dissolved in 100 mL of distilled water, adding glycerol (0.4%, w/v) and each carrageenan type (0.3%, w/v). Solutions were poured in glass plates (12×12 cm2) and dehydrated at room temperature (25±1 °C) at 55±5% RH during 48-60 h. Afterward, edible films were kept in desiccators for further analysis.

2.2. Total soluble material and soluble protein

Total soluble material was determined according to the method reported by Pereda *et al.* (2012). Edible films samples (2×2 cm) were weight and immersed in 30 mL of water during 24 h. After immersion, samples were oven dried at 105 °C during 24 h to determinate the insoluble material. Total soluble material was reported as the percent of dissolved mass (dry basis) with respect to the initial film dry weight.

From the distilled water employed in total soluble material, soluble protein was determined by biuret method (Gornall *et al.*, 1949). Film soluble protein was reported according to Jangchud and Chinnan (1999):

$$\text{Soluble protein (\%)} = \frac{\text{Protein concentration in 30 mL}}{\text{Initial film weight} \times \text{\% protein in film} \times \text{\% film dry matter}} \quad (1)$$

2.3. Mechanical properties: Puncture and tension tests

Force and deformation at the breaking point was determined according to the described by Sobral *et al.* (2001). Samples were fixed in 52.4 mm diameter acrylic cells and perforated in the center with a 3 mm aluminum probe at a constant rate of 1 mm/s in a LFRA 4500 texturometer (Brookfield Engineering Laboratories, Middleboro). From time-force curves, puncture force (maximum force at film breakdown) was reported and puncture deformation was calculated as:

$$\text{Puncture deformation (\%)} = \frac{\Delta l}{l_0} = \frac{\sqrt{(D^2 + l_0^2)} - l_0}{l_0} \times 100 \quad (2)$$

Considering that the stress was perfectly distributed along the film, where D is probe displacement, l0 is the initial film length (radius of the measurement cell, 26.3 mm).

Film tensile strength and elongation percent were determined employing a Chatillon TCM 200 motorized test stand with a DFIS 200 digital force gauge according to the described by Gennadios *et al.* (1993). Films samples were cut in 100×25.4 mm and placed in the grips with an initial separation of 50 mm. Samples were stretched at a constant speed rate of 1 mm/s until breakdown. Tensile strength was calculated dividing the peak load by the cross-sectional area (film width×thickness). The elongation percent was calculated as the ratio of the extension values and the initial grip separation multiplied by 100.

2.4. Lactic acid bacteria incorporation into edible film

Lactic acid bacteria *Pedioccocus pentosaceus* UAM22, previously reported as thermotolerant and probiotic (Ramirez-Chavarín et al, 2010; Ramírez-Chavarin et al, 2013), was prepared reactivating cells in 10 mL MRS broth, incubating at 37 °C for 24 h until an optical density close to one (λ = 600 nm), containing approximately 108 CFU/mL. Cells were harvest by centrifugation at 2000×g during 20 min and washed in sterile water. Bacterial cell preparation (1%, w/v) was added to caseinate-carrageenan solution under magnetic stirring for 5 min before casting process.

2.5. Cell viability and antimicrobial activity

Viability of the lactic acid bacteria was determining in edible films stored in petri dishes at 25 °C for 7 days. Edible films were then placed in dilution flask with 100 mL of sterile saline solution homogenizing during 5 min. Serial dilutions were performed and poured on MRS plates, incubating at 37 °C during 48 h before colonies counting.

Antibacterial activity of lactic acid bacteria in caseinate-carrageenan edible films was determined by the disc diffusion technique, the Kirby-Bauer method (Valencia *et al.*, 2013). *Listeria innocua*, a non-pathogen strain, was employed instead *Listeria monocytogenes*, due the physiological similarity (Begot *et al.*, 1997). *L. innocua* was reactivated in 3 mL of brain heart infusion (BHI) media and incubated at 37 °C during 12 h before adding 7 mL of BHI broth to incubate at 37 °C during 24 h. Same procedure was followed for *Escherichia coli* and *Staphylococcus aureus*. A 6-mm diameter disc of each edible film treatments was placed in a petri dish with Meuller-Hilton or BHI agar, previously inoculated with 0.2 mL of *L. inocua*, *E. coli* or *S. aureus* strains inoculums (ca. 10^5-10^6 CFU/mL). Petri dishes were incubated at 37 °C during 24 h to determinate the bacterial growth inhibition zone around the discs. A clear

zone after incubation assumes that the edible film and the antimicrobial compound presented inhibition (Maizura *et al.,* 2007). Inhibitory activity of the edible film as lactic acid bacteria carrier was determined as the inhibition halo diameter ($\pi \times$clear zone radio^2).

2.6. Experimental design and data analysis

The effect of the different carrageenan type on caseinate edible films on films properties, cell viability and antimicrobial activity was analyzed by PROC ANOVA procedure in SAS Statistical Software version 8.0 (SAS Institute, Cary). Significantly ($P<0.05$) difference among means were determined with Duncan's mean test in same software. The strength and direction of the linear relationship between corresponding variables, edible films properties (total soluble matter, soluble protein, puncture and tensile resistance) and film entrapped lactic acid bacteria (cell viability and antimicrobial activity) was determine with Pearson's correlation analysis with the PROC CORR procedure in same software.

3.Results and discussions

3.1. Total soluble material and soluble protein

Iota carrageenan containing films presented the significantly ($P<0.05$) higher total soluble material values, and the lower ones was observed in the lambda carrageenan samples. In same manner, significantly ($P<0.05$) lower soluble protein was observed in lambda carrageenan samples, with higher values in the samples containing iota carrageenan (Table 1).

Table 1. Edible film physicochemical and mechanical properties

Carrageenan type	Total soluble material (%)	Soluble protein (%)	Puncture Force (N)	Puncture deformation (%)	Strain force (N)	Elongation (%)
Iota	72.27±12.99 a	15.36±4.82 a	12.71±6.97 b	26.31±4.02 b	0.476±0.131 c	38.75±4.56 c
Kappa	70.25± 8.37 b	11.88±5.56 b	10.49±2.24 c	24.79±3.05 c	0.548±0.143 b	69.17±2.53 b
Lambda	62.08±12.04 c	11.40±5.94 c	14.09±7.54 a	27.64±2.17 a	0.620±0.153 a	74.74±3.75 a

a, b, c Means with same letter in same row are not significantly ($P>0.05$) different

It seems that the structural differences in sulphate groups' content can explain the results. Lower sulphate groups were related to higher soluble material and higher soluble protein, since more sulphate groups (3 in lambda, 2 in kappa, 1 in iota) were related to strong electrostatic interaction with positively charged regions in caseinate, decreasing dissolubility of edible films. Caseins interactions with carrageenans are stronger when carrageenan is in the helical conformation (Langendroff *et al.*, 2000; Gu *et al.,* 2005). At the experimental conditions employed (i.e., room temperature) the conformation of kappa and iota carrageenans are temperature dependent to undergo from a coil (disordered state) to helix (ordered state) transition in solution (Černíková *et al.,* 2008), whereas lambda carrageenan is a random coil conformation (Corredig *et al.*, 2011), increasing interactions and reducing free soluble material.

3.2. Mechanical properties: Puncture and tensile tests

Caseinate edible films formulated with lambda carrageenan presented significantly ($P<0.05$) higher values of puncture force as compared to edible films formulated with kappa or iota carrageenan. Same behavior was observed in puncture deformation, where lambda carrageenan samples presented significantly ($P<0.05$) higher values (Table 1).

The temperature dependence conformation of iota and kappa carrageenan decreased their interaction capacity at the experimental conditions, resulting in lower interactions with proteins during film formation. Lambda carrageenan, with more sulfate groups and higher electric charge density besides the no temperature dependence on it conformation result in stronger attractive interaction with caseins proteins (Langendorff *et al.,* 2000). This interaction between lambda-carrageenans and

sodium caseinate could contribute to increase the protein particle size giving rise to a more open structure, increasing edible film resistant to puncture (Fabra *et al.*, 2008).

Strain force of caseinate edible films was significantly (P<0.05) higher for lambda carrageenan containing samples. The films elongation was significantly (P<0.05) higher as well for lambda carrageenan samples. Less stretchable films were obtained with iota or kappa carrageenan (Table 1).

Incorporation of polysaccharides as carrageenans increased caseinate edible films stretchability (Fabra *et al.*, 2008). Once, the electrostatic charge of each particular carrageenan rules their interaction with proteins during the film formation. The more intensive absorption of carrageenans on caseins resulted in bridges structuring a more compact network (Langendorff *et al.*, 2000; Černíková *et al.*, 2008). Higher sulphate groups content (3 in lambda, 2 in kappa, 1 in iota) were related to strong electrostatic interaction with positively charged regions in proteins. More interaction resulted in a ductile edible film with higher resistance to be distended. Puncture force, besides tension strain, express the maximum stress developed by the film under extension test (Chiralt *et al.*, 2012).

3.3. Cell viability and antimicrobial effectiveness

The viability of lactic acid bacteria for the edible films formulated with the different carrageenans was as following: iota (8×10^6 CFU)> kappa (4×10^6 CFU)> lambda (0.1×10^6 CFU). Table 2 show the results for pathogens inhibition for the different edible films elaborated with caseinate and carrageenans. In Mueller-Hilton media, for *L. inocua* the inhibition ratio was significantly (P<0.05) higher for iota carrageenan samples, and the lower radio was observed in lambda carrageenan samples.

Table 2. Inhibition halo diameter in the antimicrobial test of edible caseinate films with Iota, Kappa or Lambda.

Carrageenan type	Mueller-Hilton agar			BHI agar		
	L. innocua	***E coli***	***S. aureus***	***L. innocua***	***E coli***	***S. aureus***
Iota	1.65±0.11 a	2.29±0.10 a	0.00	1.54±0.10 a	1.69±0.12 a	0.00
Kappa	1.56±0.12 b	2.12±0.09 b	0.00	1.45±0.11 b	1.56±0.12 b	0.00
Lambda	1.41±0.10 c	2.00±0.05 c	0.00	1.34±0.09 c	1.47±0.13 c	0.00

a, b, c Means with same letter in same row are not significantly (P>0.05) different

Same tendency was observed for *E. coli* as well, with no inhibition for *S. aureus*. In BHI agar, iota and kappa carrageenan incorporation into caseinate edible films resulted in significantly (P<0.05) higher inhibition halo radius, with the lower one in lambda carrageenan samples. The anti-Listerial effect of lactic acid bacteria has been attributed to several mechanisms, from acidification due to lactic acid production (Bredholt *et al.*, 2001), competence for nutrients (Vermeiren *et al.*, 2006), bacteriocins production (Katla *et al.*, 2002) or even to non-producing bacteriocins strains (Alves *et al.*, 2006). Probably same mechanism can be applied to E. coli, although at the experimental conditions with the employed thermotolerant lactic acid bacteria strains *S. aureus* was not inhibited.

Lower solubility and tougher structure (in lambda carrageenan samples) seems to be related to lower lactic acid bacteria viability and diminution of antimicrobial capacity. Table 3 presents the correlation coefficients, irrespectively of carrageenan type, for the physical and mechanical parameters of edible

films against their capacity to maintain lactic acid bacteria viable and to inhibit pathogens.

Table 3. Pearson's correlation coefficients and significance among variables, irrespectively of carrageenan type.

Variable	Total soluble material (%)	Soluble protein (%)	Puncture force (N)	Puncture deformation (%)	Tensile strength (N)	Elongation (%)	Cell viability ($\times 10^6$ CFU)	Inhibition halo radius (mm)
Total soluble material (%)	1.0000	0.6329 <0.0001**	0.7002 <0.0001**	0.6776 <0.0001**	0.6736 <0.0001**	0.3107 0.132*	-0.3905 <0.0001**	0.0676 0.5986•
Soluble protein (%)		1.0000	0.8954 <0.0001**	0.9448 <0.0001**	0.4537 0.0002**	-0.1735 0.1740•	0.3486 <0.0001**	0.3322 0.0078**
Puncture force (N)			1.0000	0.8489 <0.0001**	0.3780 0.0023**	-0.1707 0.1810•	-0.2985 0.0200*	-0.3689 0.0029**
Puncture deformation (%)				1.0000	0.6084 <0.0001**	0.0907 0.4796•	0.1388 0.0869•	0.1928 0.1300•
Tensile strength (N)					1.0000	0.7665 <0.0001**	-0.5824 <0.0001**	-0.3784 0.0022**
Elongation (%)						1.0000	-0.9135 <0.0001**	-0.6726 <0.0001**
Cell viability ($\times 10^6$ CFU)							1.000	0.5706 <0.0001**
Inhibition halo (mm)								1.0000

** highly significant (P<0.01), *significantly (P<0.05), •not significantly (P>0.05)

The correlation among mechanical properties was coherent, since the positive and highly significant (P<0.01) relationship between soluble material and soluble protein. In same manner, edible films solubility (total soluble material and soluble protein) presented a highly significant (P<0.01) correlation with the films resistance to perforation, being higher (high correlation coefficient value) with soluble protein. For stretch out resistance (tensile strength and elongation), there was a highly significant (P<0.01) relation of tensile strength with solubility properties. Total soluble matter was not significantly (P>0.05) with films elongation, but there was an inverse significantly (P<0.01) correlation of soluble protein with the film stretch capacity. Elongation was not significantly (P>0.05) with both puncture parameters, that presented a highly significant (P<0.01) correlation with tensile strength. These results indicate that the interactions during the filmogenic process of sodium caseinate with the different carrageenans had as consequence distinct solubility characteristics, more over of proteins. More soluble films (proteins released) in water were more resistant to puncture and tension. These properties are important to consider since edible films solubility controls the diffusion release of bioactive compounds to food surface (Quirós-Sauceda *et al.*, 2014), and mechanical resistance is important to maintain edible film integrity during process and handling before

applying to foods (Tanada-Palmu and Grosso, 2003).

Regarding to the correlation amid solubility and mechanical properties with lactic acid bacteria in edible films, total soluble material was highly significant ($P<0.01$) correlated inversely with cell viability, whereas soluble protein presented highly significant ($P<0.01$) correlation with both cell viability and a bigger inhibition halo. The more soluble protein in edible films resulted in better survive of the employed strain *P. pentosaceus*, since these had been reported as thermotolerant, this is, these lactic acid bacteria had an enhanced response to stress (survival during and after edible film process). For mechanical properties, only puncture force presented inverse and significantly ($P<0.05$) and highly significant ($P<0.01$) correlation, respectively, with cell viability and inhibition halo. Both tensile parameters presented an inverse highly significant ($P>0.01$) correlation with both cell viability and inhibition halo. This means that the more ductile or tougher edible film structure restrain lactic acid bacteria. Although the incorporation of lactic acid bacteria into biopolymer films can modify barrier or mechanical properties, application of edible films assures the food protection against moisture changes or mechanical damages (Sánchez-González *et al.*, 2014).

Finally, there was a highly significant ($P>0.01$) correlation between cell viability and inhibition radius. The different properties of edible films, as result of the protein-polysaccharide interaction, different type of carrageenan, affected the viability and metabolism of lactic acid bacteria in their antimicrobial activity. Migration or liberation of antimicrobial depends on the electrostatic interactions between the component and the polymer side chains, ionic osmosis, and possible structural changes induced by the presence antimicrobial compound and the edible film environmental factors like temperature and storage time (Cha and Chinnan, 2004; Lin and Zhao, 2007).

4. Conclusions

In caseinate edible films the type of carrageenan type had an influence on their solubility and mechanical properties, parameters that are very important to consider in the handling and application of edible films as bioactive carrier. Probiotic thermotolerant lactic acid bacteria are able to survive edible film casting process, being viable to be applied throughout the edible film as bioactive packaging. Lambda carrageenan resulted in less soluble and tougher edible films, due to their higher sulfate group content and higher interaction with proteins. Higher solubility and hence a less ductile film structure enhanced *P. pentosaceus* bacteria viability and its antimicrobial activity *in vitro*, at least for *Listeria* and *E. coli*. The solubility and structural characteristics of caseinate edible films can be manipulated depending on carrageenan type employed, to enhance their capacity to active packaging for probiotic bacteria.

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