Research Article

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Characterization and antimicrobial properties of food packaging methylcellulose films containing stem extract of Ginja cherry

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Abstract

BACKGROUND: Food contamination and spoilage is a problem causing growing concern. To avoid it, the use of food packaging with appropriate characteristics is essential; ideally, the packaging should protect food from external contamination and exhibit antibacterial properties. With this aim, methylcellulose (MC) films containing natural extracts from the stems of Ginja cherry, an agricultural by-product, were developed and characterized.

RESULTS: The antibacterial activity of films was screened by the disc diffusion method and quantified using the viable cell count assay. The films inhibited the growth of both Gram-positive and Gram-negative strains (*Listeria innocua*, methicillin-sensitive *Staphylococcus aureus*, methicillin-resistant *S. aureus*, *Salmonella Enteritidis*, *Escherichia coli*). For the films with lower extract content, effectiveness against the microorganisms depended on the inoculum concentration. Scanning electron microscope images of the films showed that those containing the extracts had a smooth and continuous structure. UV–visible spectroscopy showed that these materials do not transmit light in the UV.

CONCLUSION: This study shows that MC films containing agricultural by-products, in this case Ginja cherry stem extract, could be used to prevent food contamination by relevant bacterial strains and degradation by UV light. Using such materials in food packaging, the shelf life of food products could be extended while utilizing an otherwise wasted by-product.

Keywords: edible food packaging; cherry stem extract; antimicrobial activity; methylcellulose; agricultural by-products

INTRODUCTION

Microbial food contamination is a widespread problem that is causing growing concern in modern society. Food may come into contact with pathogenic agents from external sources; at the same time, foodborne pathogenic agents can also be developed by the food itself through degradation/decay. To avoid and/or minimize this problem, the use of appropriate packaging is essential, since it acts as a barrier that can protect fresh food from contamination. Moreover, it can also reduce food contact with atmospheric agents such as oxygen and water vapour, hence minimizing spoilage.

In recent years, increasing attention has been given to packaging films made of edible and/or biodegradable materials such as polysaccharides and protein-based materials.[1] The main advantages of such materials are their biocompatibility, aesthetic appearance, non-toxicity and edibility[2–4]; in most cases their functional properties (i.e. mechanical and/or barrier properties) are comparable to those of plastic materials.[1] In addition to this, these materials are also biodegradable; their use, therefore, reduces the amount of waste produced. This is an issue that has become very important in recent years for many industrial areas, including food production, since 30% of all generated waste comes from this area.[5]

The incorporation of antimicrobial agents in food packaging films has also been extensively studied. Indeed, significant progress has been made in this field, as several packaging materials with antibacterial properties have been developed using either traditional plastic matrixes or biodegradable edible ones.[6–12]

The use of natural products such as plant extracts and/or essential oils as antimicrobial agents has also been investigated.[7,13–15] These extracts or oils often contain compounds such as polyphenols or terpenes with antimicrobial properties.[16–18] Moreover, being of natural origin, they are perceived as being safer by consumers and are therefore more acceptable. The use of otherwise wasted agricultural by-products has obvious economic and environmental advantages.

Ginja cherry (variety *Prunus cerasus* L., Rosaceae) is a native fruit of Portugal. Because of its bitter taste, it is not used for direct consumption, but it is used among other processed products

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to produce a traditional alcoholic Portuguese liquor, Ginjinha. The production of this liquor generates large amounts of natural solid waste, such as stems and leaves from the harvesting of Ginja cherries, whose disposal is an additional cost to the manufacturer.

Previous studies showed that extracts from both stems and leaves were rich in valuable compounds such as polyphenols and terpenes and exhibited antioxidant properties[19] and antibacterial activity.[20,21] In particular, stem extracts in ethyl acetate were most active towards both Gram-positive and Gram-negative strains.

Because of their antibacterial properties, these extracts can be used as additives in food packaging films. The study described here is on the incorporation of natural extracts of Ginja cherry into a matrix of methylcellulose (MC). MC was chosen as it is a material already employed to manufacture films for food packaging,[13] and studies have been published on its use in combination with natural antibacterial compounds.[22,23] The antibacterial properties of the films were tested towards different strains, both Gram-positive and Gram-negative. Moreover, film morphology and physical properties (i.e. weight, UV and IR transmittance) were also assessed. The results demonstrate the potential of using agricultural by-products for such applications.

EXPERIMENTAL

Extract preparation

Ginja cherry stem extracts were prepared as previously described in more detail.[21] Briefly, dried and ground stems were placed in contact with ethyl actetate (40 mL g^{-1} stems) under agitation at a constant temperature of 30 °C for 24 h. Subsequently, the stems were separated from the solvent by filtration and the filtrate was evaporated to dryness in a rotary evaporator (Büchi, Flawil, Switzerland) at 45 °C. The dry extract was then redissolved in 4% (v/v) dimethyl sulfoxide (DMSO)/water (60:40 v/v) to a final concentration of 0.15 g mL^{-1}.

MC film production

To make the films, 0.144 g of MC and 0.35 mL of 50% (v/v) glycerine were dissolved in a total volume of 12 mL (water and extract); this corresponds to an MC concentration of 1.2 g per 100 mL. Variable amounts of extract were added to obtain solutions with extract concentrations of 3, 4 and 5% (w/v), designated as samples MC3, MC4 and MC5 respectively. A control sample containing no extract was also prepared (sample MCC). During the process, care was taken to avoid the formation of lumps and to obtain a smooth and homogeneous gel.

The solutions were poured into flat glass dishes and left to dry at 37 °C in a controlled atmosphere (relative humidity of 30%), protected from light and dust, until constant weight was achieved. After drying, the films were cut into 1 cm discs; all subsequent tests were performed on these discs.

Film characterization

To determine the extract content in each film, all discs were weighed. The extract concentration was calculated from the difference in weight between the films containing the extract and the control film with no extract.

Film thickness was measured using a micrometer (MI20, Adamel Lhomargy, Rossie en Brie, France). Six measurements were performed on different parts of the films; the average value was calculated for each film, with the corresponding standard deviation.

Film surfaces were studied with a Hitachi S-4100 scanning electron microscope (SEM) at 25 kV; samples were coated with gold prior to analysis. UV spectra were obtained using a Helios-α instrument (Thermo Spectronic, Reading, UK), while for IR spectra a Mattson 7000 FT-IR spectrometer with a Specac Golden Gate diamond ATR was employed, using Winfirst software.

Film colour was evaluated with a portable CR-400 Chroma Meter (Minolta, Osaka, Japan). The CIELab colour scale was used to determine the lightness (L), redness ($+a^*$)/greenness ($-a^*$) and yellowness ($+b^*$)/blueness ($-b^*$) of the films. Film samples were measured on the surface of a white standard plate with colour coordinates $L = 97.59$, $a = -0.07$ and $b = 1.89$. Film colours were expressed as the total difference in colour, ΔE, calculated with the formula

$$\Delta E = \left[(L_{\text{film}} - L_{\text{control}})^2 + (a_{\text{film}} - a_{\text{control}})^2 + (b_{\text{film}} - b_{\text{control}})^2\right]^{1/2}$$

For each film, three pieces were measured; on each piece of film, three readings were taken.

Microorganisms and growth conditions

The microorganisms used in this study were isolated from food and clinical samples. The Gram-negative microorganisms used were *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 10145) and *Salmonella Enteritidis* (ATCC 3076). The Gram-positive microorganisms used were methicillin-sensitive *Staphylococcus aureus* (MSSA, ATCC 25923), methicillin-resistant *S. aureus* (MRSA, sample recovery from Instituto Português de Oncologia do Porto, IPO), *Listeria innocua* (11288 NCTC), *Enterococcus faecium* (CCUG 34441, Gutenberg University) and *Enterococcus faecalis* (LMGS 19456 5002). Culture/maintenance of the microorganisms was performed in Mueller-Hinton Agar (Sigma Aldrich, Gillingham, UK) at 37 °C for 24 h.

Antimicrobial activity

Disc diffusion assay

Prior to testing the antimicrobial activity of the discs, an overnight liquid culture of the chosen microorganisms was prepared, with an optical density adjusted to 0.2 at $\lambda = 610$ nm, corresponding to about 10^8 colony-forming units (CFU) mL^{-1}. The film discs of 1 cm diameter were sterilized by placing them under UV light for 10 min on each side.

The inoculum was spread homogeneously onto a Mueller-Hinton Agar plate with a sterile swab; control and extract discs were placed on the medium and incubated at 37 °C for 24 h. After incubation, the diameter of the inhibition halo around the films was measured. Each experiment was performed in triplicate and run twice.

Viable cell count assay

The protocol for the viable cell count assay was the same as that used by Ramos *et al.*,[10] with some modifications. Overnight liquid cultures of the chosen microorganisms were prepared and diluted with peptone water to either $10^4/10^5$ or $10^6/10^7$ CFU mL^{-1}, depending on the desired concentration of inoculum. The 1 cm diameter discs, sterilized as described above, were placed in sterile vials; 200 µL of liquid inoculum was placed over each disc. The films were left in contact with the solution for different times (0, 1, 2, 5 and 8 h) at 37 °C. After incubation, 1.8 mL of peptone water was added to each vial; the solution was homogenized and successively diluted at appropriate factors. Aliquots (20 µL) of each

Table 1. Film thickness, weight and extract content

Parameter	MCC	MC3	MC4	MC5
Thickness (mm)	0.083 ± 0.054a	0.341 ± 0.084b	0.396 ± 0.068b	0.418 ± 0.087b
Weight (mg)	4.93 ± 1.95a	10.56 ± 2.37b	13.03 ± 2.49b	14.29 ± 1.53b
Extract content per disc (mg g^{-1} film)	—	377.57 ± 9.17a	460.84 ± 4.02b	482.92 ± 3.71c

Different letters in the same row indicate that samples are statistically different.

Table 2. CIELab coordinates for films prepared with different extract amounts

Parameter	MCC	MC3	MC4	MC5
L	90.35 ± 0.48a	52.86 ± 0.66b	43.12 ± 1.69 c	35.65 ± 1.08d
*a**	−0.95 ± 0.04a	−2.91 ± 0.17b	−0.85 ± 0.10a	0.94 ± 0.05c
*b**	5.22 ± 0.02a	41.55 ± 3.55b	28.87 ± 0.53c	21.42 ± 1.86d
Δ*E*	—	52.24 ± 1.00a	52.81 ± 1.15a	57.07 ± 1.00b

Different letters in the same row indicate that samples are statistically different.

diluted solution were plated on Muller-Hinton Agar plates; after incubation at 37 °C for 24 h, colonies were counted.

RESULTS

Film characterization

The films were produced with different solution concentrations (3, 4 and 5% w/v); several physical characteristics such as film thickness, weight and colour were evaluated.

Table 1 gives the data for the thickness of all films, including the control. The results show that the higher the percentage of extract in the film, the greater the film thickness, because the extract solution is denser than water. Films also appeared firmer visually with increasing extract concentration.

The film weight and extract content for each formulation are also listed in Table 1. It can be seen that these parameters increase with increasing solution concentration, as expected.

In Table 2 the CIELab coordinates and the total difference in film colour are reported. All three parameters *L*, *a** and *b** are affected by the addition of the extract. Indeed, increasing the extract concentration causes a decrease in film lightness and a shift in colour towards red and blue, i.e. positive and smaller *a** and *b** values respectively. The total difference in colour, Δ*E*, is the same for samples MC3 and MC4 (values non-statistically different, $P > 0.05$); sample MC5, on the other hand, has a bigger Δ*E* value.

Figure 1(a) shows the UV spectra of samples MCC and MC3. Considering the control, the film shows high transmittance in both the visible and UV ranges (λ = 400–800 and <400 nm respectively) owing to its transparency. The extract-containing film, on the other hand, shows much lower transmittance; in particular, in the UV region the transmittance is very close to zero. Figure 1(b) shows the spectrum of sample MC3 on an enlarged scale; it is possible to see the more complex transmittance features in the visible region due to its colour (see above). The spectra of the films prepared from 4 and 5% (w/v) solutions showed similar features (data not shown).

Figure 2 shows the IR spectra of samples MCC and MC3. The spectrum of the control sample shows all peaks belonging to MC-glycerol films as previously reported in the literature.[24,25] The most important ones are the signals at 924, 1035, 1107 and 1110 cm^{-1} (C—O and C—C bonds present in the cellulosic structure), at

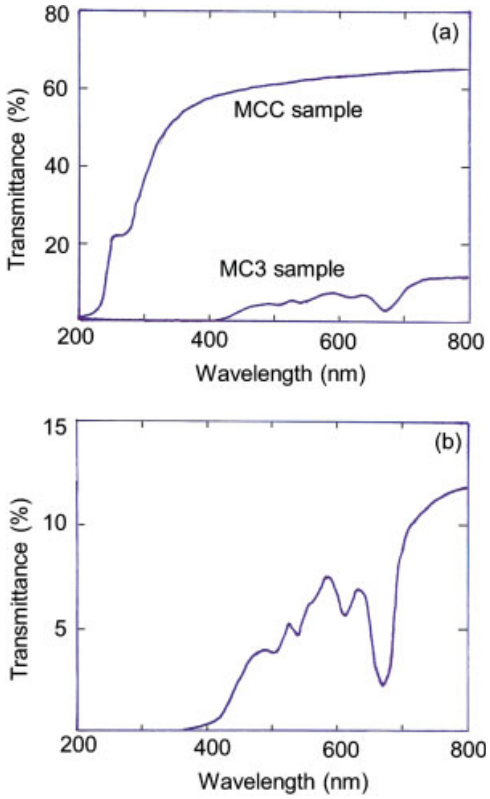

Figure 1. UV spectra of (a) samples MCC and MC3 and (b) sample MC3 on a more expanded scale.

2883 and 2935 cm^{-1} (C—H bonds of methyl groups, symmetric and asymmetric stretching) and in the range 1320–1450 cm^{-1} (in-plane and out-of-plane CH_2 bending) and the broad peaks at about 3340 cm^{-1} (OH stretching vibration) and 1645 cm^{-1} (bound water vibration).

From Fig. 2 it can be seen that the film containing the extract does not have particularly different features, as the same peaks are detected. The only clear difference is in the OH stretching vibration peak, which is stronger; this may be due to a higher

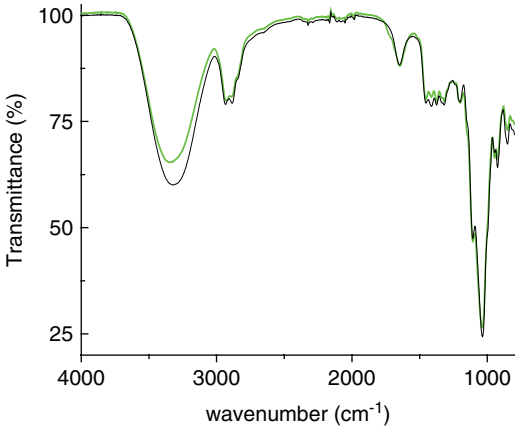

Figure 2. IR spectra of samples MCC (lighter line) and MC3 (darker line).

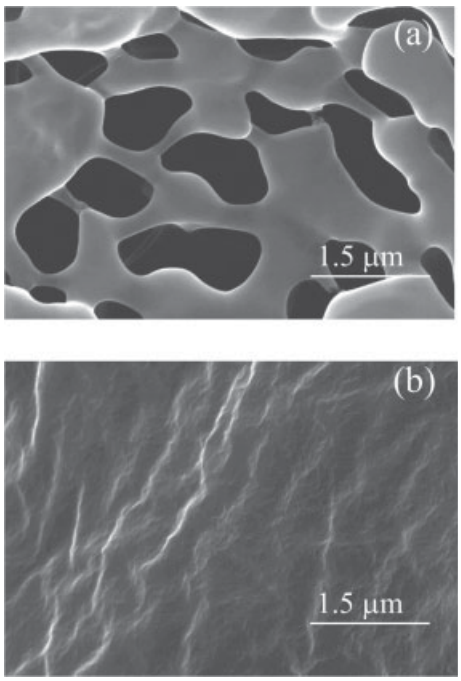

Figure 3. SEM images of samples (a) MCC and (b) MC3.

moisture content of the extract-containing films compared with the control. As mentioned in 'Introduction', the extracts are rich in terpenes and polyphenols; their concentration in the films, however, is much smaller than that of MC and consequently no IR signals characteristic of these molecules are detected.

The SEM images of samples MCC and MC3 are shown in Figs 3(a) and 3(b) respectively. The presence of the extract changes the film morphology completely. The structure of the control film is discontinuous, with pores of dimensions greater than 1 µm. In contrast, when the extract is added, the film is continuous and no pores can be observed. The film surface looks smooth, without irregularities. This indicates that the distribution of the extract in the film itself is homogeneous, without agglomeration and/or extract segregation.

Antimicrobial activity

A preliminary screening of the antimicrobial activity of the films on sample MC5 (extract content 482.92 mg g^{-1} film) was performed by the disc diffusion assay. Figures 4(a) and 4(b) show examples of tests for MRSA and *P. aeruginosa* respectively, where an inhibition halo is present for the Gram-positive strain but not for the Gram-negative one. The values of the inhibition halos measured for the different strains are reported in Table 3. Indeed, inhibition of the growth of several microorganisms, both Gram-positive (MSSA, MRSA, *L. innocua*) and Gram-negative (*S. Enteritidis*), was observed.

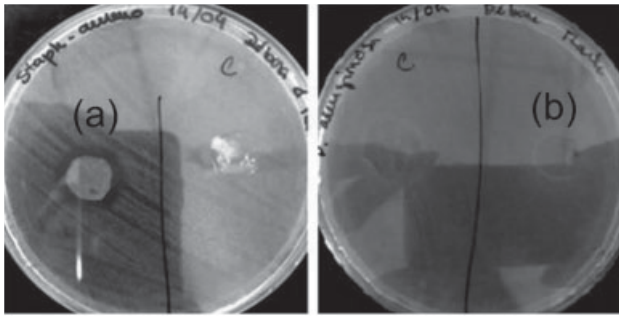

Figure 4. Disc diffusion test results for sample MC5 with (a) methicillin-resistant *Staphylococcus aureus* (positive) and (b) *Pseudomonas aeruginosa* (negative). C, control sample MCC.

Table 3. Diameter of inhibition zone for sample MC5 on different bacterial strains

Strain type	Microorganism tested	Inhibition halo (mm)
Gram-positive	*L. innocua*	14.3 ± 0.4
	MSSA	15.0 ± 2.0
	MRSA	14.0 ± 0.0
	E. faecalis	—
Gram-negative	*P. aeruginosa*	—
	S. Enteritidis	21.5 ± 0.7
	E. coli	—

Considering the encouraging results of the disc diffusion test, a more systematic investigation of the antibacterial properties of these samples was performed using the viable cell count assay; tests were done on samples MC3 and MC4 (extract contents 377.57 and 460.84 mg g^{-1} film respectively). Two series of experiments were run with different inoculum concentrations of approximately $10^4/10^5$ and $10^6/10^7$ CFU mL^{-1} respectively. Three examples of Gram-positive microorganisms, MSSA, MRSA and *L. innocua*, and two examples of Gram-negative microorganisms, *S. Enteritidis* and *E. coli*, were selected.

The results of these tests on sample MC3 are shown in Fig. 5 (graphs in the left column, corresponding to an inoculum concentration of $10^4/10^5$ CFU mL^{-1}). The films inhibited the growth of all selected Gram-positive strains; in fact, a reduction in bacterial colonies of about four orders of magnitude can be observed for all three microorganisms. This decrease is almost immediate for MRSA (Fig. 5(e)), as the bacterial population decreases markedly after only 1 h of contact with the film; longer times of 2 and 5 h are necessary for *L. innocua* (Fig. 5(a)) and MSSA (Fig. 5(c)) respectively. For the Gram-negative strains, extract-rich films are effective towards *S. Enteritidis* (Fig. 5(g)), with a decrease in viable cells similar to that seen for MSSA. In the case of *E. coli*, on the other hand, an inhibitory effect on the bacterial population was observed only for the longest contact time of 8 h (Fig. 5(i)).

When more concentrated inoculum solutions were used (approximately $10^6/10^7$ CFU mL^{-1}), the films still showed an

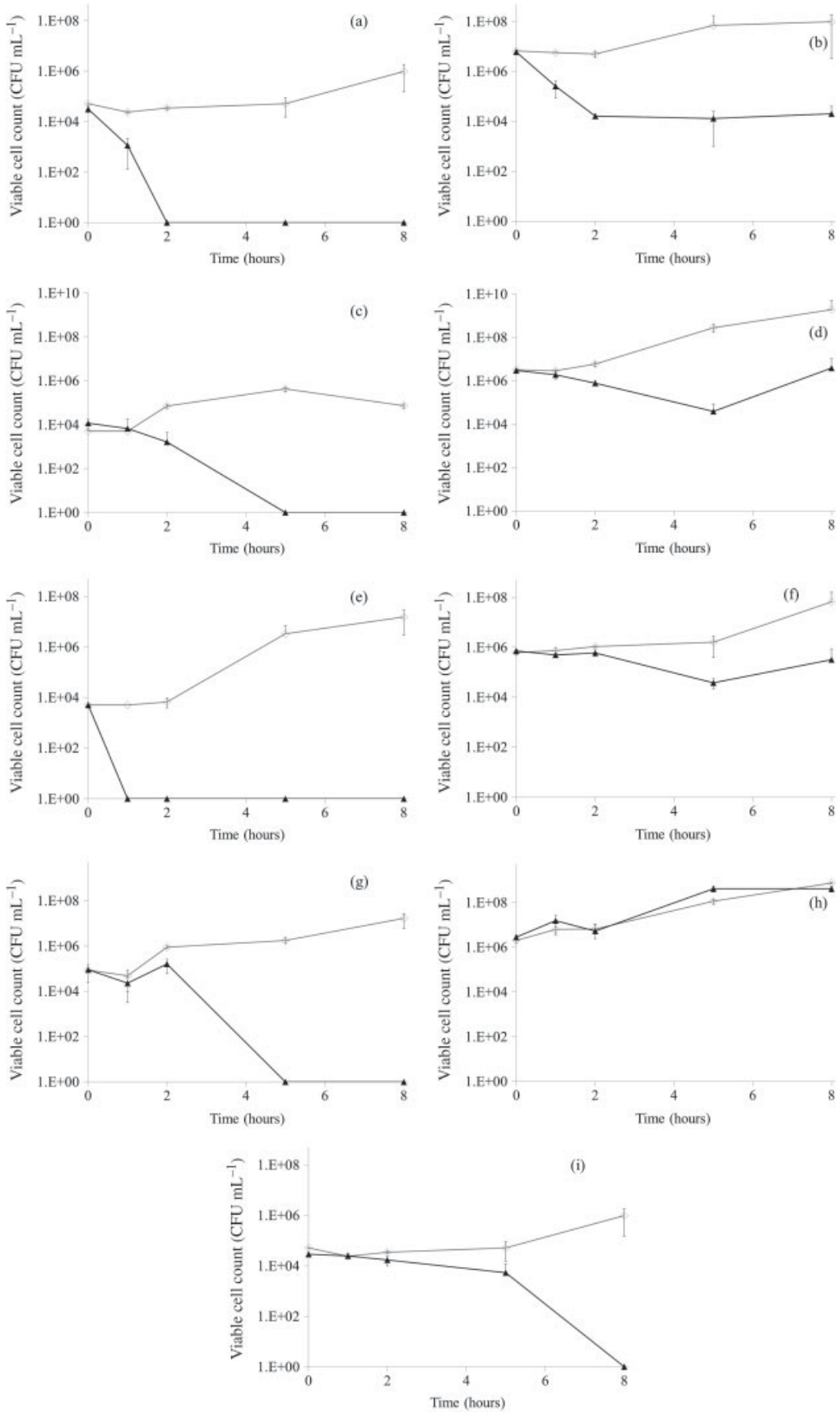

Figure 5. Growth curves of selected bacterial strains in contact with samples MC3 (▲) and MCC (◊). Inoculum concentrations: graphs on left (a, c, e, g, i), $10^4/10^5$ CFU mL^{-1}; graphs on right (b, d, f, h), $10^6/10^7$ CFU mL^{-1}. Bacterial strains: (a, b) *Listeria innocua*; (c, d) methicillin-sensitive *Staphylococcus aureus*; (e, f) methicillin-resistant *S. aureus*; (g, h) *Salmonella Enteritidis*; (i) *Escherichia coli*.

inhibition effect on the Gram-positive strains (Fig. 5, graphs in the right column). In fact, the number of viable cells decreased by about four orders of magnitude for *L. innocua* (Fig. 5(b)) and three orders of magnitude for both MSSA (Fig. 5(d)) and MRSA (Fig. 5(f)). For these last two strains, a successive increase in the bacterial population can be observed for longer times (more than 5 h of contact time). This could have occurred because, for this higher inoculum concentration, not all of the population was inhibited; consequently, the microorganism could partially recover.

For the Gram-negative microorganisms, on the other hand, no effect could be seen; in fact, no difference was observed between the films with the extract and the control for *S. Enteritidis* (Fig. 5(h)). The *E. coli* strain was not tested, considering that the extract only showed an effect on the more diluted inoculum after a much prolonged contact time.

The same tests were performed for the films containing extracts at 4% (w/v) concentration (sample MC4); in this case, complete inhibition was observed also for the Gram-negative strains with both diluted and concentrated inoculum solutions (data not shown).

DISCUSSION

The addition of extracts to a film matrix is likely to modify its properties and characteristics. In the study reported here, some of the parameters measured were affected, while others were not.

The film colour changed, as detailed in 'Results'. This is due to the colour of the extracts themselves, which are brown/green. Compared with the control film, the colour of the extract-containing films shifted towards red; this is due to the combined effect of the extracts themselves and the MC matrix. Changes in colour with the addition of natural extracts have already been reported in the literature;[26,27] indeed, plant extracts often have a colour themselves owing to their composition.

The colour of the film, as well the transmittance in the UV range, is a very important parameter for food packaging materials. Light can have a detrimental effect on food, as some of the components can be degraded by light exposure.[28] Lipids containing polyunsaturated fatty acids (PUFA), for instance, can be severely damaged by light exposure in the range 400–500 nm owing to oxidation.[29] A recent study reports that the degradation can be greatly reduced by the use of a low-transmittance packaging material.[30]

As described in 'Results' and shown in Fig. 1, the films with extracts show no transmittance in the UV and very low transmittance at $\lambda < 500$ nm; with these characteristics, they could be very effective as light-protective packaging materials.

The morphology of the films was also modified with the incorporation of the extracts, as shown by the SEM images in Fig. 3. Sample MC3 showed a smooth and continuous structure, with no formation of cracks in the film. These results differ from some literature data. For instance, Bahram *et al.*[31] studied the incorporation of cinnamon essential oil in whey protein isolate films and reported that the inclusion of the essential oil caused the formation of cracks in the films. Our results indicate that that the drying of the extract does not damage the film structure, even if it modifies it. The formation of a continuous structure could be due to the higher density of the extract-containing MC solution used to prepare the films; this may have led to the formation of a more compact film.

Previous work done with cellulose-based films and additives of natural origin confirmed that the presence of such additives can affect the film structures. Perone *et al.*,[32] for instance, observed that the addition of rosemary oil to hydroxypropyl methylcellulose (HPMC) led to the formation of denser films. In their case, however, they also observed a segregation of the oil, which was not observed in our work (see Fig. 3(b)). The formation of such a continuous structure could be due to the higher density of the extract-containing MC solution used to prepare the films; this may have led to the formation of a more compact film.

Although the permeability properties of these materials were not tested, some general considerations can still be made. Tests performed on HPMC films showed that the addition of anthocyanin caused a significant decrease in oxygen permeability.[30] It is therefore likely that similar results could be observed for the films in this study, especially considering their compact structure. Other features (e.g. the hydrophilic nature of the matrix and/or the additives), however, may also have a determinant role. Films with lower oxygen permeability would be more suitable for the packaging of foods that could be degraded by contact with oxygen, such as lipids[30] in meat products.

The comparison of the IR spectra of the control and extract-containing films did not show any significant difference, with no additional peak detected in the films with the extract incorporated. This feature may indicate that no modification of the film backbone structure was caused by the extracts; this means that the extract inclusion was obtained with a physical process and without the formation of new chemical bonds.

Previous tests performed on these extracts in liquid form demonstrated their antibacterial properties;[21] the results shown here confirm that this activity was not compromised when the extracts were included in the MC matrix. The protocol used here for the film preparation did not involve either high temperature or high pressure, so no extract degradation took place. This preservation of the extract composition and properties was also observed when a different preparation method was employed, provided that extreme pressure and temperature were not used.[33] This indicates that, potentially, these extracts could be included in different materials and still show effective antibacterial properties if an appropriate film preparation protocol is followed.

In liquid form the extracts were more active towards Gram-positive bacteria; the same behaviour was also observed here. One of the main components of these extracts is linalool.[21] Literature reports exist of several packaging materials in which linalool is used for its antibacterial behaviour; polyethylene and starch were studied, for instance, and linalool was shown to be effective as an antibacterial agent.[11,34,35] This study confirms that linalool can also be used in an MC matrix.

As reported in the literature,[11] it is often difficult to compare the antibacterial activities of different materials owing to the different protocols followed. This comparison is even more difficult when antibacterial compounds such as natural extracts or essential oils are used, because of their complex composition and the resulting synergistic effect.[36] Keeping this in mind, however, a comparison with other literature data can still be attempted. When natural extracts were added to films for food packaging, the range of concentrations in the film preparation solution was never higher than 5% (w/v), because higher extract concentrations could have caused deterioration of the packaging properties. Moreover, higher extract concentrations may have resulted in a greater degree of migration of some of the extract components into the food items.[14,15,22] In this work the same extract concentration range was considered in order to avoid these potential problems.

However, these concentrations were sufficient to give the materials good antibacterial properties.

Sample MC3 induced complete bacterial inhibition only for the Gram-positive strains and for the lower inoculum concentration (Fig. 5). When a film with higher extract content was used (MC4), on the other hand, complete inhibition was always observed in all cases. Considering this, films with different composition should be considered depending on the application, the possible level of bacterial contamination that the food could develop, and the expected shelf life of the product. Food items such as fresh and frozen meat, fish products or fresh fruits could be protected, preventing the superficial growth of pathogens and contaminants.

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