Research Article

Effect of pH and Mexican Oregano (*Lippia berlandieri* Schauer) Essential Oil Added to Carboxymethyl Cellulose and Starch Edible Films on *Listeria monocytogenes* and *Staphylococcus aureus*

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Received 19 January 2018; Revised 5 April 2018; Accepted 15 April 2018; Published 9 May 2018

Academic Editor: María B. Pérez-Gago

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The aim of this work was to evaluate the effect of pH and Mexican oregano essential oil (MOEO) added to carboxymethyl cellulose (CMC) and starch (S) edible films on *Listeria monocytogenes* and *Staphylococcus aureus*. CMC and S edible films were formulated with different concentrations (0%, 0.25%, 0.50%, 0.75%, and 1%) of MOEO at different pH (5, 6, or 7). Antimicrobial assay was performed. Inhibition curves were fitted to the Fermi model. Significant differences ($p < 0.05$) were found in t_c (time to reduce 50% of microbial population) and a (slope of the curve around t_c), being lower at acidic pH. For *L. monocytogenes*, CMC films exhibited a higher antimicrobial effectiveness (0.50% of MOEO) compared to S films which need a higher concentration of MOEO (0.75%). *S. aureus* was inhibited with CMC films at 0.50% MOEO and pH 5 and 6. Microbial modeling has allowed estimating key intrinsic factors as pH and MOEO concentration with the synergistic effect against two important food-borne pathogens.

1. Introduction

Researchers around the world are investigating the use of essential oils to protect food from microbial growth. The antimicrobial activity of essential oils and plant extracts is well known for a long time, and many research results have been published against food-borne pathogens [1] and about their low toxicity to mammals, fewer environmental effects, and low cost, which make them more attractive than synthetic antimicrobials [2]. Based on their traditional use, these extracts obtained from plants are first recommended, due to their broad-spectrum antimicrobial action and low side effects treatments [3]. Essential oils also have the additional effect of maintaining the quality of the food during storage, including sensorial, nutritional, and functional properties [4, 5].

Carvacrol (5-isopropyl-2-methylphenol) and thymol (2-isopropyl-5-methylphenol) are the major components of the essential oils of some species belonging to the Lamiaceae family including oregano, and these are phenolic compounds; isomers of monoterpenes exhibit significant antimicrobial activity in vitro [6, 7]. The WHO recognized thymol and carvacrol as GRAS compounds for consuming as long as they not exceed $50 \text{ mg} \cdot \text{kg}^{-1}$ [6].

Natural antimicrobial compounds effectiveness depends on the type, genus, species, and strain of the target microorganism and some food intrinsic and extrinsic factors such as pH, temperature, water content, atmospheric composition, and initial microbial load, with pH being one of the most important factors due to antimicrobial dissociation [3].

Bacterial susceptibility to essential oils increases with a reduction in food pH, since at low pH the hydrophobicity of the oil increases more easily allowing the dissolution of the membrane lipids' target bacterium [8].

Currently, research has been focused on the use of edible films due to their safety regarding consumption and friendly relation with the environment, and coating materials being used currently include polysaccharides (cellulose derivatives, starch, chitin, and gums), proteins (soy, milk, gelatin, corn zein, and gluten), and lipids (oils, waxes, and resins) [9]. Among them, carboxymethyl cellulose (CMC) and starch (S) are the most widespread and economic biomaterials. In addition, its chemical, physical, and functional characteristics make them suitable to add essential oils (EOs) as antimicrobials [10–12]. Several works in the literature focus on the evaluation of the antibacterial effect of EOs and propose them as an interesting option to ensure the safety and quality of minimally processed foods and extend their shelf life [13, 14]. Moreover, there are evidences that EOs incorporated to edible films can inhibit a large variety of food-borne pathogens [13, 15–19]. The main advantage of using EOs in a polymer matrix coating is a slow diffusion of the antimicrobial agent, leaving high concentrations of the active compound in contact with the food surface where contamination has occurred or is likely to occur, for a long period [20]. The aim of this work was to evaluate the effect of pH and Mexican oregano essential oil concentration added to CMC and S edible films on *Listeria monocytogenes* and *Staphylococcus aureus*.

2. Materials and Methods

2.1. Essential Oil and Chemical Characterization. Mexican oregano (*L. berlandieri* Schauer) essential oil (MOEO) was provided by CiReNA (Natural Resources Research Center of Salaices, López, Chihuahua, Mexico), which was obtained by vapor distillation for 4 h with a Clevenger-type apparatus. MOEO was analyzed with the GC-MS (TurboMass Gold Autosystem XLTM; PerkinElmer, Norwalk, CT) with a splitless injector and an FID detector, equipped with a capillary column (30 m × 0.25 ID × 0.25 μm). Helium was used as the carrier gas for a total run time of 30 min. The obtained spectra were compared with the respective mass spectra of pure compounds and with the mass profile of the same compounds available from the US National Institute of Standard Technology (NIST) library.

2.2. Edible Film Preparation. Films were made according to the Bertuzzi et al. [21] methodology with some modifications. High amylose corn starch (2 g) containing 75% apparent amylose (CPI Ingredients, Mexico) or 0.5 g of carboxymethyl cellulose (CMC) with medium molecular weight (CPI Ingredients, Mexico) and viscosity 400–800 cP in 2 wt.% in water (25°C; Brookfield) was prepared separately in previously sterilized 10 ml of 0.25 N sodium hydroxide and 10 ml distilled water by stirring for 60 min. Then, sorbitol (Aldrich Chemical Co., Milwaukee, WI) 0.5% (v/v) was added as a plasticizer, and the pH was adjusted to 5, 6, or

7 with phosphoric acid (1 N). MOEO was incorporated at 0.00%, 0.25%, 0.50%, 0.75%, or 1.00% (v/v) final concentration. Finally, to prepare the films, 7 ml of each solution was poured into sterile Petri dishes (60 mm diameter), dried (0.35 kg/cm² vacuum at 30°C for 12 h), and stored in sealed Petri dishes (4°C) until analysis [22].

2.3. Microbial Strains of *L. monocytogenes*. ATCC 7644 was provided by the Laboratorio de Salud Pública from Secretaría de Salud, Puebla, Mexico, and *S. aureus* ATCC 2913 was obtained from the Food Microbiology Laboratory of Universidad de las Américas Puebla. Both cultures were kept refrigerated at 5°C in slant tubes with Trypticase soy agar (TSA) (Merck, Mexico City, Mexico). A loopful of a stock culture was transferred to 10 mL of Trypticase soy broth (Merck, Mexico City, Mexico) and then incubated for 18 h at 35°C.

2.4. Antimicrobial Assay. Edible films antimicrobial assays were performed according to the Sánchez-González et al. [13] methodology with some modifications. The TSA medium was poured into Petri dishes (60 mm diameter), and after the culture medium solidified, different edible films (55 mm diameter) were placed on the agar surfaces. Then, properly diluted overnight cultures (10⁶ UFC/ml) from each strain were inoculated on every plate. Plates were incubated at 37°C, and microbial counts on edible films were examined at 0, 15, 30, 45, 60, and 120 min. For this purpose, edible films (10 g) were aseptically removed from Petri dishes and placed in sterile plastic bags with 90 ml of saline peptone (Aldrich Chemical Co., Milwaukee, WI). Bags were homogenized for 2 min in a Stomacher blender (400 Circulator; Seward, United Kingdom). Serial dilutions were prepared, and then, poured TSA plates were incubated at 37°C for 24 h before colonies were counted. All tests were run in triplicate.

2.5. Modeling and Statistical Analysis. Survival curves were generated from experimental data by plotting N/N_0 (where N is the number of CFU/mL at a given time and N_0 is the initial number of CFU/mL) versus treatment time. Data were fitted to the Fermi model [23] from which the biological parameters a and t_c were estimated by nonlinear regression using the KaleidaGraph 3.51 program (Synergy Software, Reading, PA, USA):

$$x = \frac{1}{\{1 + \exp(t - t_c/a)\}}, \quad (1)$$

where x is the survival fraction (N/N_0), t is the time (min) at which the sample was taken, t_c is the time (min) needed to reduce 50% of microbial population, and a is the slope of the curve around t_c [24].

Fermi biological parameters were analyzed by using analysis of variance (ANOVA). Significant values were subjected to mean analysis by the least significant difference, using a significance level of 95%. Statistical analyses were performed with Minitab 17 software (Minitab Inc., PA, USA, 2010).

FIGURE 1: Inhibition curves of *Listeria monocytogenes* at different concentrations of Mexican oregano (*Lippia berlandieri* Schauer) essential oil and pH values added to CMC (a) (0.25%/pH 5 (■), 0.25%/pH 6 (●), 0.50%/pH 7 (▲), and 0.75%/pH 7 (◆)) and starch (b) (0.25%/pH 5 (□), 0.50%/pH 5 (○), 0.50%/pH 6 (△), 0.75%/pH 6 (◇), and 0.75%/pH 7 (x)) edible films.

3. Results and Discussion

MOEO chemical analysis showed that the two major compounds were thymol (2.452 g/ml) and carvacrol (0.456 g/ml), whereas *p*-cymene, 1,8-cineole, and γ -terpinen were in very low concentrations. Survival curves for both microorganisms are presented in Figures 1 and 2. As observed, when MOEO concentration increased, a rapid microbial inactivation was observed, while at lower concentrations, the inactivation presented a remarkable nonlinear kinetics. Bermúdez-Aguirre and Corradini [25] proposed that the estimation of equivalent treatments requires a proper description of the microbial

FIGURE 2: Inhibition curves of *Staphylococcus aureus* at different concentrations of Mexican oregano (*Lippia berlandieri* Schauer) essential oil and pH values added to CMC (a) (0.25%/pH 5 (■), 0.25%/pH 6 (●), and 0.75%/pH 7 (◆)) and starch (b) (0.50%/pH 5 (□), 0.75%/pH 5 (○), 0.75%/pH 6 (△), 1.00%/pH 6 (◇), 0.75%/pH 7 (x), and 1.00%/pH 7 (◆)) edible films.

inactivation kinetics. For this reason, survival curves were adjusted with nonlinear regression using the Fermi model to predict microorganisms behavior with $R^2_{adj} = 0.942 \pm 0.02$ and $R^2_{adj} = 0.927 \pm 0.11$ for *L. monocytogenes* and *S. aureus*, respectively. This model describes a sigmoid decay and generates two biological parameters (a and t_c) between pH and MOEO concentration (Table 1). Moreover, the Fermi model can explain and predict microbial reduction in all tested pathogens and is suitable to describe inactivation in similar matrices and under different operating conditions in the future [26]. Significant differences ($p < 0.05$) were found in a and t_c values. Thus, lower Fermi parameters values were

TABLE 1: *Listeria monocytogenes* and *Staphylococcus aureus* Fermi model biological parameters values at different concentrations of Mexican oregano (*Lippia berlandieri* Schauer) essential oil and pH values added to CMC and starch edible films.

Microorganism	Film	pH	% EO	t_c (min)	a	Fit R^2_{adj}
<i>L. monocytogenes</i>	CMC	5	0.00	—	—	—
		5	0.25	7.59 ± 1.94^a	0.23 ± 0.07^a	0.992
		5	0.50	*	*	*
		5	0.75	*	*	*
		5	1.00	*	*	*
		6	0.00	—	—	—
		6	0.25	46.65 ± 8.37^b	11.61 ± 2.09^b	0.995
		6	0.50	*	*	*
		6	0.75	*	*	*
		6	1.00	*	*	*
		7	0.00	—	—	—
		7	0.25	—	—	—
		7	0.50	188.18 ± 9.58^c	58.09 ± 5.52^d	0.886
		7	0.75	70.42 ± 9.25^d	17.83 ± 4.23^b	0.992
		7	1.00	*	*	*
	Starch	5	0.00	—	—	—
		5	0.25	56.27 ± 7.28^b	21.56 ± 4.76^c	0.894
		5	0.50	7.59 ± 1.94^a	0.23 ± 0.07^a	0.992
		5	0.75	*	*	*
		5	1.00	*	*	*
		6	0.00	—	—	—
		6	0.25	—	—	—
		6	0.50	243.33 ± 12.45^c	74.32 ± 8.65^a	0.917
		6	0.75	38.48 ± 5.78^b	10.93 ± 2.78^b	0.979
		6	1.00	*	*	*
		7	0.00	—	—	—
		7	0.25	—	—	—
		7	0.50	—	—	—
		7	0.75	57.41 ± 7.34^b	45.38 ± 9.78^d	0.833
		7	1.00	*	*	*
<i>S. aureus</i>	CMC	5	0.00	—	—	—
		5	0.25	9.24 ± 2.01^a	1.29 ± 0.01^a	0.909
		5	0.50	*	*	*
		5	0.75	*	*	*
		5	1.00	*	*	*
		6	0.00	—	—	—
		6	0.25	68.62 ± 8.41^b	16.30 ± 4.05^b	0.996
		6	0.50	*	*	*
		6	0.75	*	*	*
		6	1.00	*	*	*
		7	0.00	—	—	—
		7	0.25	—	—	—
		7	0.50	—	—	—
		7	0.75	78.60 ± 9.14^b	15.11 ± 3.48^b	0.982
		7	1.00	*	*	*
	Starch	5	0.00	—	—	—
		5	0.25	—	—	—
		5	0.50	—	—	—
		5	0.75	95.31 ± 11.41^c	56.72 ± 7.81^c	0.851
		5	1.00	9.24 ± 2.01^a	1.29 ± 0.01^a	0.909
		6	0.00	—	—	—
		6	0.25	—	—	—
		6	0.50	—	—	—
		6	0.75	163.52 ± 19.67^d	71.16 ± 7.87^d	0.902
		6	1.00	50.05 ± 8.45^b	25.82 ± 6.39^b	0.964
		7	0.00	—	—	—
		7	0.25	—	—	—
		7	0.50	—	—	—
		7	0.75	173.59 ± 14.33^d	$64.75 \pm 7.25^{c,d}$	0.853
		7	1.00	72.86 ± 9.14^b	19.41 ± 4.81^b	0.982

⁺ t_c is the time needed to reduce 50% of microbial population; a is the slope of the curve around t_c . —, Growth. * Inhibition. Means followed by a different superscript letter within a column for each concentration are significantly different ($p < 0.05$).

obtained at pH 5 and MOEO concentration of 0.25%, suggesting that antimicrobial treatments are more effective at lower pH values for both microorganisms, and the *a* value helped to evaluate bacteriostatic effects that were achieved at pH 6 at lower MOEO concentrations. For *L. monocytogenes*, CMC films exhibited higher antimicrobial effectiveness than starch films at 0.50% and 0.75% MOEO at pH 5 and 6. *S. aureus* showed more resistance to edible films, that is, only for CMC films at 0.50% and pH 5 and 6. Moreover, it can be observed that, for both kinds of edible films, pH values of 5 and 6 are more effective than 7.

Some reports showed the antimicrobial effect of MOEO on *L. monocytogenes* and *S. aureus* with lower concentrations; however, these reports are in vitro studies and with direct application. One inconvenience of EO direct application on surfaces by dipping, powdering, or spraying is that antimicrobial compounds can be neutralized or diffuse rapidly from the surface into the product [30]. Our results demonstrate that edible films can serve as carriers releasing EO onto the surface controlling bacterial growth and reducing diffusion into the agar since the EO chemically forms part of the structure of the film and interacts with the polymer and the plasticizer [31]. The difference between CMC and starch polymers is the release of the antimicrobial compounds (mainly thymol and carvacrol) present in MOEO. In this report, the kind of polymer chain and pH affects the release of antimicrobial MOEO compounds due to many factors such as electrostatic interactions, osmosis, structural changes, and environmental conditions [7, 32]. Therefore, CMC releases more active compounds. pH is one of the most important parameters to prepare edible films since different pH values tend to solubilize the polymer to form the film-forming solutions [28]. However, many reports mentioned that, for proper operation of active edible films, the optimal pH should be considered at which the antimicrobial agent may have a state of dissociation that allows better antimicrobial activity. In this case, two main components of MOEO are thymol and carvacrol whose pH dissociation values are acidic (4.5 and 5.5, resp.) [33, 34]. Acevedo-Fani et al. [35] reported that thymol and carvacrol molecules can bind to membrane proteins of microorganisms by hydrophobic interactions. Thus, adequate pH values can change the membrane permeability and disintegrate the outer membrane of Gram-negative bacteria releasing lipopolysaccharides. Moreover, these authors pointed out that EO effectiveness is influenced by the sensitivity of the microorganism.

4. Conclusion

Microbial modeling allows estimating the effects of key intrinsic factors such as pH on CMC and starch edible films added with MOEO, showing a synergistic effect between EO concentration and pH, so these films might have the potential to inhibit the surface growth of pathogenic microorganisms in food by being suitable carriers of EO in different food applications. Therefore, future investigations should be conducted for the application of CMC and starch as edible coatings with MOEO in food to confirm their antibacterial effect and mechanical and sensory properties.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The authors acknowledge financial support from the Teacher Improvement Program (PROMEP), Mexican Ministry of Public Education (Research Project 5967).

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