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Bacterial Inactivation by Using Plastic Materials Activated with Combinations of Natural Antimicrobials

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Abstract: Natural antimicrobials have gained interest as possible inhibitors of biofilm formation. The aim of the present study was to determine the efficacy of antimicrobials derived from essential oils (carvacrol, thymol) plus bacteriocin AS-48 immobilized on two plastic supports (low density polyethylene and polyethylene–polyamide films) on bacterial inactivation. The polyethylene–polyamide vacuum-packaging plastic film activated with a combination of thymol plus enterocin AS-48 was the most effective in reducing the concentrations of viable planktonic and sessile cells for *Listeria innocua*, *Lactobacillus fructivorans*, *Bacillus coagulans*, and *Bacillus licheniformis*. Results from the study highlight the potential of polyethylene–polyamide film activated with thymol plus enterocin AS-48 for reducing the viable cell concentrations of spoilage Gram-positive bacteria and *Listeria* in both planktonic and sessile states.

Keywords: phenolic compounds; bacteriocin; biofilm; activated packaging

1. Introduction

Biofilms are defined as microbial communities embedded in a polysaccharide matrix formed at the interface between a solid surface and the fluid that surrounds it [1]. Biofilms show increased resistance to disinfection processes used in the food industry [2], and are persistent sources of contamination due to the release of planktonic cells and detachment of biofilm portions by mechanical forces. The process of biofilm formation has been studied in detail, and it has been found that, in most cases, it passes through a series of stages that comprise the initial binding of the bacteria to the substrate in a labile manner, followed by an irreversible binding, in which adherent molecules are involved formation of microcolonies, maturation of the biofilm, and, finally, dispersion of planktonic cells from the mature biofilm [3,4]. Several strategies have been proposed for the control of biofilm formation, such as the use of bacteriocins, bacteriophages, or antimicrobial compounds of diverse nature [5–7].

Bacteriocins are antibacterial peptides that have a relatively narrow spectrum of bactericidal action on strains of the same or related species [8]. At present, there is a large body of evidence on the possibilities and limitations of bacteriocins for application as biopreservatives in foods [9–14]. Enterocin AS-48 is a circular bacteriocin with potent antimicrobial activity, whose structure, genetic determinants, and mode of action are well known [13,14]. The activity of this bacteriocin against pathogenic or spoiling bacteria in foods of plant origin (raw vegetables, fruit juices, prepared foods, canned products, etc.), alone or in combination with other antimicrobials (including essential oils and their bioactive components), has been studied [13,14].

One of the alternatives to prevent the growth of pathogenic or spoiling microorganisms in foods is the use of coatings activated with antimicrobials [15,16]. In this way, the antimicrobial is released

more slowly, which decreases its inactivation through interaction with food components, providing longer protection and, at the same time, requiring lower concentrations of antimicrobial. Furthermore, activated films serve as a barrier against cross-contamination of the food with exogenous bacteria, and they may also be used for food packaging under a modified atmosphere.

Currently, there is strong interest in the use of natural antimicrobials, such as essential oils and their active components, in the control of antibiotic-resistant bacteria, as well as in the control of biofilm formation. In fact, natural antimicrobial compounds may be a good alternative to combat bacterial biofilms [17–19]. In addition, it is proven that some antimicrobial substances interfere with the formation of biofilms, such as the enterocin AS-48 adsorbed on polystyrene plates against *L. monocytogenes* and *Bacillus cereus* [20,21]. Similarly, some active components of essential oils are able to inhibit the formation of biofilms even when used at subinhibitory concentrations. The antimicrobial activity of enterocin AS-48 can be potentiated by carvacrol and thymol [12,13]. The aim of the present work was to study the effect of surface activation of plastic films with natural antimicrobials (enterocin AS-48, carvacrol, thymol) on the viability of food spoilage and potentially pathogenic Gram-positive bacteria in planktonic and sessile states.

2. Materials and Methods

2.1. Activation of Plastic Squares

Carvacrol and thymol were from Sigma-Aldrich (Madrid, Spain). Enterocin AS-48 was obtained by cation exchange chromatography as described elsewhere [22]. Low-density polyethylene (LDPE) stomacher plastic bags (Seward Ltd., Worthing, UK) and commercial polyethylene–polyamide (PEA) vacuum-packaging film with textured dots (available from local supermarkets) were selected for the study. Plastic squares (1.5 cm × 1.5 cm) were activated directly by immersion (1 h at room temperature with gentle shaking) in solutions (20% ethanol in water) containing enterocin AS-48 (40 µg/mL) plus carvacrol or thymol (0.375%). The activated films were allowed to dry overnight at room temperature in a biosafety cabinet (Telstar, Madrid, Spain). The plastic materials, LDPE and PEA, and the concentrations of antimicrobials used in this study, were selected from preliminary trials in which squares prepared from different commercially available plastic films were activated with carvacrol, thymol, enterocin AS-48, and their combinations, at different concentrations, and then deposited on bacterial lawns and further inspected by growth inhibition measured from the edge of the plastic squares (data not shown).

2.2. Effect of Activated Films on Planktonic and Sessile Viable Cell Concentrations

Plastic squares activated with antimicrobials were introduced into sterile 50 mL Falcon tubes containing 5 mL culture media inoculated with 100 µL from an overnight culture of the bacteria to be tested (in parenthesis, final cell density expressed as log CFU/mL): *Bacillus licheniformis* LMG 19409 (1.70), *Bacillus coagulans* CECT 12 (4.40), *Lactobacillus fructivorans* CECT 4671 (5.95), and *Listeria innocua* CECT 4030 (as a surrogate of *L. monocytogenes*) (7.24). Inoculated cultures containing plastic squares without antimicrobials were used as controls. All samples were incubated at 22 °C (in order to simulate temperature abuse conditions) with gentle shaking. The culture media used were De Man, Rogosa, and Sharpe (MRS, Scharlab, Barcelona, Spain) for lactobacilli and trypticase soya broth (TSB, Scharlab) for the rest of the bacteria tested. At regular intervals of incubation (24, 48, and 72 h), aliquots were taken from the cultures for determination of viable planktonic cells by serial dilution and plating on TSA or MRS agar (Scharlab).

In parallel, plastic squares were transferred under aseptic conditions to a new Falcon test tube, and rinsed with 5 mL sterile saline solution to remove unattached bacterial cells. After removal of the washing solution, attached cells were recovered in 5 mL sterile saline solution by sonication for 1 min in a sonicator bath (Mod 3510, Branson, Danbury, CT, USA), followed by vortex shaking according to the protocol previously developed in our research group [20]. The concentration of viable cells in

the resulting suspension was determined by serial dilution and plating. All assays were performed in triplicate.

2.3. Statistical Analysis

The statistical significance ($p < 0.05$) of differences in viable counts between samples was determined by a paired *t*-test.

3. Results

Viable cell counts obtained from bacterial cultures, carried out with plastic films activated or not with antimicrobials, are shown in Table 1. *Listeria innocua* was able to form biofilms on both plastic films without antimicrobials, with no statistical differences between LDPE and PEA control films. The activated LDPE films, containing either carvacrol or thymol in combination with enterocin AS-48, were effective in reducing the levels of viable Listeria below the detection limit in the cultured broth (planktonic state) for all sampling points. Furthermore, no viable sessile cells were detected at 24 and 72 h incubation. Also, viable counts obtained for activated LDPE films at 48 h were significantly lower ($p < 0.05$) by 5.03–5.33 log cycles, compared to the control plastic films without antimicrobials. PEA film activated with carvacrol plus enterocin AS-48 reduced viable counts in both planktonic and sessile states below detectable levels at 24 and 72 h. It also reduced viable counts significantly ($p < 0.05$) at 48 h incubation for both the planktonic and sessile states by 6.54 and 4.09 log cycles compared to the corresponding untreated controls. PEA film, activated with thymol plus enterocin AS-48, was the most effective, since no viable cells were detected from either planktonic or sessile state samples during the whole sampling period.

Tests with *L. fructivorans* indicated that all activated films were able to reduce viable counts of this bacterium in the planktonic state significantly ($p < 0.05$) compared with the controls. This effect was greatest for PEA films at 24 h, since viable cell concentrations were reduced below detectable levels. *L. fructivorans* showed a stronger capacity for biofilm formation on PEA control films, with viable counts 2.00 log cycles higher ($p < 0.05$) that LDPE films at 72 h incubation. All activated plastic films were able to inhibit biofilm formation for 24 and 48 h, but not for 72 h. Yet, the concentrations of viable cells recovered from the activated films after 72 h were significantly lower ($p < 0.05$) by 2.71–4.52 log cycles, compared to non-activated control plastic films.

Viable counts of planktonic *B. coagulans* cells were significantly lower ($p < 0.05$) for activated LDPE films compared to controls, and were also lower ($p < 0.05$) in samples containing LDPE films activated with carvacrol compared to thymol. *B. coagulans* showed a greater capacity to form biofilms on PEA control films compared to LDPE control films, as shown by the significantly higher ($p < 0.05$) cell concentrations recovered from PEA control films at all sampling points. For the activated LDPE films, sessile cell counts at 24 h only were significantly lower ($p < 0.05$) than controls for the films activated with carvacrol plus bacteriocin, but not for the thymol-activated films. However, viable counts were reduced below detectable levels at 72 h for both carvacrol- and thymol-activated films, and only some viable cells were detected in the thymol-activated films at 72 h. Activated PEA films showed higher efficacy in reducing both planktonic and sessile *B. coagulans* viable counts, and viable cells only were detected at some points during storage. Remarkably, the PEA film activated with thymol plus bacteriocin was the most effective, since no viable cells were detected for any of the samples.

B. licheniformis grew more slowly in the control samples compared to the rest of the bacteria tested. In the control samples incubated with activated plastic films, viable counts for planktonic cells were below detection levels for most of the samples. The exception was 48 h samples incubated with LDPE film activated with carvacrol plus enterocin AS-48, in which viable counts (1.48 log CFU/mL) were significantly lower ($p < 0.05$) than controls by 4.45 log cycles. For the sessile state, viable counts from control PEA films were non-significantly higher than LDPE film. In the case of films activated with antimicrobials, no viable cells were detected for any of the films and storage points.

Table 1. Effect of plastic films activated with carvacrol or thymol in combination with enterocin AS-48 on the concentrations of viable planktonic and sessile cells.

Bacteria and Treatment	Planktonic Cells (log CFU/mL)			Sessile Cells (log CFU/cm^2)		
	24 h	48 h	72 h	24 h	48 h	72 h
Listeria innocua						
LDPEC	9.17 ± 0.07	9.32 ± 0.03	8.82 ± 0.04	7.94 ± 0.17	7.93 ± 0.22	7.86 ± 0.12
LDPECvc-AS-48	<1.0 †	<1.0 †	<1.0 †	<1.11 †	2.90 ± 0.34 †	<1.11 †
LDPEThy-AS-48	<1.0 †	<1.0 †	<1.0 †	<1.11 †	2.60 ± 0.05 †	<1.11 †
PEAC	8.91 ± 0.10	8.92 ± 0.18	8.85 ± 0.10	8.22 ± 0.31	7.97 ± 0.17	8.03 ± 0.19
PEACvc-AS-48	<1.0 †	2.38 ± 0.15 †	<1.0 †	<1.11 †	3.88 ± 0.08 †	<1.11 †
PEAThy-AS-48	<1.0 †	<1.0 †	<1.0 †	<1.11 †	<1.11 †	<1.11 †
Lactobacillus fructivorans						
LDPEC	7.51 ± 0.15	8.45 ± 0.07	9.51 ± 0.18	4.88 ± 0.08	5.77 ± 0.22	5.41 ± 0.27
LDPECvc-AS-48	3.34 ± 0.13 †	4.15 ± 0.21 †	5.50 ± 0.17 †	<1.11 †	<1.11 †	2.70 ± 0.36 †
LDPEThy-AS-48	3.50 ± 0.20 †	3.54 ± 0.20 †	4.48 ± 0.28 †	<1.11 †	<1.11 †	2.42 ± 0.28 †
PEAC	7.78 ± 0.11	8.56 ± 0.18	9.45 ± 0.07	5.33 ± 0.21	6.89 ± 0.32	7.41 ± 0.37
PEACvc-AS-48	<1.0 †	3.48 ± 0.15 †	5.47 ± 0.18 †	<1.11 †	<1.11 †	2.89 ± 0.46 †
PEAThy-AS-48	<1.0 †	3.50 ± 0.14	4.47 ± 0.10	<1.11 †	<1.11 †	3.73 ± 0.25 †
Bacillus coagulans						
LDPEC	6.12 ± 0.17	8.19 ± 0.27	8.30 ± 0.12	4.03 ± 0.19	6.62 ± 0.25	6.98 ± 0.28
LDPECvc-AS-48	2.47 ± 0.07 †	1.30 ± 0.14 †	2.20 ± 0.24 †	1.88 ± 0.20 †	<1.11 †	<1.11 †
LDPEThy-AS-48	4.93 ± 0.23 †	4.46 ± 0.23 †	3.32 ± 0.21 †	3.52 ± 0.32	<1.11 †	1.11 ± 0.19 †
PEAC	6.00 ± 0.16	8.35 ± 0.21	8.11 ± 0.16	5.95 ± 0.14	8.27 ± 0.18	8.97 ± 0.28
PEACvc-AS-48	<1.0 †	1.48 ± 0.25 †	1.60 ± 0.27 †	<1.11 †	2.04 ± 0.22 †	<1.11 †
PEAThy-AS-48	<1.0 †	<1.0 †	<1.0 †	<1.11 †	<1.11 †	<1.11 †
Bacillus licheniformis						
LDPEC	3.0 ± 0.08	5.93 ± 0.14	6.40 ± 0.19	2.17 ± 0.20	4.34 ± 0.17	4.75 ± 0.28
LDPECvc-AS-48	<1.0 †	1.48 ± 0.11 †	<1.0 †	<1.11 †	<1.11 †	<1.11 †
LDPEThy-AS-48	<1.0 †	<1.0 †	<1.0 †	<1.11 †	<1.11 †	<1.11 †
PEAC	3.70 ± 0.19	6.65 ± 0.18	7.22 ± 0.31	1.96 ± 0.10	5.53 ± 0.28	5.40 ± 0.25
PEACvc-AS-48	<1.0 †	<1.0 †	<1.0 †	<1.11 †	<1.11 †	<1.11 †
PEAThy-AS-48	<1.0 †	<1.0 †	<1.0 †	<1.11 †	<1.11 †	<1.11 †

Note: LDPE, low density polyethylene from Stomacher® 80 Biomaster closure bag; PEA, polyethylene–polyamide vacuum-packaging film with textured dots; C, control films not activated with antimicrobials; Cvc-AS-48, films activated with carvacrol plus enterocin AS-48; Thy-AS-48, films activated with thymol plus enterocin AS-48; †: Significantly lower ($p < 0.05$) compared to the corresponding untreated control.

4. Discussion

According to results from the present study, carvacrol and thymol applied on plastic films, in combination with enterocin AS-48, showed strong antibacterial activity, reducing the concentrations of viable planktonic and sessile bacterial cells. Enterocin AS-48 dissipates the membrane potential of Gram-positive bacteria [12]. Carvacrol and thymol cause structural and functional damage to cell membranes [23]. The antimicrobial compounds used in the present study adsorbed best to LDPE and PEA films according to preliminary tests in which different plastic films were surface-activated with antimicrobials, and then tested for growth inhibition on plates seeded with bacteria (data not shown).

Results obtained on planktonic cells could be explained by diffusion of the antimicrobials adsorbed on the plastic film surfaces into the medium. According to the results obtained on planktonic cells, PEA film was the best carrier for immobilization of the tested antimicrobials. This plastic film is widely used for vacuum-packaging of foods. LDPE and PEA differ in surface roughness, which could explain the results obtained on antimicrobial activity and total viable cells. In particular, viable counts for sessile cells in control PEA films were higher compared to control LDPE films for *L. fructivorans* and for *B. coagulans*, suggesting that surface roughness might facilitate biofilm formation by these two bacteria.

It is also worth noting that, among the four bacteria included in the study, *L. innocua* was the most sensitive to the antimicrobials tested. Previous studies have shown that *L. monocytogenes* is highly sensitive to enterocin AS-48, with a minimum bactericidal concentration around 1 µg/mL [24]. However, another study reported that *L. monocytogenes* was much more resistant to this bacteriocin in

the sessile state [20]. In the present study, *L. fructivorans* was much more resistant in the planktonic state compared to *Listeria*. However, *L. fructivorans* was also highly sensitive in the sessile state. This could be explained assuming that the concentration of antimicrobials was higher at the surface of the plastic films compared to the liquid medium. The endospore-former, *B. coagulans*, was apparently more resistant than *B. licheniformis*. The observed difference in resistance could also be related to the lower number of cells found in cultures of *B. licheniformis*. However, both bacteria were very sensitive to the combination of thymol and AS-48 immobilized on PEA film. By contrast, LDPE film was much less effective on the higher cell density culture of *B. coagulans*, suggesting that the type of plastic support also has an influence on the efficacy of the activated film.

One of the main causes of food contamination is the formation of bacterial biofilms, because biofilms represent a persistent reservoir of bacteria and provide extra protection against disinfection [25]. Results from the present study clearly indicate that activation of plastic films with a combination of phenolic compounds and bacteriocin AS-48 caused strong reductions in the concentrations of viable sessile cells. This inhibitory effect was associated, in most cases, to a decrease or complete inactivation of planktonic cells as well. However, in some cases, no viable sessile cells were detected even in the presence of a relatively high residual population of planktonic cells, as in the case of *L. fructivorans* at 24 and 48 h incubation. These results are important, since biofilm formation may protect bacterial cells from antimicrobial substances by creating a diffusion barrier. One explanation for the observed reduction of viable sessile cell counts in the present study would be bacterial killing by the antimicrobials as they diffuse from the surface of the plastic films. Previous studies showed that enterocin AS-48 inhibited biofilm formation by *L. monocytogenes* and *B. cereus* [20,21]. Other studies have shown that bacteriocins can inhibit biofilm formation by human pathogenic bacteria [26–29]. Regarding essential oils, several studies have reported on their inhibitory effects on biofilm formation by bacterial pathogens [30–34]. Carvacrol, cinnamaldehyde, and thymol were reported to inhibit gene activation by quorum sensing, and this was related to inhibition of biofilm formation [18,35,36]. Incorporated in plastic films for food packaging, carvacrol and cinnamaldehyde were reported to inhibit biofilm formation [18]. Results from the study indicated that the combination of enterocin AS-48 with thymol was superior to carvacrol in reducing viable cell concentrations for both planktonic and sessile cells.

Altogether, results from the present study highlight the strong antimicrobial activity of combinations containing enterocin AS-48 and carvacrol or thymol adsorbed on LDPE and PEA films, and their efficacy in reducing the concentrations of both planktonic and sessile cells of the Gram-positive bacteria tested.

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