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Inhibition of *Salmonella Enterica* and *Listeria Monocytogenes* in Tofu by Activated Plastic Films

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Abstract: *Transmission of food borne pathogens through the food chain is a matter of concern. In the present study, tofu sausage slices were used as model for testing the antimicrobial activity of low-density polyethylene (LDPE) films surface-activated with tyrosol singly or in combination with p-hydroxybenzoic acid or a bacteriocin preparation from Enterococcus faecalis. Tofu slices challenged with cocktails of Listeria monocytogenes and Salmonella enterica strains were vacuum packaged in LDPE films activated with antimicrobials and stored at 10 ºC for one month. Best results against the two foodborne pathogens tested were obtained when tyrosol (1.5%) was used in combination with p- hydroxybenzoic acid (0.5%). Results from the present study highlight the potential of tyrosol for development of active packaging with antibacterial activity.*

Keywords: *Active packaging; Tyrosol; Bacteriocin; Listeria; Salmonella.*

1. INTRODUCTION

Tofu (soybean curd) is a popular food in Asian countries. Its consumption has been increasing in Western countries in recent years, especially by people with health concerns. Tofu is considered a healthy part of a well-balanced diet, as it is low in calories and rich in protein, iron, calcium, magnesium, and group B vitamins [1]. Usually, packaged tofu is sold in supermarkets, where it is normally found refrigerated (4 − 10 ℃). In Western countries, packaged tofu is also sold in other formats such as sausages for vegetarian consumers. Unpackaged tofu is sold only in open markets, typically by independent vendors who usually display the product under conditions prone to microbial contamination [2, 3]. Transmission of food borne pathogens through the food chain is a matter of concern. A recent study reported the presence of *Salmonella* spp. in 7% of unpackaged tofu samples in Bangkok [4]. Several incidents of tofu recalled due to *Listeria monocytogenes* contamination were also reported [5, 6].

The application of films or coatings activated with antimicrobial substances is an attractive approach to avoid transmission of food borne pathogens through the food chain. A large variety of materials, ranging from plastic films to coating solutions based on cellulose, pectin, starch, alginate, chitosan, or proteins have been tested against food borne pathogens in different food systems [7-10]. The activated materials allow a gradual release of antimicrobial compounds into the food surface and at the same time act as a barrier against cross contamination of the packaged or coated food product. Natural antimicrobial compounds (such as essential oils or bacteriocins) are good candidates for preparation of activated films or coatings since they have a good acceptance by the consumers [11, 12]. Films based on methylcellulose or hydroxymethylcellulose activated with the bacteriocin nisin achieved a decrease in viable counts of *Listeria* in refrigerated tofu that was dependent on the bacteriocin concentration applied [13]. Yet, we have found no other reports in the scientific literature on application of activated films for preservation of tofu.

Antimicrobial compounds found in essential oils have the advantage over bacteriocins of being naturally active on both gram-positive and gram-negative bacteria [11]. Tyrosol is a phenolic compound resulting from hydrolysis of the olive fruit main bitter compound, oleuropein [14-16], and is found in byproducts from table olives and olive oil industry. The aim of the present study was to determine the efficacy of plastic films activated with tyrosol (an antimicrobial compound naturally

found in olives) singly or in combination with p-hydroxybenzoic acid against *Salmonella enterica* and *Listeria monocytogenes* on sliced tofu sausages stored under temperature abuse conditions.

2. MATERIALS AND METHODS

2.1. Bacterial Strains

Listeria monocytogenes strains CECT 4032, CECT 911, CECT 936, and CECT 940, and *Salmonella enterica* strains CECT 915, CECT 916, CECT 4000, and CECT 4300 were supplied by the Spanish Type Culture Collection (CECT, Burjasot, Valencia, Spain). Strains were cultivated overnight at 37 ºC on trypticase soy broth (TSB, Scharlab, Barcelona, Spain). For preparation of inocula, overnight cultures were diluted one-hundred fold in sterile saline solution. Aliquots (1 ml each) of the dilutions obtained for each strain were mixed together in a single test tube to prepare the cocktails of *Listeria* and *Salmonella* strains. *Enterococus faecium* strain VMJ53 was isolated from fresh produce. This strain produced a bacteriocin with strong anti-*Listeria* activity. *Enterococcus faecalis* S-47 was used as a bacteriocin-sensitive test strain.

2.2. Preparation of Activated Plastic Films

The antimicrobials tyrosol and *p*-hydroxybenzoic acid were supplied by Sigma-Aldrich (Madrid, Spain). Low density polyethylene (LDPE) films were supplied by Andaltec (Martos, Jaen, Spain).

The bacteriocin producer strain *E. faecium* VMJ53 was inoculated (4%, vol/vol) in phosphate-buffered BHI broth (pH 7.2, 1 liter) overnight. After removal of cells by centrifugation (3,500 x *g* for 30 min), the bacteriocin from supernatant was recovered by ammonium sulfate precipitation at 70% saturation while being kept under refrigeration for 18 h. Precipitates were collected by centrifugation (4,500 x g for 30 min) and resuspended in 20 ml phosphate buffer saline (PBS). The obtained bacteriocin concentrate was dialysed overnight using 2,000 molecular weight cut-off benzoylated dialysis tubing (SigmaAldrich, Madrid) and filtered through 0.22 µm pore size low protein binding filters (Millex GV; Millipore Corp., Belford, MA, USA) under sterile conditions. The final bacteriocin preparation was serially diluted in sterile saline solution, and the titre of bacteriocin in units of activity (U) was determined by the spot test on phosphate buffered BHA seeded with the indicator strain *E. faecalis* S-47.

For surface activation with antimicrobials, films were placed on top of a sterile glass plate (10 x 10 cm). Then, 1 ml of the antimicrobial solution to be tested was deposited on the surface of the film and spread uniformly by using a sterile 5 ml plastic pipette. The coated films were allowed to dry for 2 h in a biosafety cabinet (Telstar, Madrid, Spain). Polyethylene films (10 x 10 cm) surface-activated with solutions containing 2.5% tyrosol or a combination of 1.5% tyrosol plus 0.5 % *p*-hydroxybenzoic acid were used to prepare plastic bags. The bacteriocin concentrate from strain VMJ53 (VMJ53) was tested at 200 and 400 U/ml singly or in combination with 2.5% tyrosol.

2.3. Assay of Activated Plastic Films on Sliced Tofu Sausages

Tofu sausages were purchased at a local supermarket. The sausages were sliced (1.5 cm diameter by 0.3 cm height) with a sterile knife under aseptic conditions and surface-inoculated on one side with 10 µl of the cocktails of *L. monocytogenes* or *S. enterica* strains prepared as described above. Controls were inoculated with 10 µl sterile saline solution. Tofu slices were allowed to dry at room temperature in a biosafety cabinet for 30 min before they were vacuum packaged in the activated plastic bags (three slices per bag). Two independent replicates (each one of them in duplicate) were prepared. Foods were stored for 7 days at 10 ºC in a Peltier-refrigerated incubator (Memmert GmbH, Schwabach, Germany). At desired incubation times, bags were removed and the food content was homogenized with 10 ml buffered peptone water. The resulting homogenates were serially diluted in sterile saline solution and plated in triplicate on PALCAM agar with added supplement (Scharlab) for determination of *Listeria* and brilliant green agar (Scharlab) for determination of *Salmonella*. The average number of colonies obtained after 24-48 h incubation of the plates at 37 ºC was used to calculate the viable cell concentration (expressed as Log_{10} colony forming units (CFU)/g).

2.4. Statistical Analysis

The average data ± standard deviations from replicates were determined with Excel programme (Microsoft Corp., USA). A paired *t*-test was performed at the 95% confidence interval in order to determine the statistical significance of data (Statgraphics Plus version 5.1, Statistical Graphics Corp, USA).

3. RESULTS AND DISCUSSION

The antibacterial activity of films coated with antimicrobials is shown in Table 1. *L. monocytogenes* was able to grow in the refrigerated tofu slices. A significant increase in viable cell counts of 2 log cycles was noticed by day 7 of storage, and viable counts were close to 8 log cycles by day 15. A previous study [17] reported that *L. monocytogenes* was able to grow in the three types of refrigerated tofu products tested (plain tofu, flavored tofu, and soft pudding tofu). After storage treatment for 24 days at 4-7 ºC, *L. monocytogenes* reached 7.1-7.9 log CFU/g in the samples [17].

Salmonella enterica cells were able to grow slowly in the tofu slices stored at 10 ºC. Growth was noticed after 7 days of storage, reaching viable cell concentrations that were 3.5 logs higher after 15 days compared to time 0. We found no previous studies on growth of *Salmonella enterica* in tofu. While this bacterium has a much more limited capacity to grow under refrigerated storage compared to *L. monocytogenes*, one study showed that *S. enterica* could reach high cell concentrations (of about 6 log CFU/g) in egg yolk after storage for 7 days at 10 ºC [18]. These results agree with data obtained in the present study with tofu and outline the risk of accidental cross contamination of tofu with this foodborne pathogen.

The use of films or coatings activated with antimicrobials is a practical approach to control bacterial proliferation while at the same time preventing cross-contamination with foodborne pathogens [7]. Results from the present study showed that polyethylene films coated with 2.5% tyrosol not only inhibited the proliferation of the cocktail of *L. monocytogenes* strains in tofu slices, but also decreased the numbers of viable cells during storage (Table 1). However, inactivation of the listeriae occurred slowly, and viable counts only decreased below detectable levels after 30 days of storage. This could be attributed to a slow diffusion of the tyrosol molecules from the coating into the food matrix. Combining tyrosol with *p*-hydroxybenzoic acid improved inactivation of the listeriae. The decrease in viable cell counts was noticed from day 3 of storage, reducing viable counts below detectable levels by day 15. The bacteriocin-activated films did not decrease viable cell counts, but instead delayed growth of the listeriae (Table 1). This was noticed mostly at day 7 of storage, where viable counts in the samples coated with the bacteriocin-activated film were significantly lower ($P < 0.05$) compared to controls. However, differences in viable counts between the two bacteriocin concentrations tested were not statistically significant ($P > 0.05$). The combinations of bacteriocin and tyrosol increased anti-*Listeria* activity to some extent. Best results were obtained for the highest bacteriocin concentration tested, since viable counts for the combined treatment were significantly lower ($P < 0.05$) compared to the single tyrosol treatment at days 7 and 15 of storage.

Films activated with tyrosol inhibited growth of the cocktail of *Salmonella* strains tested. However, viable cell counts still remained above detectable levels during storage (Table 1). As in the case of *Listeria*, the combination of tyrosol and *p*-hydroxybenzoic acid was also more effective against *Salmonella*, reducing viable cell counts below detectable levels by day 30 of storage. Viable *Salmonella* counts obtained for samples coated with bacteriocin-activated films were not statistically different ($P > 0.05$) compared to control samples, indicating that the bacteriocin preparation had no effect. This was expected, since the bacteriocin preparation used had no activity on Gram-negative bacteria in preliminary tests. Bacteriocin activity may be potentiated when tested in combination with other antimicrobials, especially those that alter the bacterial outer membrane permeability [12]. However, when the bacteriocin preparation was tested in combination with 2.5% tyrosol, viable cell counts were not statistically different ($P > 0.05$) compared to the single tyrosol coating.

There are scarce reports in the scientific literature about the use of activated coatings for preservation of tofu. A previous study showed that methylcellulose and hydroxypropylmethylcellulose films activated with the bacteriocin nisin showed a concentration-dependent anti-listerial activity in tofu refrigerated at 5 ºC for 30 days [13]. The study concluded that nisin film for packaging tofu has the potential to overcome the problems associated with the growth and survival of *L. monocytogenes* and the chances of secondary contamination of opened packages in household refrigerators. According to results from the present study, the combination of tyrosol and *p*-hydroxybenzoic acid was the most effective both against *L. monocytogenes* and *S. enterica*. The combination of tyrosol and bacteriocin also seems to be potentially useful for control of *L. monocytogenes*. Tyrosol has the advantage of being abundant in natural products such as by-products from the olive oil industry, and is also a cheap compound compared to other phenolic antimicrobials.

Table1. *Survival of foodborne pathogens in slices prepared from tofu sausages vacuum-packaged in polyethylene films coated with antimicrobials, during storage at 10 ºC.*

Treatment conditions	Viable counts (Log_{10} CFU/mg) during storage time (days)				
	0	**3**	**7**	**15**	**30**
Listeria monocytogenes					
Control	3.68 ± 0.21	3.60 ± 0.34	5.64 ± 0.27	7.87 ± 0.23	8.23 ± 0.28
Tyrosol (2.5%)	3.67 ± 0.17	2.44 ± 0.37	3.16 ± 0.36*	2.89 ± 0.44*	< 1.00
Tyrosol (1.5%) + PHB (0.5%)	3.64 ± 0.27	1.60 ± 0.42*	1.30 ± 0.23*,**	< 1.00	< 1.00
VMJ53 (200 U/ml)	3.55 ± 0.19	3.14 ± 0.12	3.74 ± 0.27*	6.97 ± 0.35	7.97 ± 0.44
VMJ53 (400 U/ml)	3.47 ± 0.22	3.07 ± 0.34	3.47 ± 0.44*	6.84 ± 0.56	7.94 ± 0.27
Tyrosol (2.5%) + VMJ53 (200 U/ml)	3.27 ± 0.27	2.14 ± 0.23	2.24 ± 0.37*	2.14 ± 0.37*	< 1.00
Tyrosol (2.5%) + VMJ53 (400 U/ml)	3.14 ± 0.23	2.01 ± 0.34*	1.77 ± 0.34*,**	1.27 ± 0.33*,**	< 1.00
Salmonella enterica					
Control	3.97 ± 0.23	3.20 ± 0.12	4.77 ± 0.37	7.48 ± 0.37	8.15 ± 0.12
Tyrosol (2.5%)	3.96 ± 0.42	2.72 ± 0.49	3.20 ± 0.16*	2.69 ± 0.27*	2.23 ± 0.34*
Tyrosol (1.5%) + PHB (0.5%)	3.94 ± 0.34	2.43 ± 0.15*	2.78 ± 0.32*	2.27 ± 0.25*	< 1.00
VMJ53 (200 U/ml)	3.95 ± 0.17	3.24 ± 0.27	4.67 ± 0.44	7.27 ± 0.47	8.02 ± 0.22
VMJ53 (400 U/ml)	3.98 ± 0.25	3.22 ± 0.17	4.56 ± 0.34	7.34 ± 0.27	8.11 ± 0.18
Tyrosol (2.5%) + VMJ53 (200 U/ml)	3.90 ± 0.22	2.77 ± 0.34	3.24 ± 0.23*	2.67 ± 0.24*	2.17 ± 0.44*
Tyrosol (2.5%) + VMJ53 (400 U/ml)	3.91 ± 0.34	2.56 ± 0.44	3.17 ± 0.21*	2.44 ± 0.37*	2.11 ± 0.27*

PHB, *p*-hydroxybenzoic acid.

U, bacteriocin units.

VMJ53, bacteriocin preparation from strain VMJ53.

* Counts were significantly lower ($P < 0.05$) compared to untreated controls.

** Counts were significantly lower ($P < 0.05$) compared to the single tyrosol treatment.

4. CONCLUSION

Results from the present study highlight the potential of tyrosol as a natural preservative for development of activated coatings, singly or in combination with other antimicrobials.

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