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In vitro Antifungal Activity of Biopolymeric Foam Activated with Carvacrol

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HIGHLIGHTS

- Significant differences were observed between sensitivity of the tested yeasts against biopolymeric foam activated with carvacrol.
- *Candida zeylanoides* and *Rhodotorula mucilaginosa* were the most sensitive strains against biopolymeric foam activated with carvacrol.
- *Aureobasidium pullulans* was the least sensitive yeast strain, showing clear zone of 20.4±0.3 mm.
- The biopolymeric foam activated with carvacrol was highly effective against all the studied yeasts.

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Acronyms and abbreviations
EO=Essential Oil
MB=Mater-Bi

ABSTRACT

Background: Active packaging represents a defining strategy to improve food quality and safety of the packaged foods. This study aimed to evaluate the *in vitro* ability of commercial biopolymeric foams, namely Mater-Bi (MB), activated with 20% of carvacrol, to develop a completely biodegradable and compostable packaging to inhibit the growth of spoilage and pathogenic yeasts.

Methods: MB foams, with and without carvacrol, were produced by melt mixing and the foaming process was performed in a laboratory press. The antifungal activity of foams containing carvacrol was tested applying the disk diffusion method. Statistical analysis was done using XLStat software version 7.5.2 for Excel.

Results: Statistically significant differences ($p<0.05$) were observed between sensitivity of the tested yeasts. *Candida zeylanoides* 4G362 and *Rhodotorula mucilaginosa* ICE29 were found to be the most sensitive strains with a clear zone of 28.9±0.1 and 29.0±0.1 mm, respectively. However, *Aureobasidium pullulans* was the least sensitive yeast strain, showing clear zone of 20.4±0.3 mm.

Conclusion: This study provided, for the first time, an *in vitro* analysis of the antifungal activity of MB foams activated with carvacrol against yeasts that commonly contaminate raw materials and processed foods. In conclusion, this biopolymer was highly effective against all the yeasts used as indicators strains.

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Introduction

Nowadays, the increasing demand for chemical preservative-free food products by retailers and consumers has determined the development of efficient packaging which are able to preserve food quality, to warrant freshness and to prolong the shelf life of fresh produce (Llana-Ruiz-Cabello et al., 2015; Souza et al., 2013). Moreover,

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the increasing concerns about the environmental issues suggest the replacement of conventional plastic materials for more ecofriendly and biodegradable polymers extracted from vegetable waste and by-products (Barzegar et al., 2014; Debiagi et al., 2014). In this context, totally compostable active packaging represents a winning strategy to improve food quality and safety (Wen et al., 2016). These types of packaging are able to inhibit the growth of undesired microorganisms present on the surface of foods (Appendini and Hotchkiss, 2002), and to reduce the environmental impact of the wastes (Song et al., 2014).

Thermoplastic polymers such as Mater-Bi (MB) are among biodegradable polymers obtained by renewable sources. MB is a commercial polymer completely biodegradable and compostable obtained from starch (Elfehri Borchani et al., 2015; Scaffaro et al., 2018). Moreover, in order to prepare “all-green packaging”, the incorporation of antimicrobial agents such as plant Essential Oils (EOs) into polymers have been widely applied (Campos-Requena et al., 2017). Considering that EOs extracted from aromatic plants are classified as GRAS (Generally Recognized As Safe) by the American Food and Drug Administration, these products can be used in any foods as alternative to the chemical compounds against bacteria, yeasts, and moulds (Calo et al., 2015; Gaglio et al., 2017, 2019; Tang et al., 2018).

Carvacrol is a phenolic monoterpenes commonly present in thyme and oregano EOs presenting a large inhibition spectrum against different microorganisms (Al-Bandak and Oreopoulou, 2007; Nostro and Papalia, 2012; Requena et al., 2016; Rojas-Graü et al., 2007). Recently, Lopresti et al. (2019) investigated the antibacterial activity of MB activated with carvacrol, evidencing its ability to inhibit the growth of undesired spoilage and pathogenic bacteria such as *Pseudomonas poae* and *Listeria monocytogenes*. However, to our knowledge, no study regarding antifungal activity of MB activated with carvacrol is present in literature. Based on the above considerations, the present study was aimed to investigate the *in vitro* ability of MB activated with 20% of carvacrol against spoilage and pathogenic yeasts in order to apply this new material for packaging fresh produce.

Materials and methods

Yeast strains and growth conditions

Several yeast strains of food origin, including *Aureobasidium pullulans* AD201, *Candida intermedia* 4G137-4G307-ICE86, *C. parapsilosis* ICE214, *C. zeylanoides* 4G362, *Cryptococcus curvatus* ICE84, *Pichia fermentans* 4G140, *Rhodotorula glutinis* AD64, and *R. mucilaginosa* ICE29 were used as sensitive strains to evaluate the inhibitory ability of MB activated with 20% of carvacrol. All strains belonged to the culture collection of the Agricultural Microbiology Unit of the Department of Agricultural, Food and Forest Science, University of Palermo, Italy. All yeasts were subcultured in test tubes containing Yeast Peptone Dextrose (YPD) broth, with the following formulation per liter: peptone (Biotec, Grosseto, Italy) 20 g, glucose (Carlo Erba Reagents s.a.s., Val de Reuil, France) 20 g, and yeast extract (Oxoid, Milan, Italy) 10 g. All tubes were incubated at 25 °C for 24 h.

Preparation of the MB foam disks

Preparation of the active MB foam followed the procedure (Figure 1) described by Lopresti et al. (2019). Briefly, pellets of a commercial biodegradable polymer Mater-Bi® EF05B (Novamont S.p.A, Terni, Italy) were dried under vacuum for 4 h at 60 °C. Dried pellets were melt mixed in a Plasti-Corder PLE-330 (Brabender, Barneveld, Netherlands) for 4 min at 120 °C and 60 rpm. The process was stopped in order to add 3% w/w of sodium bicarbonate (Solvay Group, Bruxelles, Belgium) as foaming agent and then restarted for 1 min. Finally, the system was before cooled and then foamed in a laboratory press (Carver Inc., Wabash, USA) at 170 °C and 140 bar. In order to prepare the antifungal foam, 20% of carvacrol (Sigma Aldrich, Milan, Italy), was added to the MB together with sodium bicarbonate. The foaming of the antifungal system was performed as reported previously.

Antifungal activity determination

MB foams were tested for antifungal activity by applying the disk diffusion method (Seydim and Sarikus, 2006). In brief, a water agar (2% w/v) base support (Cruciata et al., 2018) was overlaid with 7 ml of the YPD soft agar (0.7% w/v) inoculated at approximately 10^7 Colony Forming Unit (CFU)/ml. Six mm diameter MB foams disks containing carvacrol were placed onto the surface of the double agar layer. Sterile filter paper disks (Whatman no. 1) of the same diameter were soaked with 10 µl of cycloeximide (0.01% w/v) and used as positive control to inhibit the yeast growth, while MB foam disks without carvacrol were used as negative control. The tests were performed in triplicate.

Statistical analysis

Antifungal activity data were subjected to One-Way Variance Analysis (ANOVA) using XLStat software version 7.5.2 for Excel (Addinsoft, New York, USA). The Tukey’s test was applied to evaluate the level of

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significance between antifungal susceptibility of each strain. $p<0.05$ was considered significant.

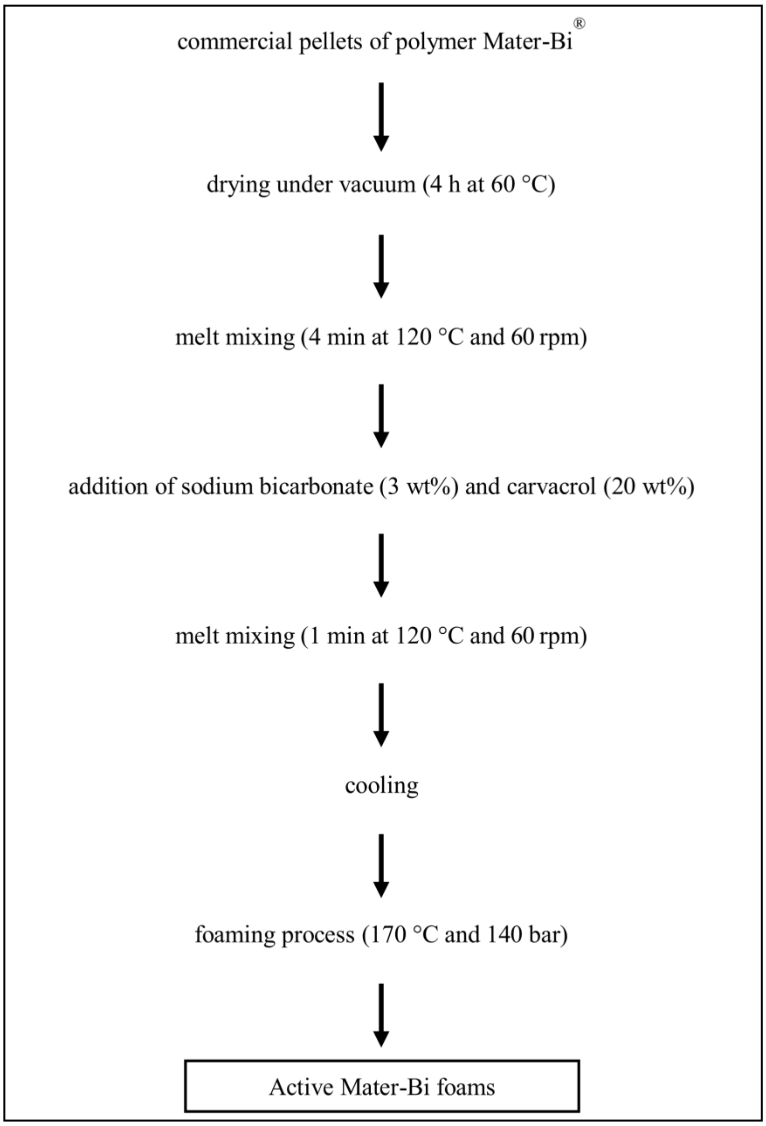

Figure 1: Flow diagram of active Mater-Bi foams production

Results

The antifungal activity of MB foams containing 20% of carvacrol analyzed in this study is shown in Table 1. The spectrum of inhibition was evaluated against spoilage and pathogenic yeasts. Statistically significant differences ($p<0.05$) were observed between the tested yeasts. The MB foams containing 20% of carvacrol were particularly effective against unicellular fungi, showing a diameter of the inhibition area around the disks higher than 20 mm for all strains tested. *C. zeylanoides* 4G362 and *R. mucilaginosa* ICE29 were found to be the most sensitive strains with a clear zone of 28.9±0.1 and 29.0±0.1 mm, respectively. However, *A. pullulans* was the least sensitive yeast strain, showing clear zone of 20.4±0.3 mm.

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Table 1: Inhibitory activity of Mater-Bi foams containing carvacrol against studied yeasts

Species	Strain	Inhibition *	Source of isolation
Aureobasidium pullulans	AD201	20.4±0.3 [g]	Wheat kernels
Candida intermedia	4G137	25.5±0.2 [de]	Ready to eat salad
Candida intermedia	4G307	25.0±0.2 [e]	Ready to eat salad
Candida intermedia	ICE86	26.3±0.1 [c]	Ice cubes
Candida parapsilosis	ICE214	23.1±0.3 [f]	Ice cubes
Candida zeylanoides	4G362	28.9±0.1 [a]	Ready to eat salad
Criptococcus curvatus	ICE84	27.6±0.1 [b]	Ice cubes
Pichia fermentans	4G140	25.9±0.1 [cd]	Ready to eat salad
Rhododotorula glutinis	AD64	27.1±0.3 [b]	Wheat kernels
Rhodotorula mucilaginosa	ICE29	29.0±0.1 [a]	Ice cubes

Results indicate the mean value of three independent assays.
Data within a column followed by the same letter are not significantly different according to Tukey's test.
* Mater-Bi foams activity is indicated by the width of the inhibition zone (mm) around the disk.

Discussion

The development of active packaging in order to prolong the shelf life of fresh foods represents one of the facing challenges of the academic and industrial researches. The *in vitro* evaluation of the antimicrobials against undesired microorganism is the first approach to study the features of active packaging in view to follow a general strategy for the food application (Llana-Ruiz-Cabello et al., 2015; Ramos et al., 2012). To our knowledge, this is the first work aimed to analyze the antifungal activity of MB foam activated with carvacrol. The *in vitro* antifungal assays of MB foam containing carvacrol revealed that this material was active against high concentrations (10^7 CFU/ml) of the sensible yeast strains indicating an applicative potential to inhibit spoilage and pathogenic yeasts.

Several studies have been previously performed on the antifungal activity of active packaging films of polypropylene containing different plant extracts such as basil (Synowiec et al., 2014), cinnamon (López et al., 2007), clove (Rodríguez et al., 2007), and oregano (Gutiérrez et al., 2009). These studies were mainly focused on the ability of these active packaging films to inhibit the growth of undesired yeasts showing lower inhibitory activity respect to our results. According to Synowiec et al. (2014), pullulan polymer coating with sweet basil extract showed considerable antifungal activity against *Rhizopus arrhizus* on apple surfaces. In another research, polypropylene and polyethylene/ethylene vinyl alcohol copolymer having 4% (w/w) of fortified cinnamon or oregano EO, revealed good growth inhibitory effect on *C. albicans*, *Debaryomyces hansenii*, as well as *Zygosaccharomyces rouxii* (López et al., 2007). Also, it was stated that the fortified cinnamon EO paraffin coating completely inhibited *C. albicans*, *Aspergillus flavus*, and *Eurotium repens* (Rodríguez et al., 2007). Gutiérrez et al. (2009) successfully developed an active packaging film coated with some plant EOs and found good antifungal activity against *C. albicans*, *D. hansenii*, and *Z. rouxii*.

Between the tested yeasts in this investigation, except for the strain *P. fermentans* 4G140 which is a spoilage yeast belonging to the *Saccharomycetaceae* (Qvirist et al., 2016), the other yeast species tested represent a risk for consumers (Novak Babič et al., 2015). In particular, all strains of *Candida* are agents of candidal infections (Van't Wout, 1996). *Candida* strains tested in this study were particularly sensitive to the active biopolymeric foams which showed a strong activity in terms of inhibition halos. The species of *Candida* used in the present research can be relevant pathogens. *C. intermedia* causes catheter-related fungemia (Ruan et al., 2010); *C. parapsilosis* is commonly associated with blood, wound, and tissue infections (Palmeira-de-Oliveira et al., 2009). In general, the pathogenic yeasts can be present in raw materials or enter the food chain during manufacturing, storage, and handling steps. For these reasons, it is evident that the use of MB foams containing 20% of carvacrol could be an effective strategy to store foods

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alternative to the use of oil-derived non-renewable polymers and to reduce the use of chemical preservatives in foods.

In the current study, we found that *C. zeylanoides* 4G362 and *R. mucilaginosa* ICE29 were the most sensitive yeast strains having a clear zone around the disk of about 29 mm diameter. Thus, they represent model strains for future *in vivo* applications to test the efficacy of the MB foams in contact with foods of different origin.

Conclusion

This study provided, for the first time, an *in vitro* analysis of the antifungal activity of MB foams activated with carvacrol against yeasts that commonly contaminate raw materials and processed foods. In conclusion, this biopolymer was highly effective against all the yeasts used as indicators strains. Further studies are necessary to produce MB foam trays incorporating carvacrol to test the *in vivo* ability of this biopolymer to inhibit the growth of the main spoilage and pathogenic microorganisms in different food model systems.

Author contributions

R.G., L.B., L.S., and F.L. designed the project of study; G.Ga. and G.Gu. conducted the experiments; R.G. analyzed the data; R.G. and L.S. wrote the manuscript. All authors revised and approved the final manuscript.

Conflicts of interest

There was no conflict of interest in this study.

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