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Active food packaging films with synergistic antimicrobial activity

Maksym Krepker [a], Rotem Shemesh [a, b], Yael Danin Poleg [a], Yechezkel Kashi [a], Anita Vaxman [b], Ester Segal [a, c, *]

[a] Department of Biotechnology and Food Engineering, Technion – Israel Institute of Technology, Haifa 3200003, Israel
[b] Carmel Olefins Ltd., P.O. Box 1468, Haifa 31014, Israel
[c] The Russell Berrie Nanotechnology Institute, Technion – Israel Institute of Technology, Haifa 3200003, Israel

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ABSTRACT

High-quality polymer nanocomposites containing a synergistic antimicrobial combination of essential oils (carvacrol and thymol mixtures) are produced and their outstanding performance as an active packaging for hummus is demonstrated. The interactive properties of carvacrol and thymol against *E. coli* are studied *in vitro*, as the combination of these essential oils (EOs), which provides a synergistic antimicrobial action, is loaded into halloysite nanotubes (HNTs) for the first time. The latter nano-scale carriers minimize the loss of the highly volatile EOs during the high-temperature polymer processing, enabling melt compounding and subsequent film production on a semi-industrial scale. The resulting films exhibit a synergistic antimicrobial activity against *E. coli*, outperforming films containing the individual EOs by both potency and shelf life. The films are also integrated into real food packaging, and their effect on *E. coli* growth in inoculated hummus is studied. Bacterial growth is reduced by seven orders of magnitude, leading to their complete eradication, while the antimicrobial performance of the control films was significantly weaker. These results demonstrate the immense potential of these films as food packaging materials to efficiently control bacteria growth in complex food systems.

1. Introduction

Microbial spoilage caused by pathogenic microorganisms, including bacteria and fungi, reduces the shelf-life of foods and increases the risk of foodborne illness and thus presents a major global concern (Böhme et al., 2012; Gram et al., 2002; Quintavalla & Vicini, 2002). As a result, new technologies are sought to provide safer food products and minimize food losses and wastage (Appendini & Hotchkiss, 2002; Irkin & Esmer, 2015; Kenawy, Worley, & Broughton, 2007; Otoni, Espitia, Avena-Bustillos, & McHugh, 2016; Scharff, 2012). Among these, active packaging, which is designed to control the development of decay and disease causing microorganisms, emerges as a promising technology for extending shelf life, maintaining food safety, reducing wastage and minimizing the risks for food-borne diseases (Han, 2005; Labuza & Breene, 1989; Malhotra, Keshwani, & Kharkwal, 2015; Vermeiren, Devlieghere, van Beest, de Kruijf, & Debevere, 1999). Despite its immense potential, antimicrobial food packaging is not used, to date, on a large scale. Currently used antimicrobial agents (e.g., oxidizing ions, such as copper or silver ions) provide only limited efficacy against microorganisms and mostly upon direct contact (Brandelli, 2015; Dallas, Sharma, & Zboril, 2011; Llorens, Lloret, Picouet, Trbojevich, & Fernandez, 2012; Rai, Yadav, & Gade, 2009). Thus, their limited effectiveness combined with concerns regarding their safety, and emergence of resistant bacteria, are the main factors limiting their common usage (Ahamed, AlSalhi, & Siddiqui, 2010; AshaRani, Low Kah Mun, Hande, & Valiyaveettil, 2008; Carbone, Donia, Sabbatella, & Antiochia, 2016; Gaillet & Rouanet, 2015; Realini & Marcos, 2014). Moreover, these antimicrobial materials do not satisfy recent consumer demand for natural and safe food products (Malhotra et al., 2015). Alternatively, essential oils (EOs), which are natural and highly effective antimicrobials against bacteria and fungi, are approved by the Food and Drug Administration (FDA) for food usage as GRAS (generally recognized as safe) (Burt, 2004; Dorman & Deans, 2000; Moreira, Alvarez, & Ponce, 2016; Vergis, Gokulakrishnan, Agarwal, & Kumar, 2013). EOs are particularly attractive compounds for incorporation into food packaging materials as they can be released as a vapor into the packaging headspace, sterilizing both the food

* Corresponding author. Department of Biotechnology and Food Engineering, Technion – Israel Institute of Technology, Haifa 3200003, Israel.
E-mail address: esegal@technion.ac.il (E. Segal).

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surface and the headspace environment (Bassolé & Juliani, 2012; Calo, Crandall, O'Bryan, & Ricke, 2015; Hyldgaard, Mygind, & Meyer, 2012; Lopez, Sanchez, Batlle, & Nerin, 2005; Martínez-Graciá, González-Bermúdez, Cabellero-Valcárcel, Santaella-Pascual, & Frontela-Saseta, 2015). Much effort is directed towards the incorporation of these sensitive compounds into commodity polymers by conventional manufacturing techniques, while minimizing their loss during high-temperature processing and maintaining their antimicrobial function (Malhotra et al., 2015; Ramos, Jiménez, Peltzer, & Garrigós, 2012; Sung et al., 2013; Suppakul, Miltz, Sonneveld, & Bigger, 2006; Valdes et al., 2015). Recently, we have demonstrated the use of Halloysite nanotubes (HNTs), which are natural aluminosilicate clay minerals (Liu, Jia, Jia, & Zhou, 2014; Lvov, Wang, Zhang, & Fakhrullin, 2015), as nanoscale carriers for carvacrol used as a model EO. Entrapment of carvacrol within HNTs allows the incorporation of this highly volatile EO into different polymer matrices at elevated temperatures of up 250 °C (Shemesh, Krepker, Nitzan, Vaxman, & Segal, 2016). The resulting nanocomposites are characterized by a higher carvacrol content within the final films in addition to the sustained release profile of the entrapped EOs in comparison to the control films with no HNTs (Shemesh et al., 2016; Shemesh et al., 2015b). Yet, possible interactions of EOs with various food components (e.g., fats, proteins and sugars) and other extrinsic factors (such as temperature and specific microorganisms) often result in inferior antimicrobial activity, requiring the use of higher dosages of antimicrobial agents (Burt, 2004). This in turn may result in undesirable negative organoleptic effects (Calo et al., 2015; Martínez-Graciá et al., 2015). Thus, exploiting synergistic interactions between several EOs or their principal constituents may increase their efficacy at lower concentrations and minimize organoleptic changes in various food products, such as hummus, which was chosen as a food model in this study (Martínez-Graciá et al., 2015). Hummus is one of the most common dishes in the Middle East, and its popularity in the United States and Europe has increased significantly over the past decade. It is prepared from cooked and mashed chickpeas (or other beans), blended with some of ingredients of choice such as lemon juice, garlic, olive oil etc. Hummus is a high moisture food with a water activity value around 0.98 and moderate acidic pH around 5.4. It is rich with nutrients which readily promote microbial growth of *Listeria monocytogenes*, *Salmonella* and *E. coli* (Ashenafi & Busse, 1991; Ashenafi, 1991). Moreover, hummus was shown to inactivate several antimicrobial agents, such as nisin and citric acid, in spite of their *in vitro* activity (Al-Holy, Al-Qadiri, Lin, & Rasco, 2006). Recent large recalls of hummus products due to bacterial contamination were recently recorded by U.S. Food and Drug Administration (FDA), highlighting the need for its safe supply chain (FDA, 2015; 2016).

Herein, we aim at developing antimicrobial LDPE films containing a synergistic combination of carvacrol (CV) and thymol (TY) and demonstrating that such systems may exert superior antimicrobial action (Campos-Requena, Rivas, Pérez, Figueroa, & Sanfuentes, 2015; Cosentino et al., 1999; Didry, Dubreuil, & Pinkas, 1993; Guarda, Rubilar, Miltz, & Galotto, 2011). Following the assessment of the interactive properties of neat CV and TY against *E. coli in vitro*, we load combinations of these EOs into the nanocarriers, producing for the first time an HNTs-based additive for polymer compounding that contains two different active compounds, which provide a synergistic antimicrobial action. Subsequently, the loaded HNTs were incorporated into LDPE matrix via conventional melt compounding using semi-industrial equipment, and the ability of HNT-entrapped volatile EOs to withstand the processing at elevated temperatures while preserving the antimicrobial activity was assessed. The antimicrobial activity of CV-TY containing LDPE films (in terms of both long-lasting and potency) was characterized *in vitro* and in a real food system (hummus spread) and compared to LDPE films containing the individual EOs.

2. Materials and methods

2.1. Materials and bacterial cultures

Halloysite Nanotubes (HNTs) were supplied by NaturalNano (USA) and dried at 150 °C for 3 h prior to use. Low-density polyethylene (LDPE), Ipethene 320, was supplied by Carmel Olefins Ltd. (Haifa, Israel) with a melt flow rate of 2 g/10 min. Carvacrol (98%), thymol (98%), Bacto agar, Luria-Bertani (LB) medium, Nutrient Broth (NB) medium, Tween-80 were purchased from Sigma Aldrich (Israel). NB bacto-agar was purchased from Becton Dickinson (USA). Preservative-free canned whole chickpeas (Pri Galil Ltd, Israel) were purchased in a local supermarket.

Escherichia coli (*E. coli*, ATCC 8739), were maintained on polystyrene beads at −80 °C. For antimicrobial susceptibility and agar diffusion assay, bacterial cultures were prepared by incubating one bead in 5 mL LB medium for 16 h at 37 °C under shaking (250 rpm). Subsequently, the culture was diluted 1:100 in fresh LB medium and incubated for an additional ~1.5 h, allowing the cells to enter their logarithmic stage. Bacterial growth was monitored photometrically by measuring the optical density at a wavelength of 600 nm (OD_{600}). The number of cells is directly proportional to the OD_{600} measurements (1 OD_{600} = 1 × 10^8 cells mL^{-1}, following calibration curve, data not shown) and bacteria concentration was calculated from the obtained OD_{600} values. For storage experiments, one bead was cultured overnight in 3 mL NB medium at 37 °C under shaking (250 rpm). On the following day, the culture was diluted 1:100 in fresh NB medium and incubated for an additional ~1.5 h, allowing the cells to enter their logarithmic stage. As the culture reached OD_{600} value of 0.6, it was diluted with 1% NB to obtain a bacterial stock suspension at a concentration of 10^4 colony forming units (CFU) mL^{-1}.

2.2. Antimicrobial susceptibility to pure carvacrol, thymol and their combinations assay

Bacterial susceptibility to pure carvacrol, thymol and their mixtures was characterized by the standard chequerboard procedure for antimicrobial susceptibility for combination of antimicrobials (Franzot & Casadevall, 1997; Pei, Zhou, Ji, & Xu, 2009). Accordingly, stock aqueous emulsions of thymol and carvacrol (at a concentration of 6400 mg L^{-1} in MillyQ water (18.2 mΩ-cm) were prepared by ultrasonication (Vibra cell VCX 750, Sonics & Materials Inc., USA). Serial dilutions of the carvacrol and thymol stock emulsions were prepared in a 96-well plate in LB medium at a concentration range of 0–350 mg L^{-1} and 0–400 mg L^{-1} for carvacrol (CV) and thymol (TY), respectively. *E. coli* culture in a logarithmic stage was added to each well to contain approximately 10^6 CFU mL^{-1} and the volume in each well to a total value of 200 μL. Control wells were similarly prepared (on the same plate) to include fresh LB medium instead of *E. coli* culture. The plate was sealed with SealPlate® (Excel Scientific Inc., Victorville, CV, US) and incubated at 37 °C for 16 h under gentle shacking in a microplate reader (Varioskan Flash, Thermo Scientific, USA). Absorbance measurements were recorded at 600 nm every 3 min. The MICs of CV and TY were considered as the lowest concentration that completely inhibited bacterial growth after 16 h (Gutierrez, Barry-Ryan, & Bourke, 2008). The summed fractional inhibitory concentration (ΣFIC) index (Lambert, Skandamis, Coote, & Nychas, 2001) of CV and TY in a combination was defined as the sum of individual FIC indices of CV and TY: