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Development of a moisture-activated antimicrobial film containing ground mustard seeds and its application on meat in active packaging system

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ABSTRACT

An antimicrobial cellulose acetate film containing finely ground mustard seeds was developed. The effect of the film properties on the allyl isothiocyanates (AITC) formation and release into the headspace was investigated. Less porous structure and larger thickness caused slower moisture absorption and consequently a slower AITC formation and release rate. For its application in food, the film was tested on whether the concentration of AITC was sufficient to inhibit bacterial growth in ground beef with different fat contents. The AITC concentration for the low-fat ground beef was lower in the product and higher in the headspace compared to medium fat ground beef. The shelf life was extended by 3.7 and 0.6 days for low fat and medium fat ground beef respectively. Besides a novel way to control the compounds' release to the packaging system, this study also shows the importance of the food composition for tailoring the effective active packaging. (150 words).

1. Introduction

Spoilage bacteria and microbial contamination are major issues for food industries in maintaining quality and safety of food products (Roodhuyzen, Luning, Fogliano, & Steenbekkers, 2017). Antimicrobial packaging is being developed by incorporating specific compounds into the packaging material to protect the food from spoilage bacteria during storage. The inhibition of spoilage can be done either by direct contact between packaging material and foods, or by indirect contact, in which the antimicrobial packaging releases a volatile antimicrobial agent into the headspace of the packaging (Quintavalla & Vicini, 2002; Yildirim et al., 2017). The volatile antimicrobials, e.g. essential oils (Rao, Chen, & McClements, 2019) and allyl isothiocyanates (AITC) (Luciano & Holley, 2009) have a greater antimicrobial activity at a lower concentration on the inhibition of microbial growth than the non-volatiles that are normally applied by direct addition into food products (MejÍA-Garibay, Palou, & LÓPez-Malo, 2015; Suhr & Nielsen, 2003). The direct addition of compounds causes a quick interaction between the compounds and food components, which can negatively affect the food sensory characteristics (Kapetanakou & Skandamis, 2016). The types of antimicrobial compounds, the release of compounds into the headspace, and its interaction with food products should be considered to effectively inhibit the bacteria.

AITC is a lipophilic volatile with strong antimicrobial properties that effectively inhibits the growth of a wide range of microorganisms (Kurek et al., 2017; Quiles, Manyes, Luciano, Manes, & Meca, 2015). For application in an antimicrobial packaging system, an active carrier is required to release the AITC into the packaging headspace where the AITC can effectively suppress the bacteria growth in the food surface. Mustard seeds can be an active carrier that can release AITC in a packaging system. Mustard seeds are a good natural source of sinigrin (Sharma, Rai, & Prasad, 2018), a precursor for the AITC formation, and contain also an endogenous enzyme, called myrosinase (Nakano, Yamada, Bednarek, Nishimura, & Hara-Nishimura, 2014). Myrosinase and sinigrin are located in different cells; Myrosinase in myrosin cells and sinigrin in s-cells (vacuoles) (Kissen, Rossiter, & Bones, 2009). The AITC formation occurs once the myrosinase enzymatically interacts with the sinigrin after cell disruption, e.g. through fat extraction or milling process, in the presence of water (Oliviero, Verkerk, Van Boekel, & Dekker, 2014). The moisture is essential to trigger the AITC formation (Chen, Chen, Xu, & Yam, 2019). In a packaging system, the packaged (model) foods can vaporize the moisture that can be absorbed by ground mustard seeds to cause a release of AITC into the headspace. Such a packaging system is defined as a triggered release system created to prevent the premature release of volatiles until the release is triggered by an internal stimulus in a closed packaging system (Chen et al., 2019).

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The incorporation of the antimicrobial agent into the packaging polymer prevents the premature excessive release of the AITC (Appendini & Hotchkiss, 2002; Fang, Zhao, Warner, & Johnson, 2017). Methods with the incorporations of pure AITC into film polymers, like an encapsulation of AITC /β-cyclodextrin complex encapsulated in electrospun nanofibers (Aytac, Dogan, Tekinay, & Uyar, 2014) and encapsulation of AITC in poly(lactic-co-glycolic acid) nanoparticles (Encinas-Basurto et al., 2017), have been studied to control the AITC volatility. These methods resulted in a sustained release of AITC due to a slower diffusion rate of AITC through the packaging material. These researches mainly investigated the release of volatiles using pure (chemical) AITC in the package. In the current study, ground mustard seeds were used as a natural source of AITC to be incorporated into the packaging polymer. This method can potentially enable a triggered release system by moisture by controlling both the AITC formation and release, which is not possible in the incorporation of pure AITC in the packaging materials.

For the application of an antimicrobial package on food products, in-depth knowledge of the food, e.g. food composition and structure is important because interactions between the applied antimicrobial compounds, the package, and the food components are possible. The interactions affect the antimicrobial activity of the compound against the bacteria growing on, and in, a food product (Keppler et al., 2017). For example, AITC that was added into a food model system containing whey protein isolate caused covalent binding between AITC and the protein, and no antimicrobial effect of AITC on the bacteria was observed (Keppler et al., 2017). Other components, like fat, could also affect the distribution of AITC between the fat phase and other phases in food due to its solubility in the fat phase (Giroux, Perreault, & Britten, 2007; Tsao, Yu, Friesen, Potter, & Chiba, 2000). Besides the factors influencing the AITC release, the understanding of the effects of fat influencing the absorption of AITC by food products could help to improve the growth inhibition of spoilage bacteria by antimicrobial packaging.

In this study, ground mustard seeds were applied in a hygroscopic polymer film made of cellulose acetate (Białopiotrowicz & Jańczuk, 2002), to be used as a triggered release system in a packaging system. The current study aims to develop moisture activated antimicrobial film containing ground mustard seeds and to understand the release mechanism of AITC from the ground mustard seeds incorporated in the film, by investigating the effect of thickness of the film, temperature, and relative humidity, on AITC release in the packaging system. This study also investigated the antimicrobial effect on ground beef. The effect of the fat content on the AITC release from the film and absorption by the food and the resulted antimicrobial effects against the spoilage bacteria in the ground beef was studied. The shelf life extension of ground beef containing different fat content was also predicted by modelling the microbial growth. This study shows that active packaging design can be optimized by tailoring the properties of active sources and food products to reach the desired and extended shelf life of food products.

2. Materials and methods

2.1. Materials

Mustard seeds were purchased from Natuurproduct.com, Jacob Hooy Brown. Lean ground beef (around 9 % fat) and normal ground beef (around 19 % fat) with the same production dates were supplied from a local supermarket, Albert Heijn. Cellulose acetate (39.8 wt% acetyl) used as film polymer and glycerol (≥99.5 %) were purchased from Sigma Aldrich. Sterile Cryovial was from Simport Scientific and Peptone physiological salt solutions (PFZ) were from Tritium Microbiology. Disposable inoculation loops were from VWR International. Other chemicals used in this study were of analytical grade and purchased from Sigma Aldrich or Merck.

2.2. Film preparation by solution casting

Films incorporating mustard seeds were produced by using a solution casting method modified from Gonçalves et al. (2019). To obtain a varying thickness of the film, different weights of cellulose acetate (300, 700, 1100, and 1500 mg) were used with 500 mg of ground mustard seeds with particle sizes <100 μm (Bahmid, Pepping, Dekker, Fogliano, & Heising, 2020). For the control, 700 mg of cellulose acetate was used. The cellulose acetate was solubilized in 25 mL acetone as a solvent in 50-mL Duran flasks and then stirred for 10 min using magnetic stirrers at room temperature until well dissolved. Glycerol (3 mL) acted as a plasticizer was also added to the solution and stirred for 10 min until well dissolved. Finally, the ground mustard seeds (500 mg) were slowly added into the solution while stirring continuously, and then the Duran flasks were closed tightly with caps to avoid evaporation of the solvent during stirring. The stirring was stopped after 3 h when the mustard particles, glycerol, and cellulose acetate turned into a homogenous mixture. The mixtures were subsequently poured into glass Petri dishes (diameter 100 mm) and stored in a fume hood for 3−5 h to evaporate the solvent at room temperature. Afterward, the formed films were removed from the Petri dishes and then cut in pieces with a size of 2 by 2 cm. Each film was weighed and the thicknesses were measured using a caliper, with 0.001 mm precision, at four points for each side of the films. To prevent premature release of AITC during storage before its usage, the films were stored in an impermeable glass Duran flask at −20 °C until usage.

2.3. Confocal microscopy and scanning Electron microscopy

The distribution of ground seeds in the film was measured by confocal microscopy. The confocal microscopy was used to characterize the phase composition of the substrate in the film polymer (Lattante, Perulli, & Anni, 2014). Fluorescent dye calcofluor white (0.002 wt%) were prepared to stain the films containing the ground seeds and also the control film (without incorporation of ground seeds). The dye mixture (30 μL) was added to the films (2 × 2 cm) that were placed on a glass microscope slide. The films were visualized by a confocal laser scanning microscope (LSM) type 510 (Zeiss, Oberkochen, Germany) using 405 nm blue diode laser for calcofluor white. All pictures were analyzed with the ZEN blue edition (Carl Zeiss Microscopy).

The surface of the films was investigated by scanning electron microscopy (SEM). The films were snap-frozen in liquid nitrogen and subsequently freeze-dried for SEM analysis. A film was attached on SEM sample holders using carbon adhesive tabs (EMS, Washington, USA), sputter-coated with a 15 nm thick layer tungsten (EM SCD 500, Leica, Vienna, Austria) and subsequently analyzed with a field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, Netherlands) with SE detection at 2 kV and 6.3 pA.

2.4. Experimental packaging design with a mustard seeds-incorporating film

Glass vials (10 mL) were used to simulate an impermeable package, which was adapted from the previous study (Bahmid, Heising, Fogliano, & Dekker, 2020). A 2-by-2 cm film (with varying thickness) was put inside the glass vials containing water or a saturated salt solution (1 mL) by attaching the film under the closed lid. The vials containing a cellulose acetate film without ground mustard seeds were used as a control. The vials were held under water for a minute to ensure there was no leakage in the package. Different liquids were added into the flasks to study the effects of relative humidity (RH) on the AITC release; Milli-Q water for RH 100 % and a saturated salt solution (Natrium Chloride, NaCl) for RH 75.5 % (Cerisuelo et al., 2012). Besides, the vials were stored at different temperatures (5, 10, and 20 °C). Each sample was prepared in a new batch for each daily measurement. To understand how moisture can be an internal stimulus for AITC release in the

packaging system, the moisture absorption of the films, sinigrin content in ground mustard seeds, and AITC concentration in the headspace were measured for 4 days.

2.4.1. Determination of moisture absorption into the film

Moisture plays a role in the hydrolysis reaction of sinigrin and therefore provokes AITC formation. The films were weighted using a balance (Mettler A-150, Columbus USA) to measure the moisture absorption of the film. The percentage of moisture absorption was calculated using the following Eq. (1);

$$Wa = \frac{W_t - W_o}{W_o} x100\% \quad (1)$$

where W_a is the percentage of absorbed moisture (%), W_t is the weight of the film in different time points (g) and W_o is the initial weight of the film (g).

2.4.2. Determination of sinigrin content in the film

The films are firstly cut in pieces of about 0.2−0.3 cm to enlarge the surface of the films, so the solvent can penetrate the films to extract the sinigrin content of the film. The sinigrin was extracted from the small pieces of film using the method described in the previous study (Bahmid, Heising, et al., 2020). The extracted solutions were collected in an HPLC vial and analyzed using HPLC (Thermo Scientific UHPLC focused, Dionex UltiMate 3000) with a C18 reversed-phase HPLC column (Merck; Lichrocart 100 LP18). The measurement method and software settings of the HPLC was according to Bahmid, Pepping, et al. (2020). To quantify the sinigrin concentration in the films, the ratio of sinigrin to glucotropeolin (as an internal standard) areas were multiplied with the mol of the glucotropeolin per weight of the films and the relative response factor (RRF) of sinigrin (1.053).

2.4.3. Determination of Allyl isothiocyanate in the headspace

The AITC concentrations in the headspace were measured with Gas Chromatography - Flame Ionization Detection (GC-FID) (Thermo-Scientific Focus GC), using direct injection. A sample of 500 μl gas was taken out of the headspace of the 10 mL-vial containing the film and then manually injected into the GC apparatus. The method and software performed in the GC apparatus were the same as described in the previous study (Bahmid, Heising, et al., 2020). To calculate the concentration of AITC in the headspace, the obtained area was converted to concentration (μg/L) by using the calibration quantified using pure AITC (Allyl isothiocyanate, 97 %) dissolved in Hexane, with the range of used concentrations 1–1000 ppm.

2.5. Experimental design of antimicrobial packaging containing the mustard seeds-incorporating film and ground beef

The antimicrobial effect of the film incorporated with ground mustard seeds was tested to a real food, ground beef. Antimicrobial packages with ground beef were prepared as described in Section 2.4. The film thickness used in this experiment was the thickest film produced with 1500 mg of cellulose acetate. As shown in Fig. A1 in the supplementary materials, 1 g of extra-lean ground beef (around 9 % fat content) or medium fat ground beef (around 19 % fat content) was taken out of the package under aseptic conditions, weighed, given a flat-round shape and placed in the bottom part of the sterilized glass vials. The films were attached under the lid of the vials. The vials without films were used as control. These preparations were conducted under an aseptic condition in a disinfected safety flow cabinet to ensure no cross-contamination during the preparation of samples and packaging. Glasses and pipettes used in the experiments were sterilized at 121 °C for 15 min and the flow cabinet was disinfected under UV light and using ethanol (70 %) before sample preparations. The vials were then kept at refrigerated temperature (around 5 °C). The measurement of AITC concentration in the headspace and ground beef and microbial growth of the ground beef was conducted for 10 days.

2.5.1. Determination of AITC in the headspace and ground beef

For headspace AITC, the measurement was conducted as described in the previous part 2.4.3. The AITC concentrations in the ground beef were measured by using the adapted method from Marton and Lavric (2013). The ground beef was taken out from the flask and then added into 10 mL hexane in 15-mL tubes. The mixtures were vortexed for 5 min and then centrifuged at 2627 *g* at 20 °C for 5 min. The supernatant (1 mL) was filtered using a PTFE 45 um filter (Phenomenex) into brown HPLC vials. The samples were measured using GC-FID in conjunction with a 10 μL-syringe cemented needle (Hamilton Microliter), connected to an autosampler (Thermo-Scientific, Waltham, MA, USA, TriPlus Autosampler). The method of measurement using the GC apparatus was the same as described in Section 2.4.3.

2.5.2. Microbiological analysis of ground beef

Non-selective plate count agar (PCA) was used as a medium to count the bacteria of the ground beef. The pipettes and other materials used in the experiments were sterilized at 121 °C for 15 min and the flow cabinet was disinfected under UV-light and using ethanol (70 %) before the analysis was started. The ground beef was taken out of the packaging (glass vial) and then put in a sterile *Stomacher* bag. Sterile peptone water (9 mL) was added into the bag to achieve 10^{-1} dilution. Afterward, the content in the Stomacher bag was homogenized for 1.5 min at 260 rpm by using Stomacher® *400 Circulator Lab Blender*. After the ground beef was completely homogenized, the homogenate was transferred into a sterile *Greiner* tube. A 1-mL sample was diluted in 9 mL of peptone physiological salt solutions (PFZ) up to 10^{-6} and 100 μl of each dilution was plated out on PCA plates. The plates were incubated at 30 °C for 48 h and then the colonies were counted. Only the plates containing 20–300 colonies were counted to determine Log_{10} CFU/mL.

2.5.3. Kinetic modeling of microbial growth and determination of ground beef shelf-life

The kinetics of microbial growth of the spoilage bacteria in ground beef was described by using using the Gompertz model Eq. (2) modified by Zwietering, Jongenburger, Rombouts, and van' t Riet (1990)):

$$ln\frac{N}{N_o} = A_s.\ exp\left[- exp\left(\frac{\mu_{max}.e}{A_s}.(\lambda - t) + 1\right)\right] \quad (2)$$

where N (CFU/g) is the number of bacteria at time t, N_o (CFU/g) is the total population of bacteria at time 0, A_s is asymptotic value, μ_{max} is maximum specific growth rate (h^{-1}), ƛ is lag time (hours) and t is time (hours).

The shelf life of the ground beef was determined by using the Eq. (3) derived from the Eq. (2) as the following equation

$$t_s = \lambda - \frac{A_s.\left\{ln\left[- ln\left(\frac{\ln(N_s) - ln(N_o)}{A_s}\right)\right] - 1\right\}}{\mu_{max}.e} \quad (3)$$

where t_s is the shelf life of a product, N_s is the maximum number of spoilage bacteria at the end of the shelf life of the food. The level of spoilage bacteria at the end of the shelf life of ground beef (N_s) was defined to be 10^7 CFU/g (Koutsoumanis, Stamatiou, Skandamis, & Nychas, 2006).

2.6. Statistical analysis

All data of headspace AITC concentration, sinigrin content, and the total population of bacteria was performed in triplicate. The data of the survival ratio (N/N_0) of total bacteria was fitted with the non-linear regression model in Eq. (2) by minimizing the sum of squared differences (SSR) with the Solver add-in in Microsoft Excel to estimate the

growth parameters consisting of μ_{max}, A_s, and λ. A_s is assumed to be constant for all samples. The standard deviation of the parameters and correlation amongst parameters were calculated using the macro SolverAid (Hu, Xie, Chau, & Si, 2015). Significant differences (at the level of $p < 0.05$) of the experimental data and parameters were evaluated.

3. Results and discussion

3.1. Thickness, morphology of the film, and distribution of ground seeds in the film

The thickness of the film with ground mustard seeds incorporated is shown in Table A1 in Supplementary materials. The average thicknesses of the films made using 300 mg (thinnest film) to 1500 mg (thickest film) of cellulose acetate were around 134 ± 13–293 ± 25 μm, while the thickness for the control film was around 121 ± 10 μm. The results revealed a linear increase of the thickness with the increasing content of cellulose acetate in the film.

The morphology of the thickest and the thinnest films as well as the control film without ground mustard seeds was observed using SEM (Fig. 1a–f). Fig. 1a reveals a sponge-like porous structure (white arrows) on the surface of the film control (without the addition of ground mustard seeds). The sponge-like porous structure can also be seen in the cross-section image as shown in Fig. 1d. With the incorporation of ground mustard seeds into the polymer solution, a strong reduction of the porosity on the surface was observed and the morphology of the film surface became more compact, homogenous, and smoother as shown in Fig. 1b and c. The porous structure indicated by white arrows was possibly due to the evaporation of acetone during the drying process. The sponge structure is also known as "breath-figure" structures on the surface obtained from the evaporation of the solvent droplets on cellulose acetate-glycerol (as plasticizer) solution of the film (Furtado, Hilamatu, Balaji, Ando, & Petri, 2020; Kalaycıoğlu et al., 2020). The smoother surface and low porosity in the film containing ground mustard seeds are in line with the results obtained by Rajeswari, Christy, Swathi, and Pius (2020) who investigated the effect of the addition of polysaccharide (fine powder of sodium alginate and carrageenan) on the film formation. The ground mustard seeds were well embedded in the cellulose acetate, so the film was relatively smooth, flat, and had an unbroken matrix in the absence of pores.

The film with 1500 mg cellulose acetate (threefold more than the ground seeds) into the film led to a nonhomogeneous structure of the film. In the cross-section image of the films containing the ground seeds, Fig. 1f depicts two distinct layers; a layer of cellulose acetate polymer for the bottom part and a layer of a mixture of ground mustard seeds and the cellulose acetate for the upper part. These bilayers did not appear in the film with a lower amount of cellulose acetate (Fig. 1e). The bilayers were possibly due to the excessive amount of cellulose acetate polymer added into the mixture, resulting in a separate homogenous layer of cellulose acetate. In general, the results show that the ground mustard seeds could be used as a filler of the pores in the cellulose acetate.

Fig. 1g–i shows the images of confocal microscopy of the films. The black flakes indicated with yellow arrows represent the ground mustard seeds phase dispersed in the films. Without the addition of ground mustard seeds, no black flakes were expectedly found in the images (Fig. 1g). In the samples with ground mustard seeds, the flakes were visible and homogenous in almost the whole area of the film (Fig. 1h–i). More black flakes can be seen in the film in Fig. 1h, which was expected since the film contains less cellulose acetate and a relatively higher content of ground mustard seeds.

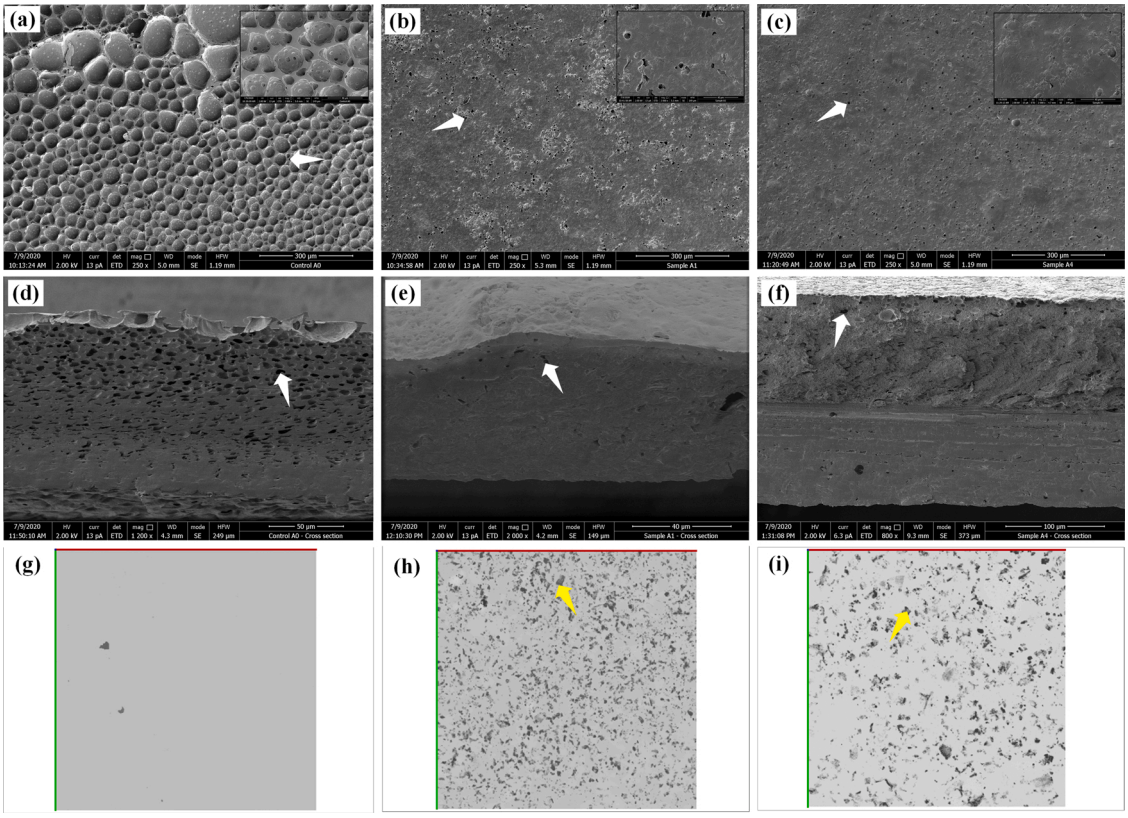

Fig. 1. Morphology of film surfaces **(a-c)** and film cross-section **(d-f)** measured using SEM, and confocal laser scanning micrographs of ground mustard seeds dispersions **(g-i)** in the films measured using confocal microscopy. **Left, middle, and right** pictures depict the control film made with 700 mg of cellulose acetate and without ground mustard seeds, the film with 500 mg mustard seeds produced with 300 mg of cellulose acetate, and the film with 500 mg mustard seeds produced with 1500 mg cellulose acetate, respectively. White and yellow arrows point to the porous structure and black flakes indicating ground mustard seeds distribution in the film, respectively.

3.2. Moisture absorption, sinigrin content remaining in the film, and AITC release in the headspace

Fig. 2a depicts the percentage of moisture absorbed by the film with and without ground mustard seeds monitored for 5 days at 100 % RH and 5 °C. The thickest film (made using 1500 mg cellulose acetate) absorbed moisture up to 15 % at day 1, which was half of the percentage of moisture absorbed by the thinnest film (made using 300 mg cellulose acetate). The relative decreasing content of cellulose acetate in the film with ground seeds increased the moisture absorption, but the control film had the highest percentage of moisture absorption. At day 1, the percentage of water absorption increased to 40 %. The absorption of the moisture in the film might be related to thickness (Table A1 in supplementary materials) and surface roughness (Fig. 1). Valente, Polishchuk, Burrows, and Lobo (2005) found that the percentage of moisture absorption did not depend on the cellulose acetate content, but it was a function of thickness. Furthermore, they also observed that the percentage of water sorption was slightly higher for the membrane containing a more porous surface structure, which was consistent with our results. The porous structure of the control film shown in Fig. 1d might cause the moisture to easily penetrate through the porous structure to the intrinsic structure of the film, while the smoother surface, more compact structure and low porosity (Fig. 1b and c) in the film containing ground seeds stimulate slower moisture absorption. This result confirms the capability of the film in absorbing the vaporized water from the (model) food that could trigger the enzymatic hydrolysis of sinigrin in the film to form and then release AITC into the headspace.

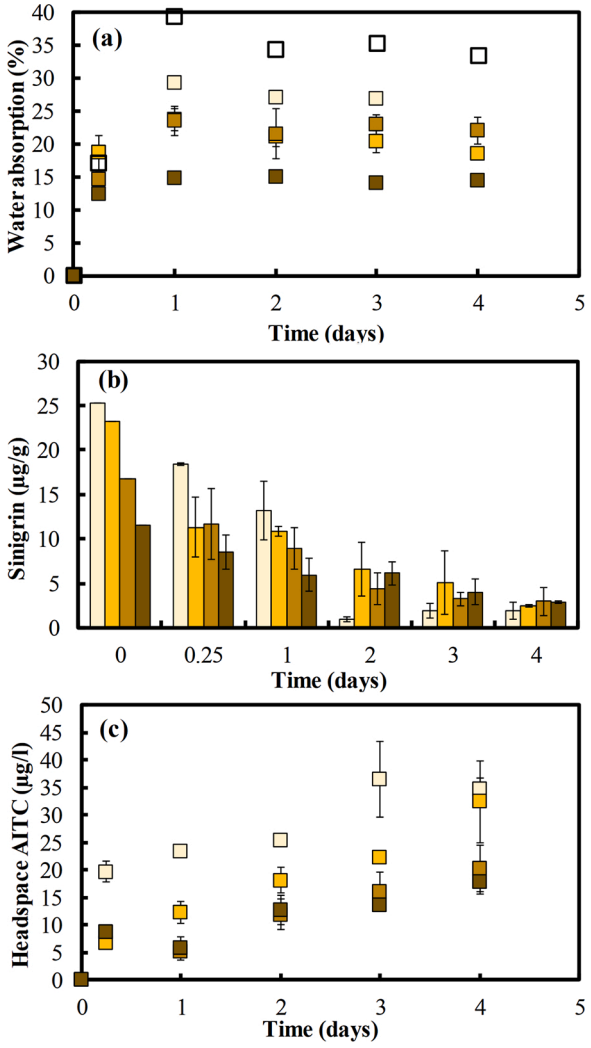

Fig. 2. (a) percentage of moisture absorption of the film, **(b)** the sinigrin content remaining in the ground mustard seeds, and **(c)** the concentration of Allyl isothiocyanate in the headspace with storage at 5 °C. The control film (700 mg of cellulose acetates) without ground mustard seeds (□); the film with mustard seeds produced with 300 mg (□); using 700 mg (□); 1100 mg (□); and 1500 mg (□) of cellulose acetates.

Fig. 2b shows the sinigrin content remaining in the ground mustard seeds. The sinigrin content of ground seeds in the thinner film reduced more quickly than that in the thicker film. From day 0–2, the sinigrin content of ground seeds in the thinnest film dropped from 25 µg/g to less than 2 µg/g, compared to that in the thickest film, reducing from 12 to only 7 µg/g. The faster reduction of sinigrin was related to the higher percentage of moisture diffusing into the thinner film. A higher amount of water absorbed in the film stimulates the enzymatic hydrolysis of sinigrin resulting in the formation of a higher amount of AITC.

The concentration of AITCs in the film and the headspace are a consequence of the sinigrin degradation in the film and the release of the formed AITC. In Figure A2. Supplementary materials, AITC concentration in the thinnest film peaked at 0.85 µg/g after 3 h and quickly decrease to reach almost the same level of AITC content of the thickest film after 4 days, while the AITC remaining in the thickest film kept increasing to 0.3 µg/g for 2 days and then followed a gradual decrease. As a result, the thicker film resulted in a lower AITC concentration in the headspace (Fig. 2c). After 4 days the AITC concentration in the headspace of the package with the thickest film just reached around 20 µg/L which was equal to the concentration for the packaging with the thinnest film after 6 h. A lower AITC concentration in the headspace of the package containing the thicker film was related to the slower degradation of sinigrin because of limited moisture absorption and/or a slower AITC release rate because of the higher polymer thickness as was also found by Seo et al. (2012); the release rate of allyl isothiocyanates from a sachet of low-density polyethylene (LDPE) increased significantly with reduced LDPE thickness. In general, the AITC release in the headspace was influenced by the morphology and thickness of the film.

The results show that moisture absorption triggered AITC formation in the film and its release in the headspace. The steps of diffusion and reaction mechanisms in the triggered release packaging system are proposed to be; (1) the absorption and diffusion of water vapour through the film, (2) enzymatic sinigrin hydrolysis and AITC formation, (3) the diffusion of AITC through the film, (4) the release of AITC into the headspace, and (5) the possible absorption of AITC by the (model) food as discussed in the next sections.

3.3. The effect of temperature and relative humidity on AITC release

The AITC concentrations in the headspace at different temperatures and film thickness are given in Fig. 3a and b. The AITC release and degradation from the thinnest films at 20 °C were faster than the AITC release and degradation from the thicker film and at lower temperatures. For example, at 20 °C AITC concentration in the packaging containing the thinnest film peaked at around 150 µg/L in 6 h and followed a quick decrease to almost 0 µg/L at day 4 (Fig. 3a), and at 5 °C the AITC kept increasing to 40 µg/L until day 4. This is consistent with the result obtained by Seo et al. (2012) with pure AITC who found a more rapid release of AITC from an LDPE sachet at 25 °C than that of 4 °C. The faster release of AITC is resulted from the higher sinigrin hydrolysis as an act of myrosinase activity at higher temperature as shown in Figure A3 in the supplementary materials. The higher myrosinase activity degrades the sinigrin more quickly to form more AITC at higher temperature (Bahmid, Dekker, Fogliano, & Heising, 2021).

Fig. 3c and d depict the effects of relative humidity to understand the importance of moisture to AITC release in the packaging system. A slower rate of AITC release and less AITC concentration in the headspace at 75.5 % RH was observed compared to 100 % RH. Lower water-vapor presence in the packaging system causes less water being absorbed by the film and then less sinigrin degradation to form less AITC, resulting in

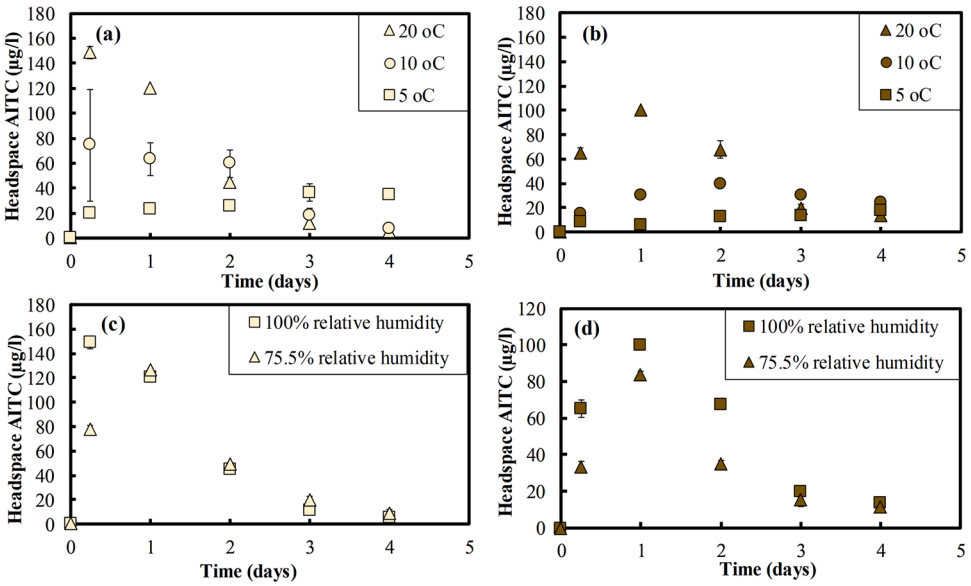

Fig. 3. The effect of **(a,b)** temperature and **(c,d)** relative humidity on the concentration of AITC released from the thinnest films made using 300 mg of cellulose acetate **(left)** and the thickest films made using 1500 mg cellulose acetate **(right)**.

a slower release of AITC to the headspace. Understanding the effects of the relative humidity gives an insight into which food products this packaging might be effective. For example, this packaging system might not be effective to pack dried food because the low amount of water vapor might not be enough to humidify the packaging. Consequently, a limited effect of the addition of the antimicrobial film in the packaging system is expected. The obtained results of the effect of RH emphasize the role of moisture to trigger the AITC formation and release in a food package.

3.4. The antimicrobial effectiveness of the developed film in a ground beef package

3.4.1. Partitioning of AITC between the packaging headspaces and the ground beef

Fig. 4 depicts the concentration of AITC from the thickest film in the headspace and the ground beef at 5 °C. Generally, the AITC concentration increased continuously in the headspace for 4 days and in the ground beef for 3 days and then decreased gradually. A higher AITC concentration in the headspace was observed in the packaging containing extra-lean ground beef (9 % fat) compared to the packaging with medium-ground beef (19 % fat) (Fig. 4a). This result was opposite to the results of the AITC concentration in ground beef (Fig. 4b), showing a lower absorbed AITC concentration in the presence of less fat in ground beef (9 % fat). This opposite trend can be explained by the solubility of AITC in a higher fat content of ground beef. In a similar packaging system Wang et al. (2020) found that higher fat content in ground beef enables more carvacrol to penetrate ground beef due to the higher solubility of carvacrol in the fat phase.

3.4.2. The effect of AITC on microbial growth in the ground beef

The total bacterial count in ground beef is shown in Fig. 5. For control samples, the total bacteria in extra-lean ground beef reached the maximum (almost 9 Log_{10} CFU) faster than that in the medium ground beef. The AITC release from the film reduced bacteria around 3 log_{10} after 6 days and 4 log_{10} after 8 days for the lean ground beef, while for medium ground beef reduced the bacteria only a log_{10} CFU after 8 days.

Kinetic modelling of bacterial growth in the ground beef was used to understand the growth rate and lag phase of the bacterial growth. The used kinetic model was the Gompertz model (Eq. (2)) which showed a good fit to the observed data (Figure A4. Supplementary materials). The value of asymptotic parameters of the model was set as a constant for the different samples since the maximum total bacterial count was assumed to be similar in the different ground beef samples. For the control samples, Table 1 depicts that medium ground beef has a significantly more prolonged lag phase of the bacterial growth and slower growth rate than those of the extra-lean ground beef. The higher fat content in ground beef caused a 2 days longer shelf life for the medium ground

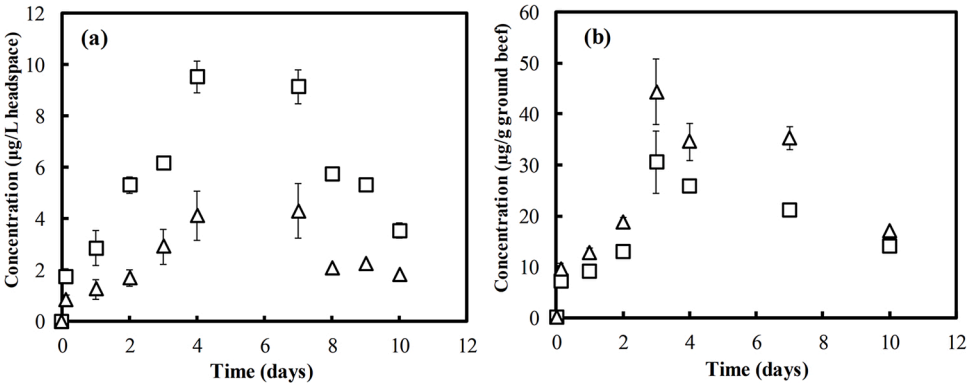

Fig. 4. The AITC concentration in **(a)** the headspace and **(b)** ground beef containing (□) 9 % fat and (△)19 % fat.

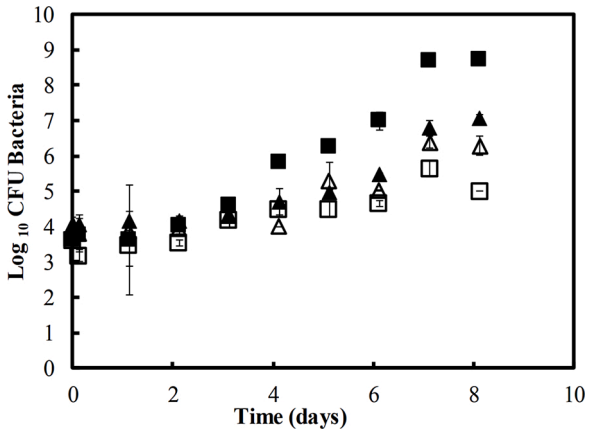

Fig. 5. The curve of bacterial growth in (■) extra-lean ground beef and (▲) medium ground beef for the control group, and (□) extra-lean ground beef and (△) medium ground beef for the antimicrobial packaging.

Table 1
The estimated parameters of the Gomperts model as calculated by fitting the model to observed PCA count data of the ground beef and the predicted shelf life (at 7 $\log_{10}$ CFU).

Samples	As ($\log_{10}$ CFU)	Lag phase (h)	μmax (h^{-1})	Shelf life (days)
Extra-lean ground beef (control)		52.45 ± 7.23^{a}	0.09 ± 0.01^{c}	3.84 ± 0.56^{a}
Extra-lean ground beef	15.53 ± 2.71	83.40 ± 19.32^{ab}	0.04 ± 0.01^{a}	7.52 ± 1.38^{b}
Medium-ground beef (control)		93.10 ± 11.11^{b}	0.07 ± 0.01^{bc}	5.28 ± 0.65^{ab}
Medium-ground beef		102.14 ± 13.12^{b}	0.06 ± 0.01^{ab}	5.84 ± 0.73^{b}

Values represent means ± standard deviation. Small letters indicate significant differences between the samples.

beef. The fat in food products is well-known as a growth-restricting factor (Wilson et al., 2002), so the larger amounts of fat reduced the microbial growth, like L. *monocytogenes,* at 4 °C, leading to a longer lag phase and lower maximum specific growth (Verheyen et al., 2018).

Table 1 shows that the film caused extended lag phases for both extra lean and medium ground beef, and reductions of the growth rate of microorganisms in extra lean ground beef by about 0.05 h^{-1} and by around 0.01 h^{-1} for the medium ground beef. Therefore, the shelf life of extra-lean ground beef was significantly extended by around 3.5 days and it could be concluded that the application of this antimicrobial package almost doubled the shelf life of the lean ground beef. The medium ground beef had an extended shelf life of 0.56 days. These results confirm the effect of food composition on AITC concentrations and its antimicrobial efficacy in the packaging system, where the higher AITC concentration was observed in the headspace for the ground beef with lower fat (Fig. 4). Initially, the film releasing 1−2 μg AITC per liter headspace of packaging containing both types of ground beef (Fig. 4) was able to reduce the total bacterial count by 0.5–1 $\log_{10}$ CFU for 6 h (Fig. 5a). However, the obtained AITC concentration (around 5 μg/L) during 4 days in the packaging might not be sufficient to effectively suppress the bacterial inhibition in medium ground beef, because the MIC of AITC against *P. fragi* is around 6.05 μg/L (Bahmid, Heising, et al., 2020). Whilst the AITC concentration was increasing to 10 μg/L (exceeded the minimum inhibitory concentration) during 4 days in the headspace of the packaging with extra-lean ground beef. The AITC was able to prolong the lag phase and reduce the growth rate in the extra lean ground beef. These results show that the concentration of the compounds should be above the MIC of the bacteria. Otherwise, the compounds might have limited antimicrobial effect on packaged food.

On the other hand, despite the higher AITC concentration in high-fat ground beef as shown in Fig. 4, the AITC could be solubilized mostly in the fat phase of the ground beef, where the AITC is not effective to inhibit the bacteria in the ground beef. The solubility of AITC in the fat phase limits the interaction between AITC and the bacteria growing in the aqueous phase of the food as also shown for other lipid-soluble antimicrobial compounds (Wang, Heising, Fogliano, & Dekker, 2020). From the results of this study, it can be concluded that the food composition does not only influence the antimicrobial film properties but also influences the AITC concentration in different phases of the package and this can affect the bacterial growth in the food products. Other factors might also affect the effectiveness of AITC against the bacteria in ground beef, as the ground beef contains for example proteins that might covalently interact with the AITC (Keppler et al., 2017), which was not considered in this study. For commercial issue and application of packaging system, there is a possibility that the food can have a contact with the packaging film during distribution, resulting in unexpected production of other compounds that can influence the effectiveness of the AITC in the packaging system. This direct contact could be benefit to trigger the AITC formation before the AITC releases into the headspace as long as the packaged food does not continuously block the film releasing the AITC into the headspace, leading to AITC migration only to the contact food area. This lab-scale experiment is required to upgrade to an industrial scale to investigate the optimal condition in a real food packaging system.

4. Conclusion

This study shows the development of a moisture-activated antimicrobial film containing ground mustard seeds. The properties of the active carrier (ground mustard seeds incorporating film), storage conditions (RH and temperature), and food composition (fat content) influenced the AITC concentration in different phases of the package, which then affected the bacterial growth and shelf life of ground beef. The higher cellulose acetate content and the thicker films reduced the moisture absorption and in turn decreased the rate of AITC formation and release into the packaging system. The formation of AITC in the ground mustard loaded into the film polymer was triggered by the vaporized moisture absorbed by the film and was therefore influenced by the rate and amount of absorbed moisture. The formation and release of AITC were also dependent on the storage conditions where higher temperatures and relative humidity enhance the rate of AITC release into the packaging system. Controlling those factors in the packaging system is important to result in a sufficient and sustained AITC concentration in the packaging system. The packaging system with the thickest film (made using 1500 mg cellulose acetate) was tested to study the effects of the fat content of ground beef influence the AITC concentration and absorption and the antimicrobial effect of AITC against the bacterial growth in the ground beef. The packaging with ground beef with lower fat content gave a higher AITC concentration in the headspace, but a lower AITC concentration in the ground beef. However, the shelf life of the low-fat ground beef can be very effectively extended by 3.68 days, compared to a limited extension of only 0.56 days of the shelf life of the medium ground beef. In this case, differences in fat composition cause the efficiency of food packaging from very effective to barely noticeable. Besides showing the novel way to control compound release, these results give an insight that tailoring the effective active packaging system should not only consider the properties of the active carrier but also the storage conditions and food characteristics.

CRediT authorship contribution statement

Nur Alim Bahmid: Conceptualization, Methodology, Investigation, Data curation, Visualization, Formal analysis, Writing - original draft, Funding acquisition. **Matthijs Dekker:** Conceptualization, Supervision, Writing - review & editing. **Vincenzo Fogliano:** Conceptualization,

Supervision, Writing - review & editing. **Jenneke Heising:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.fpsl.2021.100753.

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