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Physical properties and antifungal activity of bioactive films containing *Wickerhamomyces anomalus* killer yeast and their application for preservation of oranges and control of postharvest green mould caused by *Penicillium digitatum*

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ABSTRACT

This study assessed the ability of two bio-based films, obtained from sodium alginate (NaAlg) and Locust Bean Gum (LBG), to protect the viability of *Wickerhamomyces anomalus* cells and control the growth of *Penicillium digitatum*. The effect of microbial cell incorporation on physical properties of the developed films was evaluated in terms of barrier, mechanical and optical properties. Furthermore, the application of these two matrices as bioactive coatings was investigated in order to evaluate their efficacy in preserving the postharvest quality of 'Valencia' oranges and inhibiting the growth of *P. digitatum* on artificially inoculated fruits. Results showed that NaAlg and LBG films were able to maintain more than 85% of the initial *W. anomalus* yeast population and that the developed films incorporating the killer yeast completely inhibited the growth of *P. digitatum* in synthetic medium. Likewise, NaAlg and LBG coatings enriched with *W. anomalus* yeast were effective at reducing weight loss and maintaining firmness of 'Valencia' oranges during storage, and reduced green mould in inoculated fruits by more than 73% after 13 days.

Keywords: Sodium alginate, Locust Bean Gum, *Wickerhamomyces anomalus*, Film properties, Bioactive coating, Postharvest quality

1. Introduction

Citrus fruits are among the most consumed fruits in the world, mainly because of their high content of vitamin C and other bioactive compounds including flavonoids and phenolic acids (Widmer and Montanari, 1996), known for their beneficial effect in reducing risk of cancer and cardiac diseases incidence (Attaway, 1994). However, during harvesting, postharvest handling, transportation and storage, citrus fruits are susceptible to injury and colonization by various fungi. *Penicillium digitatum* (Pers.) Sacc., the causal agent of green

mould, has been reported to be one of the most devastating postharvest fungal pathogens of citrus fruits. This mould may cause 60-80% decay under ambient conditions (Moscoso-Ramírez et al., 2013) which results in severe economic losses, especially for exporting countries. Over the last few decades biological control, using antagonistic microorganisms including yeasts, yeast-like fungi and bacteria, has emerged as one of the most promising treatments for controlling wound-invading postharvest pathogens (Janisiewicz and Korsten, 2002). Among microbial antagonists, yeasts have received particular attention as potential biocontrol agents against postharvest diseases in fruits, mainly because of their high inhibitory capacity, rapid colonization of fruit wounds (Rosa-Magri et al., 2011) and simple nutritional requirements enabling them to colonize dry surfaces for long periods of time (El-Tarabily and Sivasithamparam, 2006). Moreover, in contrast to filamentous fungi, yeasts do not produce allergenic spores or mycotoxins, reinforcing their safe use for human consumption purposes (Fan and Tian, 2000). In the last decade, several scientific studies have demonstrated the efficacy of antagonistic yeasts as biocontrol agents against many phytopathogenic fungi including species of *Penicillium* (Bautista-Rosales et al., 2013; Manso and Nunes, 2011; Platania et al., 2012; Restuccia et al., 2006; Wang et al., 2010).

Among a wide variety of antagonistic yeasts particular attention has been directed toward the use of yeasts that exhibit a killer phenotype (K+) for controlling postharvest decay in fruits, due to their ability to secrete extra cellular protein toxins designated as killer proteins or killer toxins. These proteins have the potential to kill other species of yeasts, moulds and pathogenic bacteria through different mechanisms, including the hydrolysis of the major cell wall component β-1,3-glucans (Izgü and Altinbay, 2004; Muccilli et al., 2013), the ion leakage by ion channel formation on the cytoplasmic membrane of the target cell, and the inhibition of DNA synthesis (Schmitt and Breinig, 2002). Among the killer species, *Wickerhamomyces anomalus* (previously named *Pichia anomala*) has been reported to

produce high levels of killer toxins with a wide spectrum of killing activity and a relatively high stability, compared with toxins of other killer yeasts (Wang et al., 2008). Furthermore, *W. anomalus* has been granted Qualified Presumption of Safety (QPS) status by European Food Safety Authority (EFSA), which may authorize its use as a novel microorganism in food preservation (Sundh and Melin, 2011).

In the last decade, several studies have demonstrated the efficacy of *W. anomalus* as a biological control agent against different postharvest phytopathogenic fungi. In this sense, Lima et al. (2013) reported a significant reduction in the incidence of infection, for up to 6 days after inoculation, when assessing the efficiency of *W. anomalus* (strain 422) for the biocontrol of the anthracnose disease caused by *Colletotrichum gloeosporioides* in papaya. Similar effects were obtained by Lassois et al. (2008) and Platania et al. (2012), when evaluating the antagonistic activity of different strains of *W. anomalus* against the fungi causing crown rot disease in banana fruit and *P. digitatum* on Tarocco oranges, respectively.

Recently, Sánchez-González et al. (2013; 2014) developed bioactive polymeric films through the incorporation of different strains of bacteriocin-producing lactic acid bacteria into polysaccharide and protein matrices. These authors demonstrated the efficacy of the different developed films at maintaining the viability of incorporated cells and controlling the growth of *Listeria innocua* on an artificially contaminated synthetic medium.

Among many polysaccharides, sodium alginate (NaAlg) and Locust Bean Gum (LBG) have been reported as potential coating components not only because of their excellent film forming properties and selective permeabilities to O_2 and CO_2 (Mikkonen et al., 2007; Oms-Oliu et al., 2008), but also for their ability to act as effective matrices for the entrapment of bioactive compounds (Aloui et al., 2014a and b).

Although the efficacy of many antagonistic yeasts against a wide variety of phytopathogenic fungi has been well documented in literature, there are only few published

data on their incorporation into coating formulations for fresh fruits (McGuire and Hagenmaier, 1996; McGuire and Dimitroglou, 1999; Fan et al., 2009). To our knowledge, no research has been reported on the use of edible coatings carrying *W. anomalus* killer yeast for controlling fungal decay and preserving postharvest quality of fruits.

The objectives of this work were to evaluate the effect of *W. anomalus* yeast incorporation on the functional and optical properties of NaAlg and LBG bio-based films, and to investigate the ability of these two polysaccharide matrices to maintain viability and antifungal potential of incorporated cells. Likewise, the application of these matrices as bioactive coatings was investigated to evaluate their effectiveness at preserving the postharvest quality of 'Valencia' oranges and controlling fungal spoilage caused by *P. digitatum*.

2. Materials and Methods

2.1. Raw materials

'Valencia' oranges (*Citrus sinensis* L. Osbeck) were purchased from a wholesale distributor located in Catania (Italy) at commercial maturity and transported to the laboratory in polystyrene boxes to avoid mechanical damage, at ambient conditions of temperature and humidity (18 °C and 75% RH). Oranges were visually selected on the basis of uniform shape, size, color, firmness and absence of mechanical injuries or fungal infection. Before coating application, selected oranges were washed with a solution of sodium hypochlorite (0.01%) for 3 min, then drained and air-dried at room temperature. Coating experiments were carried out on the same day.

NaAlg (molecular weight ~ 80,000 Da, CAS Number 9005-38-3, Sigma Aldrich, Steinheim, Germany) and LBG (molecular weight ~ 310,000 Da, Sigma Aldrich, Steinheim,

Germany) were used as coating materials. Glycerol (≥99% purity; Sigma-Aldrich) was used as a plasticizer and was purchased from Sigma Aldrich (Steinheim, Germany).

2.2. Microorganisms

W. anomalus BS 91 killer yeast strain used in this study, obtained from the DiGeSA collection (Department of Agri-Food and Environmental Management Systems, University of Catania), was isolated from naturally fermented olives and identified by sequencing the D1/D2 region of the 26S rRNA gene (Muccilli et al., 2011). The selection of this strain was based on its high antifungal activity against *P. digitatum* according to our preliminary experiments (data not shown). The killing mechanism of *W. anomalus* BS 91 was reported to be based on β-glucanase production (Muccilli et al., 2013).

Freeze dried culture of *P. digitatum* DSM 2748 was obtained from DSMZ culture collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The fungus was rehydrated following the supplier's instructions, inoculated on potato dextrose agar (PDA) (Oxoid, Basingstoke, Hampshire, England) and incubated at 25 °C until sporulation.

2.3. Preparation of the bioactive film forming solutions

Pure NaAlg and LBG film forming solutions were prepared by dissolving either NaAlg (2%, w/v) or LBG (1 %, w/v) powder in distilled water heated at 70 °C with constant agitation, until all particles were thoroughly dispersed. Glycerol (20%, w/w based on biopolymer content) was added as plasticizer to enhance film flexibility, overcome brittleness and facilitate film detachment. The mixture was then stirred overnight at room temperature, before being degassed to remove entrapped air bubbles.

W. anomalus BS 91 killer yeast suspension used for the preparation of bioactive film forming solutions, was prepared from cells grown in YPD broth (1% yeast extract, 2% peptone, 2% glucose; Oxoid, Basingstoke, Hampshire, England) for 24 h at 25 °C. The yeast culture was centrifuged at 8000×g for 10 min and the pellet was washed twice with sterile water. *W. anomalus* BS 91 cells were then incorporated into NaAlg and LBG film forming solutions, cooled to 30°C, at a concentration of 10^7 CFU/mL (~6 logs CFU/cm^2 in dry film). The concentration of antagonistic yeast in the stand alone films and film-forming solutions was chosen from results of preliminary laboratory tests (data not shown). This concentration was able to completely inhibit the growth of *Penicillium digitatum* without affecting the physical properties of sodium alginate and LBG films.

The bioactive film forming solutions were subsequently placed under magnetic stirring for 5 min.

2.4. Preparation of the stand alone coatings

A casting method was used to produce NaAlg and LBG stand-alone coatings with and without yeast cells. In order to obtain films with a similar thickness, a constant amount that provided a solid surface density of 50 g/m^2 was poured into Petri plates, and dried at room temperature for approximately 48 h. Once formed, films were peeled from Petri plates and preconditioned in climatic chamber at 25 °C and 75% relative humidity (RH), before testing.

2.5. Characterization of the stand alone coatings

2.5.1. Mechanical properties

The mechanical properties of the films including tensile strength (TS) and elongation at break (%E) were determined using the Instron Universal Testing Machine (Model 3345, USA) according to a standard method of ISO 1924-2-1994. Samples were cut into 15 mm

wide and 100 mm long strips and fixed with an initial clamp separation of 50 mm at a test speed of 100 mm/min. TS was calculated by dividing the maximum load on the film before failure by the cross-sectional area of the initial film specimen. %E was determined by dividing the extension at the moment of breakage by the initial gauge length of the samples and multiplying by 100. Ten replicates of each film type were tested.

2.5.2. Contact angle measurements

Contact angle measurements were carried out using a goniometer (Pocket goniometer PGX, Sweden) according to the sessile drop method, frequently used to estimate wetting properties of solid surfaces. Briefly, a droplet of distilled water (~1 μL) was deposited on the film surface with a precision syringe, and the angle formed between the surface of the film in contact with the drop and the tangent to the drop of liquid at the point of contact with the film surface was measured. For each film type, at least five measurements at different positions on the film surface were taken and the average was calculated.

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2.5.3. Water vapor permeability (WVP)

WVP was measured in quadruplicate according to the gravimetric method described in the AFNOR NF H00-030 standard (AFNOR 1974). Film specimens were sealed in permeation cells containing a desiccant (silica gel) to maintain a RH 0% in the cell. The permeation cells were 6.4 cm (internal diameter) by 8.9 cm (external diameter) by 4.8 cm deep with an exposed area of 26.42 cm^2. The permeation cells were placed in a controlled temperature (38 ± 1 °C) and RH (90 ± 3%) chamber fitted with a variable-speed fan to provide a strong driving force across the film for water vapor diffusion. Unsteady-state periods of 30 min were demonstrated in preliminary tests. The water vapor transport was determined from the weight gain of the cell. Changes in the weight of the cell were recorded as a function of time. Slopes were calculated by linear regression (weight changes vs. time)

and the correlation coefficient for all reported data was >0.99. The water vapor transmission rate (WVTR) was defined as the slope (g/d) divided by the transfer area (m^2). After the permeation tests, film thickness was measured and WVP was calculated as follows (McHugh and Krochta, 1994):

$$WVP = \frac{\text{WVTR . X}}{\Delta p} \quad [\text{g mm/m}^2\text{/d/kPa}]$$

Where X is the film thickness, Δp is the difference of partial water vapor pressure across the film ($\Delta p = p\ (RH_2 - RH_1)$ = 5.942 kPa, where p is the saturation vapor pressure of water at 38 °C, RH_2 = 90%, RH_1 = 0%).

2.5.4. Gloss measurement

Gloss was measured using an elcometer (Model 406L Dual 20/60° Statistical Mini Glossmeter, Novo-Soft software, England) at an incidence angle of 60° according to the ASTM D523 method (ASTM, 1999). Measurements were taken in triplicate for each sample, and three films of each formulation were considered. Results were expressed as a gloss unit (GU) referring to a highly polished surface of standard black glass with a value close to 100.

2.5.5. Viability of W. anomalus yeast cells during storage of stand alone coatings

The viability of *W. anomalus* yeast cells was evaluated in NaAlg and LBG films just after preparation and periodically (every 7 days) during a storage period of 21 days. Bioactive films stored in Petri plates inside the climatic chamber at 25°C and 75% RH, were aseptically removed and placed in sterile plastic bags containing 100 mL of sterile physiological saline (0.9% NaCl), before being homogenized for 2 min, using a stomacher blender (Bag Mixer 400, Interscience). Serial dilutions were then prepared and 0.1 mL sample of appropriate

dilutions was spread on potato dextrose agar (PDA). Colonies were counted after incubation for 48 h at 25 °C. All tests were carried in duplicate.

2.5.6. In vitro antifungal activity of NaAlg and LBG films against P. digitatum

The antifungal activity of NaAlg and LBG films against *P. digitatum* was evaluated according to the antimicrobial methodology adopted by Sánchez-González et al. (2014). Briefly, preconditioned films (with and without killer yeast) with the same diameter as the Petri plates were placed onto solid PDA surfaces previously buffered at pH 4.5 (reported as the optimal pH for toxins production and activity, according to Muccilli et al. (2013)), and inoculated with 100 µL of a conidial suspension of *P. digitatum* at a concentration of 10^5 conidia/mL. Control films without killer yeast were used as a negative control while inoculated plates that did not carry films were used as a positive control. All the plates were sealed using Parafilm to avoid dehydratation and incubated at 25 °C for 15 days. *W. anomalus* and *P. digitatum* counts on PDA plates were carried out immediately after inoculation and periodically during the storage period.

The agar was removed aseptically from Petri dishes and placed in a sterile plastic bag with 100 mL of sterile physiological saline (0.9% NaCl) and homogenized for 2 min, using a stomacher blender (Bag Mixer 400, Interscience). Serial dilutions were then prepared and 0.1 mL sample of appropriate dilutions was spread on potato dextrose agar (PDA). Colonies were counted after incubation for 48 h at 25 °C. All tests were carried in duplicate.

2.5.7. Production and diffusion of β-glucanase enzymes from bioactive films

As β-1,3-glucanase has been reported to be involved in the killer mechanism of *W. anomalus* BS 91 against *P. digitatum* (Platania et al., 2012), a plate assay on solid media was performed in order to demonstrate the ability of this yeast incorporated into NaAlg and LBG

films to produce β-glucanase enzymes, and to verify their diffusion from NaAlg and LBG matrices. Briefly, NaAlg and LBG films (with and without killer yeast) were cut into 5 mm wide and 50 mm long strips using a sterile scalpel and placed aseptically onto solid PDA surfaces buffered at pH 4.5, with a citrate buffer (0.06 M). Ten µL of a conidial suspension of *P. digitatum* at a concentration of 10^5 conidia/mL was used to inoculate the opposite edge. Plates inoculated only with *P. digitatum* were used as a positive growth control. All the plates were incubated at 25 °C. The mycelial growth was measured after 10 days of incubation and expressed as the distance (mm) from the strip to the side edge of the actively growing mould. All tests were carried in duplicate.

2.6. Application of coating treatments

Selected oranges were dipped in the different coating solutions (prepared as described above in section *2.3*) for 1 min. After coating treatments, fruits were hung up and air-dried for 24 h before being stored for 15 days at 25°C and 75% RH, to simulate retail handling and marketing conditions.

2.7. Fruit quality

Fifteen oranges for each treatment and sampling date were characterized in terms of weight loss and firmness, considered as the most determinant factors for postharvest life and quality of fresh fruits. Changes in weight and firmness were assessed at different storage times (3, 6, 9, 12 and 15 days). All the assays were carried in triplicate.

Analysis of weight loss was performed as described by Aloui et al. (2014a). Weight loss was expressed as percentage loss of the initial weight.

Fruit firmness was determined according to Aloui et al. (2014a) by measuring force at break (N) using the Instron Universal Testing Machine (Model 3345, USA) with a 5 kN load

cell and a 10 mm diameter cylindrical probe. Fifteen oranges for each treatment and sampling date were 50% compressed at a 2 mm/s deformation rate.

2.8. Control of P. digitatum *growth on oranges*

The antifungal activity of the different coating treatments was evaluated according to Aloui et al. (2014b). Briefly, oranges previously washed with sodium hypochlorite (0.01%) were injured (3 holes per fruit) with a sterile needle (3 mm diameter x 3 mm deep), then dipped in a conidial suspension of *P. digitatum* at a concentration of 10^5 conidia/mL for 1 min and dried at room temperature for 2 h. Inoculated fruits were then immersed in the different coating solutions (30 oranges for each treatment) for 1 min and air-dried at room temperature. Coatings were applied after the infection of fruits by pathogens, since most contamination is assumed to occur during harvesting, postharvest handling and transportation.

Thirty uncoated oranges were used as a control. After coating treatment, oranges were stored at simulated marketing conditions (25 °C, 75% RH) for 13 days and the disease incidence, expressed as the number of infected oranges out of the total number of fruits per treatment, was evaluated daily. Each experiment was conducted twice. An orange was considered decayed when at least one of the inoculated wounds was infected.

2.9. Statistical analysis

Results were analysed by a multifactor analysis of variance (ANOVA) with a 95% significance level using Statgraphics® Plus 5.1(Manugistics Inc., Rockville,MD, USA). Multiple comparisons were performed through 95% Fisher's LSD intervals.

Data on incidence of *P. digitatum* infection were subjected to the arcsine transformation before applying the ANOVA in order to assure the homogeneity of variances.

3. Results and discussion

3.1. Mechanical properties

The intended use of films for food packaging applications requires a clear understanding of their mechanical properties. High mechanical properties are usually required to maintain packaging integrity during shipping, handling and storage. Tensile strength (TS) and elongation at break (E%) which are the key indicators of films' strength and flexibility, respectively, are the most commonly reported responses to describe mechanical properties of biopolymer-based packaging materials.

Changes in TS and E% of NaAlg and LBG stand-alone films with and without killer yeast are summarized in **Table 1**. Pure NaAlg films exhibited significantly higher TS and lower E% values than pure LBG films ($P<0.05$). Overall, comparable mechanical properties were reported by Benavides et al. (2012) and Martins et al. (2012) for NaAlg and LBG films, respectively.

As shown in **Table 1**, no significant changes were observed in TS and E% of both NaAlg and LBG films after addition of killer yeast cells ($P>0.05$). A similar trend was reported by Gialamas et al. (2010) when studying the effect of *Lactobacillus sakei* cell incorporation on the mechanical behavior of sodium caseinate films. According to these authors, the relatively low proportion of added cells compared with the total mass of the polymeric matrix may explain their insignificant effect on the mechanical properties of the protein films. Conversely, Sánchez-González et al. (2014) reported a significant reduction in the TS of methylcellulose and sodium caseinate films due to the addition of *Lactobacillus reuteri* cells. This decrease in the film strength was ascribed to the introduction of discontinuities in the film matrix, which may reduce the cohesive forces in the polymer network, giving rise to the observed losses in terms of mechanical resistance.

3.2. Water contact angle

Water contact angle measurement is important for films intended for food packaging applications in order to better understand their wetting and adhesion properties and to define useful polymer properties such as ease of coating.

Surface hydrophobicity of NaAlg and LBG films with and without killer yeast was evaluated by measuring the contact angle of water upon the film according to the static sessile drop method. In general, films with higher contact angles values exhibit a higher surface hydrophobicity (Tang and Jiang, 2007).

Overall, LBG films showed significantly higher contact angle values than NaAlg films ($P<0.05$), suggesting a higher surface hydrophobicity (**Table 1**). This difference in wetting properties between the two biopolymer matrices may be attributed to the differences in their hydrophilic-hydrophobic ratio, amorphous-crystalline structure and polymeric chain mobility (Souza et al., 2010). Moreover, the difference in the nature and chemical structure of film-forming materials as well as the intermolecular bonds occurred during film formation may also result in diverse barrier properties (Guilbert et al., 2002).

As shown in **Table 1**, the incorporation of killer yeast cells into NaAlg and LBG film-forming solutions led to no significant changes in the contact angle values of the resulting films. Because water contact angle is reported to be directly related to the surface roughness of the film (Jayasekara et al., 2004), the added microbial cells did not induce relevant changes in the film surface morphology, and thus in the hydrophilic/hydrophobic character of the film-forming material, probably due to their relatively low proportion compared to the total dry matter of the polymer matrix.

3.3. Water vapor permeability

Edible and/or biodegradable packaging materials are often required to impede moisture exchanges between the food and the environment in order to reduce food dehydration and prevent quality loss due to mass transfer. In this sense, the WVP should be as low as possible to minimize weight losses and maintain both firmness and appearance of coated products during distribution and storage.

WVP results of NaAlg and LBG films with and without killer yeast are recorded in **Table 1**. NaAlg films showed significantly higher WVP values than films based on LBG ($P<0.05$), probably because of their higher hydrophilic character as revealed by the results of contact angle measurement. Overall, WVP values of NaAlg films are in the range of those obtained by Benavides et al. (2012), however higher values were reported by Martins et al. (2012) for films based on LBG. The differences in water vapor barrier properties can be attributed to different experimental conditions such as temperature, RH gradient and type and amount of plasticizer (Greener and Fennema, 1989).

As shown in **Table 1**, the incorporation of killer yeast cells into NaAlg and LBG biopolymer matrices did not significantly affect their barrier properties ($P>0.05$). In agreement with the present findings, no significant differences were observed between the WVP values of sodium caseinate films with and without *Lactobacillus sakei* cells (Gialamas et al., 2010). Conversely, Sánchez-González et al. (2014) reported that the incorporation of *Lactobacillus sakei* or *Lactobacillus acidophilus* cells into the sodium caseinate and methylcellulose films resulted in significant increase in WVP values. According to these authors, the introduction of structural discontinuities in the polymer matrix due to the presence of bacterial cells may promote the mass transfer of water molecules through the film.

3.4. Gloss measurement

Gloss is one of the most relevant optical properties to determine the suitability of films as food coatings since it has a direct impact on the appearance as well as on the consumer acceptability of coated products.

Gloss values of NaAlg and LBG films with and without killer yeast, measured at an incidence angle of 60°, are recorded in **Table 1**. Overall, NaAlg films had significantly higher gloss than LBG films ($P<0.05$), which may suggest a significant difference in the refractive index of these two biopolymer matrices. Other factors such as the physical (rheological behavior, molecular weight and intrinsic viscosity) and chemical (monosaccharide composition and minerals) properties of the film-forming materials as well as the surface morphology reached during the film drying, may also affect the optical properties of the resulting films, and therefore explain the observed difference in gloss between NaAlg and LBG films (Nussinovitch et al., 2008).

As shown in **Table 1**, no significant difference was found between the gloss of films based on pure NaAlg and LBG and those containing yeast cells ($P>0.05$). Because gloss is reported to be directly related to the surface roughness of the film, this result may suggest that the proportion of added cells did not alter the film surface morphology, and thus its refractive index.

Overall, NaAlg films exhibited higher TS and gloss values than LBG films, while the latter showed higher flexibility and barrier properties. Taking into account these results, LBG films are more suitable as potential coating materials for citrus fruits than NaAlg films due to their high flexibility which facilitates their adhesion to fruit surface. Moreover, these films showed higher water vapor barrier properties than LBG films, which make them able to prevent quality loss due to mass transfer and thus maintain both firmness and appearance of coated fruits.

3.5. Viability of W. anomalus *yeast cells during the storage of the stand alone coatings*

The viability of *W. anomalus* killer yeast incorporated into NaAlg and LBG films was assessed during a storage period of 21 days at 25 °C and 75% RH in order to compare the suitability of these two polysaccharide matrices to maintain cell growth and survival. Changes in *W. anomalus* immobilized cells viability over storage time are shown in **Figure 1**. Regardless of the biopolymer matrix, no significant changes in the viability of the incorporated killer yeast were observed during the first week of storage ($P>0.05$). This result indicates the effectiveness of both NaAlg and LBG matrices in maintaining the viability of *W. anomalus* cells, probably due to their ability to provide the nutrients needed to sustain their survival. After 14 days of storage, *W. anomalus* population was significantly reduced ($P<0.05$) by 0.75 and 0.95 log CFU/cm^2 respectively in LBG and NaAlg films, due to the progressive depletion of nutrients and the decrease of water content. ANOVA results demonstrated that no significant decreases in yeast cell viability were observed in both NaAlg and LBG films with increasing sampling time from 14 to 21 days ($P>0.05$). Moreover, no significant differences in *W. anomalus* survival rates were noted between the two polymeric matrices, which were able to maintain more than 85% of the initial yeast population, at the end of the storage period. In agreement with our findings, Fan et al. (2009) reported the ability of NaAlg films to maintain almost the half of *Cryptococcus laurentii* initial population at the end of 20-day storage period. Higher yeast survival rates (more than 75%) were recorded by these authors when glucose was added as a carbon source. Recently, Léonard et al. (2014) observed a similar positive effect of sodium alginate containing MRS broth on the survival of *Lactococcus lactis subsp. lactis* LAB3 cells. These results were in line with those reported previously by Brachkova et al. (2010) who came to the conclusion that calcium alginate films containing MRS broth were able to maintain the viability of lactic bacteria for a 6-month storage period at 4 °C. Conversely, Sánchez-González et al. (2013) reported the inability of

cellulose derivatives, particularly hydroxypropylmethylcellulose, to maintain the viability of *Lactobacillus plantarum*, which was almost null after one month of storage at 5 °C. Many factors such as the nature of the entrapped strain, the chemical composition of the film-forming material as well as the drying and storage conditions may strongly affect the survival rate of microbial cells entrapped in biopolymeric matrices, and therefore explain the difference in cell viability observed among the different results from published studies.

3.6. In vitro antifungal activity of NaAlg and LBG stand alone films against P. digitatum

The effects of NaAlg and LBG films with and without killer yeast on the growth of *P. digitatum* over an incubation period of 15 days at 25 °C are presented in **Figure 2a**. *P. digitatum* population increased gradually over time ($P<0.05$) in the uncoated PDA plates to reach a maximum level of 6.089 log CFU/cm^2 at the end of the storage period, suggesting the progressive adaptation of this fungus to its new environment. Similar behavior was observed in PDA plates coated with pure NaAlg and LBG films, although a slight decrease in the growth of *P. digitatum* was noticed during the storage period compared with the uncoated plates ($P<0.05$). Regardless of the film type, the mould growth reduction was about 0.2 log CFU/cm^2 at day 15, compared with the uncoated plates. As moulds are strictly aerobic (Ellis et al., 1993), this reduction could be attributed to a decrease in the transmission rate of oxygen through polysaccharide films, previously proved to be good barriers against gases (Baldwin et al., 1995).

As it can be seen in **Figure 2a,** NaAlg and LBG films incorporating *W. anomalus* killer yeast completely inhibited the growth of *P. digitatum* during the storage period, while at the same time favoring the growth of killer yeasts which were able to reach a level of approximately 10^6 CFU/cm^2 after only 3 days of incubation and to maintain their viability until the end of the storage period (**Figure 2b**). This result appeared to be related to the

antifungal potential of the incorporated *W. anomalus* killer yeast which is mainly attributed to its ability to secrete toxic glycoproteins with β-glucanase activity, as revealed by the results of the diffusion assay (**Figure 3**). **Figure 3** shows a clear inhibition halo in the plates carrying NaAlg and LBG films enriched with *W. anomalus* yeast, indicating the ability of incorporated cells to secrete β-glucanase enzymes which were able to diffuse from NaAlg and LBG matrices and to completely inhibit the growth of *P. digitatum* in proximity to the bioactive films (**Figure 3c and e**). Moreover, a significant reduction by more than 36% in the mycelial growth of *P. digitatum* was observed in these plates, after 10 days incubation, compared with the controls and those carrying NaAlg and LBG pure films ($P<0.05$) (**Table 2**) in which the fungus appeared to overgrow the films strips for complete colonization of the plate (**Figure 3b and d**). The antifungal potential of β-glucanase enzymes against *P. digitatum* was attributed to the ability of these killer glycoproteins to hydrolyze glucans, the most abundant component of fungal cell walls, leading to cell death by lysis and leakage of cytoplasmic components (Platania et al., 2012).

3.7. Weight loss

Changes in weight loss of both coated and uncoated oranges throughout the storage period are reported in **Table 3**. The weight loss of all samples increased gradually throughout the storage time ($P<0.05$) due to the loss of water caused by evaporation, transpiration and respiration processes. Overall, NaAlg and LBG coatings applied on 'Valencia' oranges were effective at reducing weight loss compared with the control ($P<0.05$). However, no effect of yeast cell incorporation was found on the weight loss of coated oranges ($P>0.05$), contrary to previous reports by Fan et al. (2009) who noticed a reduction in the water loss of coated strawberries upon the incorporation of *C. laurentii* into NaAlg coatings. Regardless of the coating type, weight loss reduction in coated oranges was in the range of 28-33% at day 15, compared to

the control (*P<0.05*). These results are in agreement with data shown in **Table 1** concerning WVP: indeed the inclusion of yeast cells did not cause significant changes in WVP of stand-alone films, and this result is confirmed also after application on fruits, as highlighted by water loss measurements. Previous studies have reported the beneficial effect of edible coatings on weight loss reduction of a wide range of fruits including papaya (Ali et al., 2011), grapes (Aloui et al., 2014a), strawberries (Perdones et al., 2012) and citrus fruit (Youssef et al., 2012).

3. 8. Firmness

Changes in firmness of control and treated oranges throughout the storage time are reported in **Table 4**. A firmness decrease was observed for both treated and untreated fruits during the storage period (*P<0.05*) and was due to tissue senescence and cell structure deterioration, as well as to sample water loss, occurring during fruit ripening (Xiao et al., 2010). This decrease in firmness was more pronounced for control samples which were found to lose more than 50% of their firmness at the end of the storage period (*P<0.05*). Overall, coatings based on NaAlg and LBG were effective at reducing firmness loss of 'Valencia' oranges compared with the control (*P<0.05*), irrespective of yeast cell incorporation (*P>0.05*). In contrast to these results, Fan et al. (2009) reported the higher efficacy of NaAlg coatings incorporated with *C. laurentii* yeast in maintaining strawberry firmness, compared to those based on pure NaAlg. Regardless of the coating type, firmness loss reduction in coated oranges was estimated at more than 21% at day 15, compared with the control (*P<0.05*). Previous studies have reported the beneficial effect of polysaccharide coatings on firmness retention of many fruits including papaya (Ali et al., 2011), grapes (Aloui et al., 2014a), banana (Maqbool et al., 2011) and mandarins (Valencia-Chamorro et al., 2011).

3.9. Control of P. digitatum *growth on oranges*

The effects of the different coatings on the fungal decay of inoculated oranges during storage at 25 °C are presented in **Figure 4**. After the fourth day of storage, 100% uncoated samples were infected by moulds; however a significant delay in the rate of fungal decay was observed in fruits treated with pure NaAlg and LBG, showing a maximum level of infection (100%) at day 5. NaAlg and LBG-based coatings, as well as other bio-based coatings (Sánchez-González et al., 2011; Perdones et al., 2012), might act as potential barriers to gaseous exchange, and this could explain the slight growth inhibition determined by the stand-alone coatings. As moulds are strictly aerobic and highly sensitive to CO_2 (Soliva-Fortuny et al., 2004), a decrease in oxygen permeability and CO_2 accumulation in the fruit could, thereby, affect *P. digitatum* growth in coated oranges. Results on 'Valencia' orange samples confirm the slight inhibitory effect (about 0.2 log CFU/cm^2) of both stand-alone coatings already observed in Petri plates (see paragraph *3.6* and **Figure 2a**).In agreement with our findings, Aloui et al. (2014a) have reported the efficacy of pure NaAlg coatings in reducing fungal decay caused by *P. digitatum* in inoculated grapes.

Figure 4 shows that NaAlg and LBG coatings incorporating *W. anomalus* killer yeast were effective at preventing disease incidence up to the 10th day of storage (0% disease incidence) and controlling the growth of *P. digitatum* until the end of the storage period, resulting in a fungal decay reduction of more than 73%, compared with the control and coatings formulated with pure NaAlg or LBG ($P<0.05$). In agreement with these findings, Fan et al. (2009) reported the efficacy of NaAlg coatings incorporating *C. laurentii* yeast in slowing down mould growth and reducing fungal decay incidence of strawberries by approximately 30% compared with the control at the end of the storage period. In our study, the high effectiveness at controlling fungal decay of coated oranges appeared to be related to the ability of *W. anomalus* to produce killer proteins with β-glucanase activity as revealed by

the results of the plate diffusion assay. In agreement with our findings, Platania et al. (2012) noticed a strong inhibition of *P. digitatum* growth up to the 10th day of storage (less than 15% of disease incidence), when assessing the biocontrol efficiency of *W. anomalus* strain (BS91) against the green mould caused by *P. digitatum* in Tarocco oranges. In another study, Lima et al. (2013) reported the efficacy of *W. anomalus* (strain 422) in delaying the onset of the anthracnose symptoms and reducing the intensity of disease lesions, caused by *C. gloeosporioides* in papaya. The high control efficiency of this strain was ascribed to multiple factors, including competition for space and nutrients owing its ability to colonize the fruit wounds faster than the fungus, mycoparasitism through direct physical interaction with fungal hyphae and production of enzymes that lyse the phytopathogen cell wall.

4. Conclusion

The incorporation of *W. anomalus* cells into NaAlg and LBG did not affect the functional and optical properties of the respective polysaccharide films which were able to maintain more than 85% of the initial yeast population and to completely inhibit the growth of *P. digitatum* in synthetic medium. The application of these two matrices as bioactive coatings was investigated to evaluate their efficacy in preserving the postharvest quality of 'Valencia' oranges. Our results showed that NaAlg and LBG coatings enriched with *W. anomalus* yeast were not only effective at reducing weight loss and maintaining firmness of 'Valencia' oranges during storage, but also at controlling green mould in inoculated fruits by more than 73% after 13 days. These results and the QPS status recently obtained by EFSA for the use of *W. anomalus* as a novel microorganism in food preservation, demonstrate the potential application of these bioactive coatings as effective and promising alternatives to synthetic antifungal agents for maintaining quality attributes and controlling green mould of 'Valencia' oranges.

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Figure legends

Figure 1. Survival of *Wickerhamomyces anomalus* yeast cells in sodium alginate (● NaAlg) and Locust Bean Gum (▲ LBG) films during 21 days of incubation at 25 °C and 75% RH. Mean values and LSD intervals. For each incubation time, mean values not followed by the same uppercase letter are significantly different ($P < 0.05$). For each treatment, mean values not followed by the same uppercase letter are significantly different ($P < 0.05$)

Figure 2. Effect of sodium alginate (NaAlg) and Locust Bean Gum (LBG) films either alone (■ NaAlg; ▲ LBG) or combined with *Wickerhamomyces anomalus* yeast cells (× NaAlg+Yeast; ▲ LBG+Yeast) on growth of *Penicillium digitatum* (**a**) and survival of *Wickerhamomyces anomalus* yeast cells (**b**), in contact with PDA medium incubated at 25°C. Mean values and LSD intervals. For each incubation time, mean values not followed by the same uppercase letter are significantly different ($P < 0.05$). For each treatment, mean values not followed by the same uppercase letter are significantly different ($P < 0.05$).

Figure 3. *In vitro* antagonism of *Wickerhamomyces anomalus* (*W. anomalus*) yeast incorporated into sodium alginate (NaAlg) and Locust Bean Gum (LBG) films against *Penicillium digitatum*: (**a**) control plates, (**b**) plates carrying pure NaAlg films, (**c**) plates carrying NaAlg films enriched with *W. anomalus* yeast, (**d**) plates carrying pure LBG films and (**e**) plates carrying LBG films enriched with *W. anomalus* yeast.

Figure 4. Effect of coatings formulated with sodium alginate (NaAlg) and Locust Bean Gum (LBG) either alone (■ NaAlg; □ LBG) or combined with *Wickerhamomyces anomalus* yeast cells

(■NaAlg+Yeast; ■ LBG+Yeast) on the incidence of green mould on 'Valencia' oranges artificially inoculated with *Penicillium digitatum*, coated and stored at 25ºC. Mean values and LSD intervals. For each storage time, mean values not followed by the same uppercase letter are significantly different ($P < 0.05$). For each treatment, mean values not followed by the same uppercase letter are significantly different ($P < 0.05$).

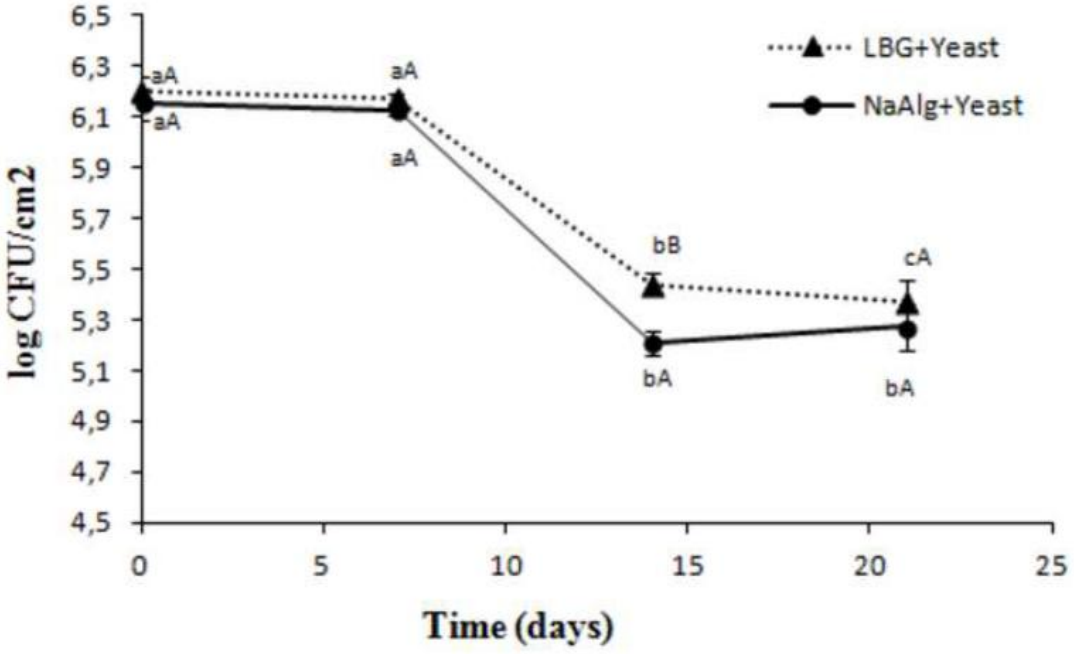

Figure 1

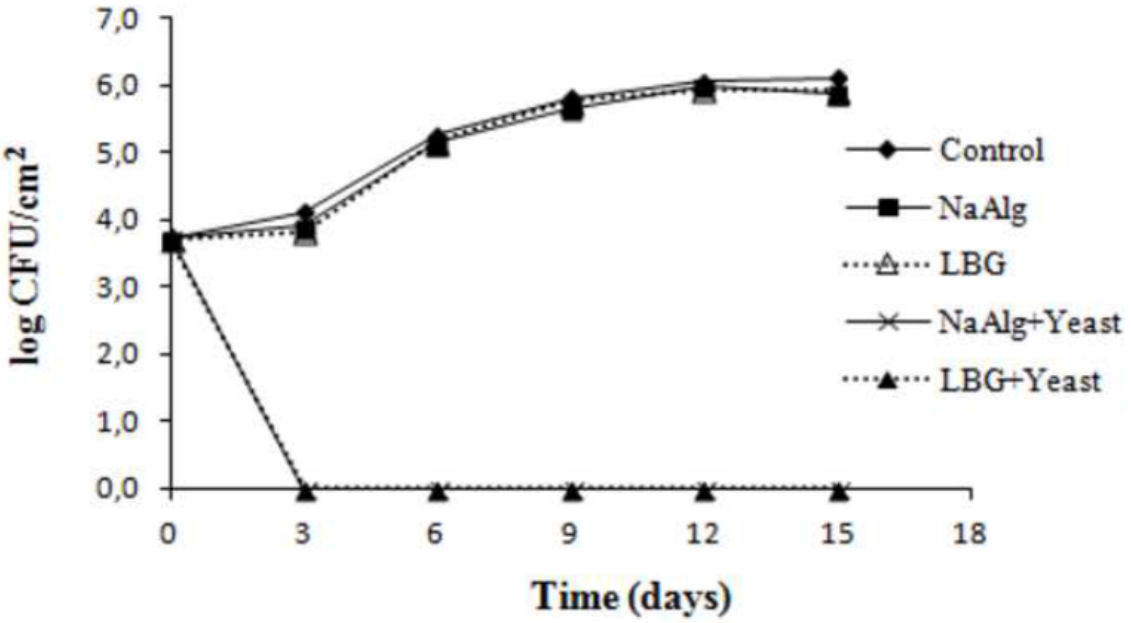

Figure 2a

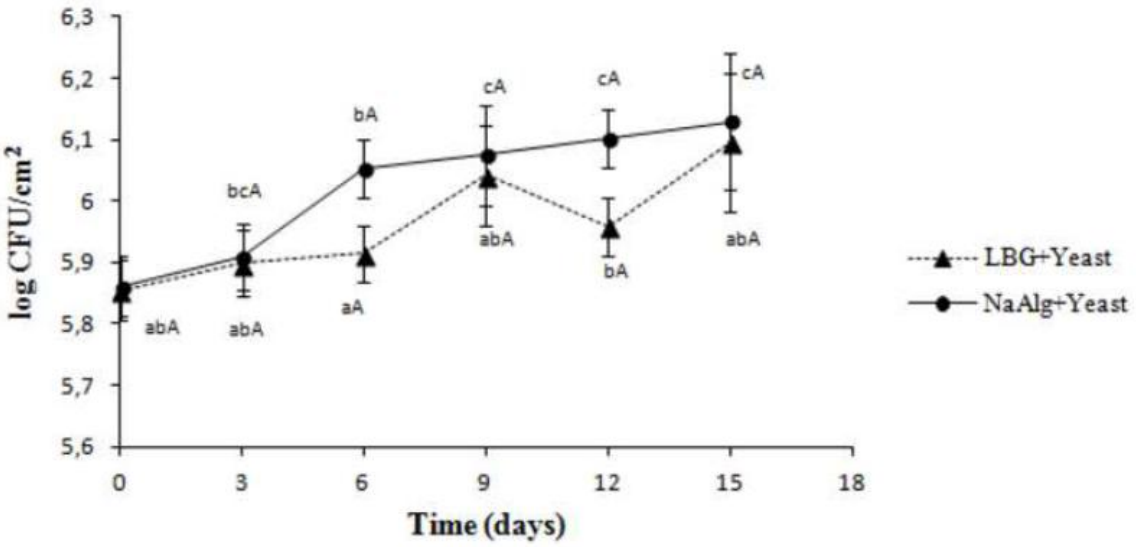

Figure 2b

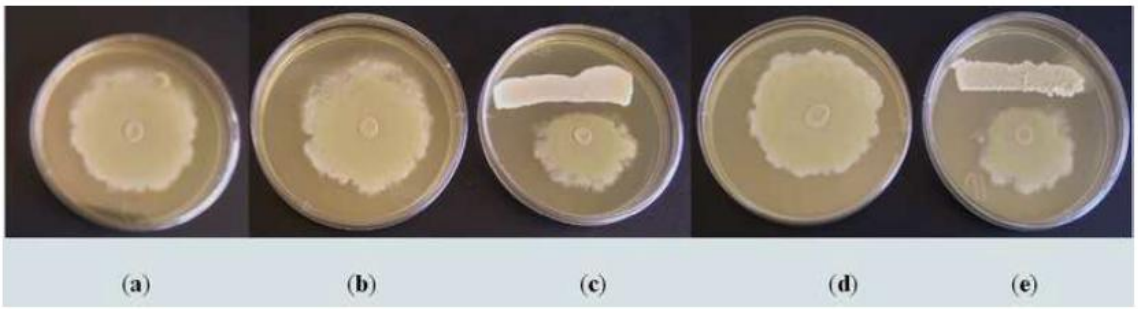

Figure 3

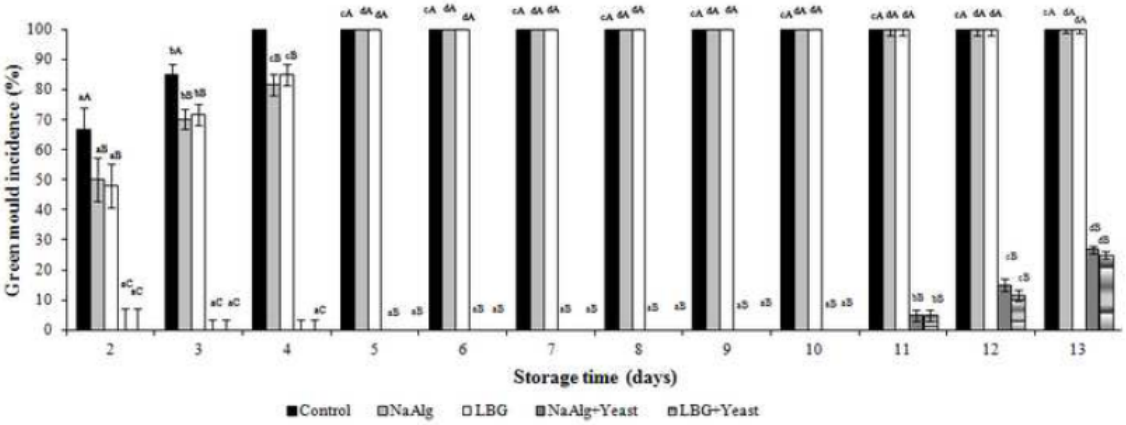

Figure 4

Table 1

Effect of *Wickerhamomyces anomalus* yeast cells incorporation on tensile strength (TS), elongation at break (%E), water vapor permeability (WVP), contact angle and Gloss of sodium alginate (NaAlg) and Locust Bean Gum (LBG) films. Mean values and standard devation.

Films	TS (MPa)	% E (%)	WVP(g mm/m^2/d/KPa)	Contact angle (°)	Gloss (GU)
LBG	11.63±1.72^a	24.15±2.92^b	1.92±0.25 ^{a}	70.23±6.35^b	13.56±1.23^a
LBG+Yeast	10.91±0.69^a	22.74±3.33^b	2.01±0.29^a	70.66±6.24^b	13.93±1.17^a
NaAlg	47.02±3.98^b	4.52±0.51^a	2.92±0.32^b	53.13±3.67^a	42.21±4.04^b
NaAlg+Yeast	46.10±2.53^b	4.58±0.47^a	3.01±0.19^b	52.09±5.82^a	43.79±6.40^b

a–b: Different superscripts within a column indicate significant differences among films ($P<0.05$).
NaAlg (pure sodium alginate film)
LBG (pure Locust Bean Gum film)
NaAlg+Yeast (sodium alginate film enriched with *Wickerhamomyces anomalus* yeast)
LBG+Yeast (Locust Bean Gum film enriched with *Wickerhamomyces anomalus* yeast)

Table 2

Mycelial growth diameter of *Penicillium digitatum* after 10 days incubation at 25°C. Mean values and standard deviation.

Films	Mycelial growth diameter (mm)
Control	57.50±0.70[a]
NaAlg	57.25± 0.35[a]
LBG	57.00± 1.41[a]
NaAlg+Yeast	35.50± 0.70[b]
LBG+Yeast	36.00± 1.41[b]

a–b: Different superscripts indicate significant differences among films at ($P<0.05$).
Control: plates seeded only with *P. digitatum.*
NaAlg (pure sodium alginate film)
LBG (pure Locust Bean Gum film)
NaAlg+Yeast (sodium alginate film enriched with *Wickerhamomyces anomalus* yeast)
LBG+Yeast (Locust Bean Gum film enriched with *Wickerhamomyces anomalus* yeast)

Table 3

Weight loss of uncoated and coated 'Valencia' oranges stored at 25 °C for 15 days. Mean values and standard deviation.

Storage time	Weight loss (%)				
	Control	LBG	LBG+Yeast	NaAlg	NaAlg+Yeast
3	1.90±0.81^{a,x}	1.24±0.21^{a,z}	1.53±0.20^{a,y}	1.45±0.17^{a,yz}	1.42±0.15^{a,yz}
6	3.62±0.36^{b,x}	2.60±0.18^{b,y}	2.63±0.31^{b,y}	2.94±0.71^{b,y}	2.52±0.33^{b,y}
9	5.83±0.51^{c,x}	3.73±0.26^{c,y}	3.46±0.42^{c,y}	3.85±1.00^{bc,y}	3.40±0.96^{c,y}
12	7.10±0.51^{d,x}	4.81±0.34^{d,y}	4.77±0.38^{d,y}	5.10±0.70^{cd,y}	4.79±0.12^{d,y}
15	8.74±0.95^{e,x}	6.26±0.52^{e,y}	5.83±0.27^{e,y}	6.00±0.85^{d,y}	6.20±0.16^{e,y}

a–e: different superscripts within a column indicate significant differences among storage time ($P < 0.05$).
x–z: different superscripts within a file indicate significant differences among treatments ($P < 0.05$).
NaAlg (pure sodium alginate coating)
LBG (pure Locust Bean Gum coating)
NaAlg+Yeast (sodium alginate coating enriched with *Wickerhamomyces anomalus* yeast)
LBG+Yeast (Locust Bean Gum coating enriched with *Wickerhamomyces anomalus* yeast)

Table 4

Force at break of uncoated and coated 'Valencia' oranges stored at 25 °C for 15 days. Mean values and standard deviation.

Storage time	Force at break (N)				
	Control	LBG	LBG+Yeast	NaAlg	NaAlg+Yeast
0	23.84±1.53^{a,x}	23.84±1.53^{a,x}	23.84±1.53^{a,x}	23.84±1.53^{a,x}	23.84±1.53$^{a.x}$
3	19.15±0.62^{b,x}	21.72±1.18^{b,y}	21.81±1.80^{b,y}	21.55±1.42^{b,y}	22.07±1.93^{b,y}
6	16.69±0.99^{c,x}	20.34±1.50^{b,y}	20.05±2.06^{bc,y}	20.66±0.94^{b,y}	20.15±0.94^{c,y}
9	14.72±0.67^{d,x}	18.29±1.56^{c,y}	18.68±1.38^{cd,y}	18.04±0.95^{c,y}	18.42±0.76^{d,y}
12	13.65±0.43^{d,x}	17.46±1.43^{cd,y}	17.74±2.12^{de,y}	17.58±1.30^{cd,y}	17.22±0.77^{de,y}
15	11.44±0.21^{e,x}	16.68±0.58^{d,y}	16.75±1.13^{e,y}	16.46±1.02^{d,y}	16.89±1.96^{e,y}

a–e: different superscripts within a column indicate significant differences among storage time ($P < 0.05$).
x–y: different superscripts within a file indicate significant differences among treatements ($P < 0.05$).
NaAlg (pure sodium alginate coating)
LBG (pure Locust Bean Gum coating)
NaAlg+Yeast (sodium alginate coating enriched with *Wickerhamomyces anomalus* yeast)
LBG+Yeast (Locust Bean Gum coating enriched with *Wickerhamomyces anomalus* yeast)

Highlights

- Sodium alginate and locust bean gum films incorporating killer yeast were developed.
- More than 85% of the initial yeast population survived after incorporation to polysaccharide films.
- Bioactive films inhibited the growth of *Penicillium digitatum* in synthetic medium.
- Coatings containing yeast reduced weight and firmness losses of 'Valencia' oranges.
- Bioactive coatings reduced green mould in inoculated oranges by more than 73%.