Responsive packaging based on imine-chitosan films for extending the shelf-life of refrigerated fresh-cut pineapple

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ABSTRACT

Reversible imine chemistry has been employed to stabilize antimicrobial liquid volatiles trans-2-hexenal (HX) and salicylaldehyde (SL) to the surface of chitosan films. The reactivity of both aldehydes to amine groups of chitosan was high and the synthesis of imine bonds was assessed by ATR-FTIR. The hydrolysis of the bonds and the release of the aldehydes depended on the pH of the medium. Films exerted in vitro microbiocidal effect against E. coli, S. cerevisae and pineapple wild yeast when were immersed at pH 3.6. Finally, films were incorporated in the double-bottom of plastic containers filled with fresh-cut pineapple maintained at 4 °C for 18 days. Juice leakage from pineapple pieces triggered the release of aldehydes to the headspace of the package. Both aldehydes extended microbiological shelf-life of fruit and slowed down browning. SL increased juice leakage from fruit and affected firmness negatively.

Keywords: Chitosan, pH-responsive films, imine bond, antimicrobial packaging, pineapple, fresh-cut, trans-2-hexenal, salicylaldehyde.

1. Introduction

Pineapple is one of the most popular tropical fruits and is often found cut and ready-to-eat in markets. Fresh-cut fruit is a minimally processed product, which provide consumers
convenient, nutritious, healthy, and fresh tasting food. Despite its benefits, fruit processing makes the product very perishable and susceptible to rapid deterioration and quality loss due to microbial growth and oxidative process on the product surface (Barth, Hankinson, Zhuang, & Breidt, 2009; Lante, Tinello, & Nicoletto, 2016). Pineapple presents an endogenous microbial population mainly consisting on yeasts (Tournas, Heeres, & Burgess, 2006) which together with browning and leaking due to minimally processing operations limits its shelf-life. Acid pH of pineapple allows the growth of lactic and acetic bacteria, moulds and yeasts, whereas the high acidity is expected to hinder the proliferation of foodborne pathogen bacteria. However, there are several works reporting the survival of these pathogens in different varieties of fresh-cut pineapple (Abadias, Alegre, Oliveira, Altisent, & Viñas, 2012; Feng et al., 2017). Moreover, pineapples have been associated with outbreaks of Escherichia coli and Salmonella spp. (Strawn, Schneider, & Danyluk, 2011).

There is a huge amount of studies regarding the use of naturally-occurring antimicrobial compounds in fresh-cut produce. In most of them the antimicrobial is applied by dipping or incorporated in an edible coating. These techniques together with refrigeration and packaging with passive or active MAP are the main tools to avoid or delay deterioration processes of fresh-cut produce (Çandır, 2017).

Advanced packaging technologies are based on the development of active packages which, among other designs, use encapsulating materials as carriers and release systems of antimicrobial volatiles. In this regard, active packaging technologies based on the sustained release of antimicrobials combine with other hurdle technologies are promising for extending the shelf life and improving the safety of fresh-cut fruit (da Rocha Neto, Beaudry, Maraschin, Di Piero, & Almenar, 2019).
Most of the studies regarding the use of antimicrobial volatile compounds in fresh-cut pineapple are based on their application by dipping/spraying or, by incorporating them into edible coatings; only a few studies have explored the application of the active compound in the headspace of the package. Thus, methyl jasmonate was embedded in cotton and applied in fresh-cut pineapple stored in jars (Martínez-Ferrer & Harper, 2005).

In this work, a new sophisticated approach consisting on the chemical immobilization of antimicrobial volatiles in the surface of a polymer film through reversible covalent bonds has been developed with the aim to stabilize the volatile molecules. The antimicrobial activity of the films is due to the reversibility of the synthetized bonds and the subsequent release of the active molecules to the surrounding environment when necessary. Currently, the use of reversible covalent bonds is being explored in different fields for multiple purposes (Huang et al., 2020) including the design of smart release systems of bioactives (Chen et al., 2020; Fadida, Selilat-Weiss, & Poverenov, 2015; Rizzo & Kehr, 2021; Tchakalova et al., 2021; Zentner, Anson, Thayumanavan, & Swager, 2019). However, their application in the design of antimicrobial responsive food packaging is practically unexplored. Therefore, the novelty of this work relies on the synthesis of pH-reversible imines to create responsive materials with antimicrobial properties that can be applied to the design of responsive packaging to extend the shelf-life of minimally processed fruit. The synthesis of imine bonds has been chosen due to their capability of breaking apart under mild acid hydrolysis.

A series of naturally-occurring aldehydes proceeding from essential oils and classified as Generally Recognized as Safe (GRAS) compounds were evaluated for antimicrobial properties against pathogen and spoilage microorganism capable of growing in fresh-cut pineapple. Those having the greatest antimicrobial activity against these microorganisms were chosen to be chemically immobilized on the surface of chitosan films through the
formation of imines. Chitosan was chosen as the polymer to stabilize the active volatiles because it has a large number of free amino groups that can be converted into imines. The modified chitosan films were characterised to verify the formation of imine bonds, and pH-reversibility of imines was also tested. The release of grafted aldehydes is based on the hydrolytic breakage of the imine bond catalysed by mild acids. In that sense, fresh-cut pineapple exudates could be the trigger to release the antimicrobial volatiles from the films. The antimicrobial effectivity of synthetized imine-chitosan films was evaluated in vitro against E. coli, S. cerevisae and pineapple wild yeast. Later on, the responsive films developed were tested in vivo on fresh-cut pineapple. For that, the films were incorporated in the design of a double-bottom package containing the fresh-cut produce. This kind of package avoids the direct contact between the fruit and the active film, whereas the double perforated bottom collects the exudate from the processed fruit and triggers the hydrolysis of imine bonds on chitosan films and the release of the antifungal volatile to the package headspace creating a biocidal atmosphere around the fruit surface. The evolution of several shelf-life parameters (firmness, pH and colour) and microbiological growth was monitored on passive and responsive packaged fresh-cut pineapple stored at 4 °C for 18 days.

2. Materials and methods

2.1. Materials

Low molecular weight (LMW) chitosan (75 – 85% deacetylation degree), glacial acetic acid and all aldehydes used in this work were provided by Sigma-Aldrich (Barcelona-Spain). Citric acid monohydrate, and disodium phosphate to carry out buffer medium at pH 7 and pH 4 was also purchased from Sigma-Aldrich (Barcelona-Spain). Hydrochloric acid 37%, ethanol 96%, di-phosphorus pentoxide and granulated sodium hydroxide of
synthesis grade were supplied by Scharlab (Barcelona, Spain). Milli-Q water was obtained by Milli-Q Plus purification system (Millipore, Molsheim, France). *Escherichia coli* (CECT 434) and *Listeria innocua* (CECT 910) was supplied by the Spanish Type Culture Collection (CECT, Valencia, Spain). The yeast *Sacharomyces cerevisae var. ellipsoideys* (NCYC 2959) was supplied by National Collection of Yeasts Cultures (NCYC). All culture medium employed were supplied by Scharlab (Barcelona, Spain).

2.2. Antimicrobial assays

2.2.1. Microorganisms and preparation of inoculums

Bacterial strains, *E. coli* (CECT 434) and *L. innocua* (CECT 910) were grown and maintained on tryptone soy broth (TSA) and incubated for 24 h at 37 °C. The yeast *Sacharomyces cerevisae var. ellipsoideys* (NCYC 2959) was used as reference. In addition, a wild yeast was isolated from a chunk of pineapple following the method reported by Amorim, Piccoli, & Duarte, (2018) and was labelled as PWY (Pineapple Wild Yeast). The PWY was used to assess the effectiveness of the active compounds on pineapple yeast population. Yeasts were grown on malt extract agar (MEA) for 72 h at 26 °C. All microorganisms were stored at 4 °C on agar medium and transferred monthly. Previous to experiments, an overnight culture was obtained. For that, a loopful of the microorganism cultured in agar was transferred to sterile broth medium and incubated for 72 h at 26 °C for yeasts and 24 h at 37 °C for bacterial strain to obtain early stationary phase of cells.

2.2.2. Antimicrobial activity of aldehydes

Previous to grafting aldehydes to the surface of chitosan films, a screening on the antimicrobial activity of several food grade aldehydes was carried out, and those with the
greatest activity were later on used for developing responsive films. Thus, the antimicrobial properties of citral (CT), citronellal (CO), trans-2-hexenal (HX), benzaldehyde (BZ), cuminumaldehyde (CU), p-anisaldehyde (AN), salycialdehyde (SL), trans-cinnamaldehyde (CN), hydrocinnamaldehyde (HC) and (S)-(−)-perillaldehyde (PL), were evaluated in vitro against *E. coli*, *L. innocua*, *S. cerevisae* and pineapple wild yeast. The micro atmosphere test was used to evaluate the antibacterial activities of the aldehydes in the vapour phase (Wang et al., 2018). For this purpose, each overnight was diluted until obtain an inoculum with $10^7$ CFU/mL. Then, 100 µL of each inoculum was spread on MEA when working with yeasts, and TSA for *E. coli* and *L. innocua*. A sterile paper disk of 25 mm was placed and fixed in the lid cover of a Petri dish and 10 µL of each aldehyde were added. The plates were sealed using parafilm to reduce losses of the aldehyde evaporated from the paper. Finally, bacterial strains were incubated at 37 °C for 24 h and *S. cerevisae* and PWY at 26 °C for 72 h. After that time, the inhibition halo was measured and a percentage of inhibition (%) calculated.

The most effective aldehydes were tested against bacterial and yeast strains to determine minimal inhibition concentration (MIC) and minimal microbicidal concentration (MMC). Following the previously described method, an antimicrobial test of vapour phase of aldehydes was carried out. Different volumes of aldehydes were dosed in a disk of paper attached on the lid of plates containing 15 mL of agar medium. A range between 0.5 to 10 µL of aldehyde per plate was assessed. Controls without volatile were also analysed. After incubation time, the diameter of the inhibition zone was measured to determinate MIC and MMC. The MIC is described as the minimal amount of volatile causing growth inhibition or retraction zone. MMC is defined as the minimal amount of volatile that completely inhibits microorganism growth. All analyses were conducted in triplicate.
2.3. Grafting aldehydes to chitosan films

Those aldehydes more active against all tested microorganisms were selected to be immobilized in chitosan films through reversible imine bonds. For that, 1.5% (w/w) of LMW chitosan with a molecular weight range of 50-190 kDa was dissolved in 0.5% (w/w) of acetic acid under continuous stirring at 50 °C until chitosan was completely dissolved. Chitosan acetate films were obtained by solvent casting method. For that, 90 g of film forming solution was poured in polystyrene plates (25 x 16 cm) and dried at 37 °C for 24 h. Then, the films with a thickness of 35±5 μm were neutralized by immersion in 0.1 M sodium hydroxide aqueous solution at room temperature for 24 h. After that, neutralized chitosan films (CS) were washed with deionized water and dried at 37 °C and stored in glass desiccators.

Aldehyde immobilization in chitosan film was carried out by a reaction between neutralized CS films and aldehydes in 96% (v/v) ethanol and hydrochloric acid was used as catalyst. CS films and aldehyde were added in a weight ratio 1:2 and the reaction took place at 60 °C during 24 h using a shaking bath. After that, reaction medium was removed and films were washed with ethanol for 24 h to remove free aldehyde from films. This operation was carried out three times. Finally, responsive chitosan films were dried and kept in a desiccator.

2.4. Spectroscopic characterization of imine bonds

Fourier transform infrared spectrophotometer with Attenuated Total Reflectance accessory (ATR-FTIR) was used to characterize the imine bonds formed in chitosan film. Infrared spectra were recorded with a Bruker Tensor 27 FTIR spectrometer (Bruker Española S.A., Barcelona, Spain). At least 32 scans were recorded in the range of 4000
to 600 cm⁻¹ for each sample and with a resolution of 4 cm⁻¹. Infrared spectrum was treated with the OPUS v. 5.0 software.

2.5. **Quantification of aldehydes grafted to chitosan films**

The amount of aldehydes respect to glucosamine units of chitosan films was measured as degree of substitution (DS, %) according to the equation proposed by Takeshita, Konishi, Takebayashi, Yoda, & Otake, (2017). For that C/N ratio of the films was determined by elemental analysis with a CHNS-O elemental analyser FlashSmart (Thermo Fisher Scientific, Waltham, MA, USA). The degree of acetylation of chitosan was also determined by using elemental analysis and according to the methodology of Kasaai, Arul, Chin, & Charlet, (1999). All test was carried out in triplicate.

2.6. **Release of aldehydes grafted to chitosan films: effect of the pH**

The effect of the pH on the hydrolysis of formed imine bonds, and subsequent release of the aldehyde molecules previously grafted to chitosan films was evaluated by gas chromatography (GC). For this purpose, 0.1 g of responsive films were placed in a glass jar with a volume of 250 mL. Films were covered with 10 mL of aqueous solution buffered at several pH (3, 4, 5, 6 and 7). Glass jars were hermetically sealed with a twist off lid, previously perforated (Ø = 1 cm) to place a septum for gas samples withdrawal from the jar headspace. Samples of 0.5 mL were manually collected after 24 h of jar storage at 23 °C, and injected into a gas chromatograph mod. 6850 Series II Network GC System (Agilent Technologies, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a Restek RTX1 capillary column (30 m of length, 0.53 mm internal diameter, and 5 μL thickness) with a flow rate 14.6 mL/min of helium as carrier gas. Injector temperature and FID temperature was fixed at 220 °C. Temperature of the oven
was from 35 to 220 °C in 21.8 min. Injected samples were run in the splitless mode. The obtained values were quantified according to a previous calibration curve with known amounts of aldehyde. Results were represented as concentration of aldehyde (mg per L_{air} of headspace per g of film). All analyses were performed in triplicate.

2.7. *In vitro* antimicrobial activity of responsive films

The antimicrobial activity of developed responsive films was tested against the same microorganisms tested for free aldehydes. A double plate system was used to carry out the *in vitro* assays as described in a previous study (Heras-Mozos et al., 2021). Briefly, a Petri dish (58 mm) with agar medium was placed into an empty Petri dish (90 mm). A volume of 60 μL of the aliquot with 10^7 CFU/mL was deposited and extended with a Digralsky handle on different agar medium depending on the microorganism as mentioned above. The responsive films (0.25 g) were placed in the empty plate and activated by embedding it with aqueous buffer solution (pH 7 and 3.6). The system was covered with a lid and sealed with parafilm. Using double Petri dish avoids direct contact between films and culture medium. Two systems, one having untreated CS film and the other one without film were used as controls. The test for *L. innocua* and *E. coli* were incubated at 37 °C for 24 h, whereas yeasts were incubated at 26 °C for 72 h. After that time, the inhibition was evaluated by visual inspection of growth surface and comparison with the control. All analyses were performed in triplicate.

2.8. Shelf-life studies of fresh-cut pineapple packaged with responsive films

2.8.1. Packaging and storage of fruit

Costa Rican ‘Tropical Gold’ type fresh pineapples (*Ananas comosus*) were purchased in a local market and were stored at 7 °C until its processing. Pineapple fruit was processed
at room temperature. To minimize possible cross contamination, the whole pineapple was sanitized by immersion in a sodium hypochlorite solution (1%) for 5 min and then, pineapple was dried. Peeling and cutting of the fruit took place aseptically on a sterile surface and sharp knives. Pineapple’s core was removed and cut into triangular pieces with a thickness of 25 mm and weight in the 10 - 12 g range. Polypropylene (PP)/ethylene-vinyl alcohol copolymer (EVOH)/PP containers of 220 cm³ were used to package pineapple pieces. The containers were provided of a double bottom to avoid direct contact between responsive films and fruit. The films (0.5 g) were placed at the bottom of the container, and a perforated plastic pad was placed between the film and 60 g of fruit. A cross-section view of the package is showed in Figure 1. The perforated double bottom system, besides to avoid direct contact between the film and the fruit, allows pineapple exudate to contact the films and thus, and triggers the release of anchored aldehyde during the storage of the fruit. A package without films was used as control.

Finally, the containers were thermo-sealed with PP film (30 μm) using a SMART 300 heat sealer for trays (ULMA. Embalaje S.C., Spain). The samples were stored at 4 °C for 18 days. Analyses of packaged pineapple were conducted at days 3, 6, 9, 12, 15 and 18 after packaging. The packaged samples were performed in triplicate.

![Figure 1. Antifungal responsive package for fresh-cut pineapple.](image)
2.8.2. Quality parameters of packaged fresh-cut pineapple

Packaged fresh-cut pineapple was evaluated for main quality parameters such as colour, pH, juice leakage and firmness.

Colour of individual chunks of pineapple was determined using a CR-300 Minolta Chroma meter® (Minolta Camera Co. Ltd., Osaka, Japan). CIELAB colour coordinates were measured, and hue angle (h°) and Chroma (C*) determined. Total colour difference values (\(\Delta E\)) were evaluated according to the equation:

\[
\Delta E = \sqrt{(L - L_0)^2 + (a - a_0)^2 + (b - b_0)^2}
\]

where \(L_0\), \(a_0\), and \(b_0\) refer to the colour values at 0 day. The results of colour were expressed as the average ± standard deviation of the values of at least 5 chunks of pineapple in both sides.

Measurements of pH were performed using a portable pH meter (Consort C830, Belgium) with a penetration pH electrode for solids (PHEL-PB5-001, Metria, Spain) to measure the pH directly in the fruit. Juice leakage was recollected from the bottom of all packages and reported as mg of exudate per g of pineapple.

Texture was evaluated with a TA.XT.plus Texture Analyzer (Stable Micro Systems, Godalming, UK). A 15-mm penetration was made with a 0.1-inch diameter cylindrical probe with a flat base (TA/0.1) at a speed of 1 mm/s. The test was carried out to determine the work to penetrate and values were expressed as N x mm.

2.8.3. Microbiological analysis of packaged fruit

2.8.3.1. Natural microbial load of pineapple

The microbiological quality of refrigerated fresh-cut pineapple packaged using responsive films was evaluated through monitoring the growth of moulds and yeast, mesophilic and psychrophilic microorganisms. The microbiological load was measured
every 3 days during 18 days of storage. For this propose, around 30 g of fruit were aseptically transferred to a sterile Stomacher bag and blended with 30 mL of 0.1% sterile peptone water. It was homogenized for 3 min in a stomacher bag using a Stomacher Blender (IUL S.L., Barcelona). Subsequently, serial dilutions were made in sterile peptone water and aliquots of each dilution were plated in Petri dishes containing several agar culture media through spread plate method. The media employed was plate count agar (PCA) to determinate the mesophilic and psychrophilic microorganism. The plates of psychrophilic were incubated at 4 °C for 10 days, whereas the mesophilic were incubated at 30 °C for 48 h. For determination of moulds and yeast counts, the dilutions were spread in Malt Extract Agar (MEA) and incubated at 26 °C for 4 days. The colonies were enumerated and results were reported as logarithm of colony forming units (CFU) per grams of fruit (log CFU/gfruit). All microbiological analysis was carried out in triplicate.

2.8.3.2. Inoculation and survival of pathogens in packaged fruit

Antimicrobial response of imine-chitosan films against E. coli and L. innocua on pineapple fruit was carried out. Containers of 60 cm³ were filled with 20 g of inoculated fruit and 0.17 g of films were placed in the bottom of the packages. Prior to the assays, inoculum of each pathogen was prepared. For that, an aliquot of 100 μL of overnight culture was transferred to 10 mL of sterile TSB tube and incubated at 37 °C until reach exponential phase with 10^6 CFU/mL. Inoculation of fresh-cut fruit pieces with pathogenic bacteria was carried out by depositing 0.1 mL of inoculum with 10^6 CFU/mL on the surface of 20 g of fresh-cut pineapple per each package. The E. coli and L. innocua suspension was distributed uniformly over the surface of pineapple pieces. Inoculated samples were maintained under sterile atmosphere for 10 min to allow the inoculum to
dry. Packaged fruit without responsive films was used as control. After that, the packages were closed and stored at 4 °C for 9 days. The microbial load in fruit was evaluated at 0, 1, 3, 6 and 9 days of storage. For that, the same process as described above was followed. The selective medium for *E. coli* enumeration was Violet Red Bile Dextrose (VRDB agar) and Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesculin Mannitol agar (PALCAM agar) was used to enumerate *L. innocua*. The plates were incubated for 24 h at 37 °C and the data were expressed as log CFU/g fruit. All test was carried out in triplicate.

2.9. Statistical analyses

All the tests were conducted at least in triplicate and represented as the average ± standard deviation. Statistical analysis of the results was performed with SPSS® computer program, v.27 (SPSS commercial software, SPSS Inc., Chicago, IL). Results were analysed applying a one-way analysis of variance (ANOVA). Means were separated using the Tukey b test with a level of significance of P ≤ 0.05.

3. Results and discussion

3.1. *In vitro* antimicrobial activity of aldehydes

Table 1 shows antimicrobial properties of the aldehydes assayed against *E. coli*, *L. innocua*, *S. cerevisae* and PWY (Pineapple Wild Yeast). No inhibitory effect was observed against the microorganisms assayed when exposed to 10 μL in vapour phase of benzaldehyde (BZ), cuminaldehyde (CU) and p-anisaldehyde (AN). *S. cerevisae* and PWY were susceptible to citronellal (CO) and perillaldehyde (PL), whereas these aldehydes did not show any inhibition on the growth of the bacteria strains tested. Citral (CT), hydrocinnamaldehyde (HC) and cinamaldehyde (CN) showed a considerable antimicrobial activity against all the microorganisms tested, with an inhibition around 40-
50%. However, the most effective aldehydes were trans-2-hexenal (HX) and salicylaldehyde (SL). HX inhibited 100% the growth of the microorganisms assayed and SL showed the same behaviour except for *L. innocua*, which caused a 58% of inhibition. Antimicrobial activity of aldehydes has been widely studied against several microorganisms (Friedman, Henika, & Mandrell, 2003). In particular, antimicrobial potential of trans-2-hexenal and salicylaldehyde have been previously evaluated against food pathogens (Kawacka, Olejnik-Schmidt, Schmidt, & Sip, 2021; Ma, Zhao, Zhao, & Xie, 2019). Thus, SL and HX were selected based on this preliminary aldehyde screening for antimicrobial activity in vapour phase. The antimicrobial effectiveness of SL and HX against tested microorganism was assessed by determining MIC and MMC. Table S1 shows the MIC and MMC values of both aldehydes for each microorganism. The aldehydes were more active against yeasts than bacterial strains, being MIC in the 0.5 – 1 and 2.5 μL/plate ranges, respectively.

**Table 1.** Growth inhibition (%) of several microorganisms exposed to 10 μL of several naturally-occurring aldehydes.

<table>
<thead>
<tr>
<th>Aldehyde</th>
<th><em>E. coli</em></th>
<th><em>L. innocua</em></th>
<th><em>S. cerevisae</em></th>
<th><em>PWY</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>39.7 ± 0.8bA</td>
<td>52.2 ± 3.3cB</td>
<td>50 ± 5.6cB</td>
<td>47.2 ± 2.8bcAB</td>
</tr>
<tr>
<td>CO</td>
<td>0aA</td>
<td>0aA</td>
<td>87.8 ± 6.7ebB</td>
<td>89.4 ± 2ebB</td>
</tr>
<tr>
<td>HX</td>
<td>100 ± 0cA</td>
<td>100 ± 0cA</td>
<td>100 ± 0fA</td>
<td>100 ± 0fA</td>
</tr>
<tr>
<td>BZ</td>
<td>0aA</td>
<td>0aA</td>
<td>0aA</td>
<td>0aA</td>
</tr>
<tr>
<td>CU</td>
<td>0aA</td>
<td>0aA</td>
<td>0aA</td>
<td>0aA</td>
</tr>
<tr>
<td>PL</td>
<td>0aA</td>
<td>63.9 ± 2.8dcC</td>
<td>49.2 ± 0.8ebB</td>
<td></td>
</tr>
<tr>
<td>AN</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
<td>0a</td>
</tr>
<tr>
<td>SL</td>
<td>100 ± 0cB</td>
<td>57.8 ± 2.2daB</td>
<td>100 ± 0fbB</td>
<td>100 ± 0fbB</td>
</tr>
<tr>
<td>CN</td>
<td>43.1 ± 1.4bA</td>
<td>100 ± 0edA</td>
<td>67.5 ± 0.8dcB</td>
<td>53.6 ± 1.9dbB</td>
</tr>
<tr>
<td>HC</td>
<td>42.2 ± 4.4bAB</td>
<td>33.3 ± 5.6bA</td>
<td>40.6 ± 1.7bAB</td>
<td>45.6 ± 0.6bcC</td>
</tr>
</tbody>
</table>

Different letters (a-f) in the same column indicate a statistically significant difference between different aldehydes. Different letters (A-C) in the same row indicate a statistically difference of same aldehyde against different microorganism.
3.2. Spectroscopic characterization of imine bonds

The formation of new imine bonds obtained by the condensation between primary amine groups of chitosan and carbonyl groups of salicylaldehyde (SL) and 2-trans-hexenal (HX) was evaluated by ATR-FTIR analysis of the new films. The IR spectra of control chitosan films, and films modified with aldehydes are represented in Figure S1. The spectra show a characteristic peak of chitosan, corresponding to the stretching of $\text{C}_6\text{-OH}$ corresponding to the glycosidic ring of the polymer. An overlapping and wide absorption band corresponding to O-H and N-H vibrations was observed from 3600 to 3000 cm$^{-1}$, and around 2900 cm$^{-1}$ appears other band which is assigned for stretching vibration of C-H of chitosan. The bands allocated to C=O stretching of amide I, and N-H bending of the primary amine were observed at 1644 and 1582 cm$^{-1}$, respectively (Barbosa, Attjioui, Leitão, Moerschbacher, & Cavalheiro, 2019). Zoom IR spectra of films in the range of 1750 to 700 cm$^{-1}$ is shown in Figure 2. The modification of chitosan with SL and HX gave rise to novel absorption bands at 1626 and 1650 cm$^{-1}$, respectively, which correspond to the formation of imine bonds (C=N) by the nucleophilic addition of aldehydes to primary amino groups of chitosan. In addition, other peaks were altered after Schiff base formation, the involvement of primary amine groups to form imines caused a reduction of the intensity of the band associated to primary amine group at 1585 cm$^{-1}$ (Iftime, Morariu, & Marin, 2017). New peaks are observed in the spectra of imine-chitosan films, which are associated to the immobilization of the aldehydes in chitosan films. Also, a new band is observed around 2900 cm$^{-1}$, especially notable in CSHX films, which is due to vibration of C-H of aliphatic chain of trans-2-hexenal (Chen et al., 2020; Prakash, Baskaran, & Vadivel, 2020). After modification with salicylaldehyde, some peaks corresponding to aromatic ring of salicylaldehyde can be observed at 1580 and 750 cm$^{-1}$ (Dos Santos, Dockal, & Cavalheiro, 2005). Also, CSSL films showed a decrease of
the broadband around 3300 cm\(^{-1}\), which could be due to an interaction between hydroxyl group of aldehyde in \textit{ortho} position with N-H and O-H groups of chitosan backbone (Iftime et al., 2017).

![ATR-FTIR spectra of chitosan films and responsive films synthezized with trans-2-hexenal (CSHX) and salicylaldehyde (CSSL) in the range of 1750-700 cm\(^{-1}\).](image)

**Figure 2.** ATR-FTIR spectra of chitosan films and responsive films synthesized with trans-2-hexenal (CSHX) and salicylaldehyde (CSSL) in the range of 1750-700 cm\(^{-1}\).

3.3. \textit{Quantification of aldehydes grafted to chitosan films}

Elemental composition, C/N ratio and degree of substitution (% DS) of chitosan films and those modified with salicylaldehyde (SL) and trans-2-hexenal (HX) is presented in Table S2. Previously, the degree of acetylation of untreated CS films was calculated, being around 16%, this involves a large amount of free amino groups where aldehydes can be anchored to form imines. Chitosan films modified with aldehydes showed an increase of C/N ratio due to the incorporation of the compound, being 5.4 for untreated chitosan films and over 9.0 for modified chitosan films. DS values of modified films showed a great degree of conversion of amino groups of chitosan films to imines, reaching values above 50% in both films, and being slightly higher for CSHX films (71.6%)
compared with CSSL films (64.3%). Trans-2-hexenal is a lineal α,β-unsaturated aldehyde, contrary to saturated aldehydes, the carbonyl group is conjugated with an alkene which makes it electrophilic at both the carbonyl carbon as well as the β-carbon. Thus, either site can be attacked by nucleophiles. The conjugated double bond makes this aldehyde more reactive, whereas its lineal structure favours a more reduced steric hindrance when it is grafted to chitosan compared with aromatic aldehydes.

3.3.1. Effect of the pH on the release of aldehydes grafted to chitosan films

The acidity of hydrolytic environments has been identified as a primary factor influencing the reversibility of imines (Xin & Yuan, 2012). Thus, the effect of the pH on the reversibility of the imine bonds created in chitosan films, and subsequent release of salicylaldehyde (SL) and trans-2-hexenal (HX) was evaluated by gas chromatography. The amount of aldehyde released to the headspace of closed hermetic jars after 24 h of immersing the films in buffered solutions of different pH is depicted in Figure 3. The results show that regardless of the aldehyde, an increasing amount is recovered in the headspace of the jars as the pH of the hydrolytic solution decreases. Thus, the greater aldehyde concentration was obtained at pH 3, reaching 5 ± 0.1 mg/L·g<sub>film</sub> of HX, and 9.3 ± 0.2 mg/L·g<sub>film</sub> of SL, whereas at pH 7 these values were 0.3 ± 0.1 and 1.1 ± 0.1 mg/L·g<sub>film</sub>, for HX and SL respectively. The latter values represent 12 and 6 % of the amount measured when the films were activated in acid medium (pH 3). Thus, the films developed are considerably stable in neutral hydrolytic environments. In this line, different acidic environments have been used to reverse imine formation and subsequently trigger the release of the active molecule. Thus, the acid microenvironment of tumour tissues (Peng et al., 2019; Tao, Liu, Zhang, Chi, & Xu, 2018), the organic acids produced by microorganisms (Neqal, Fernandez, Coma, Gauthier, & Héroguez, 2018), or
gastric juices (Chen et al., 2020) are examples of bioactive triggers. In the current work, it is expected that pineapple juice rich in organic acids triggers the hydrolysis of imine bond and the release of volatile aldehydes to exert its antimicrobial action on the fruit. Regarding the amount of aldehyde released to the medium, it was observed that, in spite of the greater amount of HX incorporated into the films (DS ~ 72% and 64% for HX and SL, respectively), the amount of HX released was lower compared with that for SL (Figure 3).

Figure 3. Concentration of aldehyde (mg/L·g<sub>film</sub>) released to the vapour phase from imine-chitosan films immersed at different pH during 24 h at 23 °C.

Thus, besides the pH of the media, the structural features of the aldehyde anchored to chitosan plays a role on the release of the molecule to the headspace of the package. Chen et al. (2020) have recently studied the stability of aldehyde-chitosan conjugates in acid medium. The authors reported that cinnamaldehyde and citral form more stable imines than citronellal and vanillin. Similar to cinnamaldehyde and citral, trans-2-hexenal is a polyfunctional substrate, containing a double bond conjugated with a carbonyl group. This feature makes the molecule more reactive than saturated aldehydes. Thus, besides
nucleophilic addition of primary amines of chitosan to carbonyl group, α, β-unsaturated aldehydes can undergo Michael addition, that is, nucleophilic attack to the electrophilic β-alkene carbon and the formation of a covalent bond which is not easily hydrolysable. Therefore, trans-2-hexenal could be also grafted to chitosan in a different way to the formation of a reversible Schiff base. The consequence would be that aldehyde molecules attached via Michael addition could not be available for release in acidic media. This rational could explain the lower release of HX from the films. Other important point to consider is that when the imine bond is hydrolysed, the aldehyde molecules migrate from the film to the water solution, and then evaporates to the surrounding atmosphere. Therefore, some physico-chemical parameters such as log P and vapour pressure at saturation of the aldehyde affect the equilibrium liquid-vapour and thus, to the amount of aldehyde determined in the headspace.

3.3.2. In vitro antimicrobial activity of responsive films

The antimicrobial activity of CSHX and CSSL was tested in vitro against *E. coli*, *L. innocua*, *S. cerevisae* and PWY (Pineapple Wild Yeast). The hydrolysis of the imine bonds was studied at pH 3.6 and 7 to evaluate the effect of the triggering solution pH on the antimicrobial activity of the films. Figure S2 shows pictures of the growth of the tested microorganisms on the agar surface in Petri dishes exposed to the vapours of HX and SL released from films after being activated at two pH values. The control that appears in the picture refers to the surface growth of microorganisms in a Petri dish that was not exposed neither to plain chitosan films nor to new films developed. It is important to point out that no differences were observed between control without film or with plain chitosan films because in the in vitro antimicrobial system assayed there was no direct contact with the growth medium. A visible decrease of microbial density on agar surface of Petri dish
corresponding to CSSL films activated at pH 7 is observed and is related to the antimicrobial effect exerted by the low amount of aldehyde released at this pH. CSSL films exerted microbicidal activity when were activated at pH 3.6, except for *L. innocua*, which is in accordance the highest values of MMC. According to the data obtained by gas chromatography, which showed that acid pH promotes the highest release of aldehyde, a greater antimicrobial activity is expected when the trigger is a buffer solution at pH 3.6. Films modified with HX (CSHX) also exerted microbicidal activity at pH 3.6 for *E. coli, PWY* and *S. cerevisae*, whereas the growth of *L. innocua* was inhibited, observing a reduction of bacterial density on surface agar. The HX release at pH 7 was fungicidal but not bactericidal. The results showed a higher susceptibility of both yeasts to the presence of aldehydes, while bacterial strains were more resistant, especially *L. innocua* as observed in the MIC and MMC data. Although the amount of HX released is lower than that for SL as discussed in the above section, HX has a potent fungicidal activity (as reflected by the low MIC values, see table S1) which explains the results obtained.

3.4. *Shelf-life studies of fresh-cut pineapple packaged with responsive films*

3.4.1. *Quality parameters of packaged fruit*

Fresh-cut pineapple is a ready-to-eat product packaged for direct consumption. When it is cut into portions, its shelf-life is compromised as a consequence of broken vegetable tissue. Figure 4 shows the visual aspect at different storage time of pineapple chunks packaged with and without responsive films. As can be appreciated, responsive films contributed to preserve the original aspect of the fruit.
The colour of the ready-to-eat cut pineapple is a quality parameter that affects consumer acceptance. Changes in the colour of pineapple chunks during storage was monitored through $\Delta E^*$, lightness ($L^*$), Chroma ($C^*$) and hue angle ($h^\circ$) parameters. Figure 5 shows $\Delta E^*$ evolution of pineapple chunks stored under passive and responsive packages incorporating CSHX and CSSL films. In general, $\Delta E^*$ increased progressively through the storage period but this increase was more pronounced for fruit stored in a passive package, and differences in colour between passive and responsive package were significant after nine days of storage.
Figure 5. Evolution of the total colour difference (ΔE*) of pineapple pieces packaged without and with imine-chitosan films of trans-2-hexenal (CSHX) and salicylaldehyde (CSSL) for 18 days at 4 °C.

The evolution of L*, Chroma and hue angle of pineapple chunks packaged in passive and responsive packages is shown in Figure 6. In all the samples, L* tended to decreased through storage which is indicative of fruit darkening. However, L* dropped from 63.9 to 46.7 after 18 days of storage in fruit packaged without responsive films. The darkening of samples packed with the active system was less accused, and only a slight decrease of L* from 63.9 to 58.4 for CSHX and to 57.0 for CSSL was observed. Decompartmentalisation and bruising of tissue lead to oxidative reactions such as enzymatic browning reaction and consequent generation of dark pigments (González-Aguilar, Ruiz-Cruz, Cruz-Valenzuela, Rodríguez-Félix, & Wang, 2004). Several authors have also reported the beneficial effect of essential oils delaying browning (Çandır, 2017; Nogales-Delgado, 2021; Zhou, Sun, Zou, Zhou, & Liu, 2021). Over time, chroma values of the samples tended to diminish indicating that the colour intensity of pineapple chunks decreased with storage, this tendency being significantly less accused in fruit that was...
stored in responsive packages. Hue angle of fruit was maintained through storage without finding significant changes between passive and responsive packaging carrying CSHX films, whereas $h^\circ$ of fruit pieces packaged with CSSL slightly increased.

**Figure 6.** Colour parameters of pineapple pieces packaged without and with imine-chitosan films for 18 days at 4°C. (CSHX: trans-2-hexenal-imine-chitosan films, CSSL: salicylaldehyde-imine-chitosan films).
The evolution of acidity of pineapple pieces was monitored through pH and is shown in Table 2. The pH of control fresh-cut pineapple slightly increased through storage, whereas changes were less accused in the samples packaged with responsive films. During fruit ripening, acidity decreases and pH increases due to conversion of organic acids into sugars (Benítez, Soro, Achaerandio, Sepulcre, & Pujolá, 2014). A delay in the consumption of organic acids could explain these results. Since polyphenol oxidase (PPO) activity depends on the pH, and PPOs from pineapple are more active around neutral pH (Nogales-Delgado, 2021), pH evolution results are in agreement with the retention of L∗ values of fruit packaged using responsive films.

Table 2. pH of pineapple packaged for 18 days at 4 °C without and with imine-chitosan films of trans-2-hexenal (CSHX) and saliciyaldehyde (CSSL).

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>3</th>
<th>6</th>
<th>9</th>
<th>12</th>
<th>15</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.7±0.1aA</td>
<td>3.8±0.2aAB</td>
<td>4.1±0.2cBC</td>
<td>4.3±0.2bCD</td>
<td>4.2±0.1bCD</td>
<td>4.4±0.1bD</td>
</tr>
<tr>
<td>CSHX</td>
<td>3.8±0.1ab</td>
<td>3.8±0.2ab</td>
<td>3.5±0.1aA</td>
<td>3.8±0.1ab</td>
<td>3.9±0.1abc</td>
<td>4.1±0.1ac</td>
</tr>
<tr>
<td>CSSL</td>
<td>3.8±0.1aA</td>
<td>3.8±0.2aA</td>
<td>3.8±0.2hab</td>
<td>3.9±0.1aA</td>
<td>4.0±0.1aA</td>
<td>4.0±0.2aA</td>
</tr>
</tbody>
</table>

Different letters (a-c) in the same column indicate a statistically significant difference between packaged samples per day. Different letters (A-C) in the same row indicate a statistically difference of same treatment at different days.

Juice leakage is together with brown discoloration, microbial and visible fungal growth the main factor limiting the shelf-life of fresh-cut pineapple (Çandır, 2017). Pineapple cultivar and the fresh-cut shape have been reported to affect juice leakage whereas neither temperature nor atmosphere modification have effect on juice leakage incidence (Marrero & Kader, 2006; Montero-Calderón, Rojas-Graü, & Martín-Belloso, 2008; Serrano, Martínez-Romero, Castillo, Guillén, & Valero, 2005). Figure 7 shows firmness and juice leakage of pineapple chunks packaged in passive containers and those employing responsive films. The amount of juice that leaked from fruit pieces increased following
a linear trend line, however the slope was much greater for fruit packaged using CSSL films. No differences were found in the juice leaked from pieces packaged in passive containers and using CSHX. Salicylaldehyde released into the headspace of the package could be more absorbed by the fruit than trans-2-hexenal, interacting with cell wall components of pineapple causing fruit leakage.

Figure 7. Firmness (bars) and juice leakage (lines) of pineapple pieces stored for 18 days at 4 °C under passive and responsive packaging (CSHX: trans-2-hexenal-imine-chitosan films, CSSL: salicylaldehyde-imine-chitosan films).

Firmness evolution of pineapple chunks stored at 4 °C for 18 days using passive and responsive packaging is displayed in Figure 7. Firmness of fresh-cut pineapple slightly decreased during the storage period, but the decrease was greater for fruit packaged with CSSL films, especially after ninth days of storage. Thus, at the end of the storage, firmness of control fruit was reduced by 19 % respect to the initial value, whereas the
firmness of fruit packaged with CSHX suffered a reduction of 11%, and the decrease was around 25% for fruit packaged incorporating CSSL films. In general, EOs or their components do not alter or exert some beneficial effect on fruit firmness and other quality parameters. However, some of them can cause adverse effects on fruit, with occurrence of phytotoxicity and acceleration of the physico-chemical and physiological changes related to ripening and senescence (Serrano et al., 2005; Taghavi, Kim, & Rahemi, 2018). In other cases, adverse effects of natural volatiles or essential oils depend on the concentration applied on the fruit (Basaglia et al., 2021; Prakash et al., 2020). In the present study, the amount of SL released from CSSL films to the headspace of the package increased juice leakage and had an adverse effect on fruit firmness.

3.4.2. Microbiological assays

3.4.2.1. Microbiological analysis of packaged fruit

Besides biochemical and physiological changes associated to processing operations such as peeling and cutting, the shelf life of fresh-cut pineapple is limited mainly by microbial and visible fungal growth (Çandır, 2017). In the current study, the natural microbial load of pineapple including the growth of moulds and yeasts, mesophilic and psychrophilic microorganisms was monitored in pineapple chunks stored for 18 days under refrigeration in passive containers without or incorporating CSHX and CSSL films. Figure 8 shows microbial counts evolution of the natural microorganisms of pineapple. The results showed a great difference on the evolution of microorganisms in passive and responsive packaged fresh-cut pineapple. The differences were more marked for the growth evolution of moulds and yeasts since these microorganisms, capable of growing at low pH, are the predominant population in fresh-cut pineapple.
Just prepared fresh-cut pineapple presented a total count of around 3.5, 2.6 and 3.6 log CFU/g\textsubscript{fruit} of mesophiles, psychrophiles, and moulds and yeasts, respectively. These data are in accordance with those reported by other authors (Bierhals, Chiumarelli, & Hubinger, 2011; Leneveu-jenvrin et al., 2020). Similar to other countries of the European Union, Spanish legislation establishes for fresh-cut produce a range of aerobic mesophilic counts from $5-6$ log CFU/g\textsubscript{fruit} on the day of processing and packaging, to $6-7$ log CFU/g\textsubscript{fruit} on the expiration date of the product, but it does not specify limits on moulds and yeasts count for ready-to-eat fruits (BOE, 2001). However visible fungal growth is one of the main causes limiting the shelf-life of fresh-cut pineapple (Chonhenchob, Chantarasomboon, & Singh, 2007). Moreover, in accordance with the microbiological standards for food not thermally processed of IFTS, (1999), around $6$ log CFU/g\textsubscript{fruit} is considered the limit of acceptance of moulds and yeasts in fresh-cut fruit. Besides the ability of fungi to spoil minimally-processed pineapple, Leneveu-jenvrin et al. (2020) reported the ability of some fungal isolates from pineapple to produce mycotoxins.

In this study, microbial flora of fruit stored without or with responsive films was maintained inside the quantitative levels of microbiological quality until the 6\textsuperscript{th} day of storage, although responsive packaged fruit had lower microbiological load. At day 9, considerable differences were found in moulds and yeasts population between passive and responsive packaged fruit. Control fruit reached a population around $6$ log CFU/g\textsubscript{fruit} which is in the limit recommended by IFST, whereas this value was 4.5 and 4 log CFU/g\textsubscript{fruit} in pineapple packaged with CSHX and CSSL films, respectively. Regarding mesophilic population, it was maintained below legislation levels of $6-7$ log CFU/g\textsubscript{fruit}, although mesophilic counts were much lower for fruit packaged with responsive films. At 12\textsuperscript{th} day of storage, mesophilic counts of control fruit were in the up limit allowed by the legislation, whereas fruit packaged with CSSL and CSHX films has count of 4 and 5
log CFU/g_{fruit}, respectively. Psychrophilic counts were 6.8 log and around 4-5 log CFU/g_{fruit} for control and fruit packaged with responsive films. Regarding moulds and yeasts counts, they were around 7 log for fruit packaged in passive system, whereas 5.3 and 4.2 log CFU/ g_{fruit} were enumerated when fruit was packaged with CSHX and CSSL films. These data correspond with the strong visual deterioration of passive packaged fresh-cut pineapple at day 12, while the other samples maintained a better appearance (Figure 4). Typically, ready-to-eat fruit become spoiled once microbial flora levels are in the range 10^7-10^8 CFU/g_{fruit}, the growth of moulds and yeasts, and bacteria produce a wide variety of enzymes and processes responsible of limiting shelf-life of foods, with changes in colour, odour and texture. At the end of the storage, moulds and yeasts enumerated in fruit packaged with responsive films did not exceed the limit recommended by IFST, whereas mesophilic and psychrophilic counts were around 5 log CFU/g_{fruit}.

Comparing responsive films, the best microbiological results were obtained employing CSSL films. However, SL caused a loss of firmness and an excess of juice leakage respect to control and fruit packaged with CSHX films (as described earlier, figure 7). Similar results of microbial inhibition were observed when a nanoemulsion containing citral was incorporated in a coating for fresh-cut pineapple (Prakash et al., 2020). The authors reported that although the best microbiological results were obtained with the higher amount of citral tested, it affected negatively to quality parameters of pineapple.

Therefore, it should be interesting to study if it is possible to find the optimal salicylaldehyde concentration necessary to retard microbial proliferation without damaging fruit texture.
3.4.2.2. Growth and survival of pathogens inoculated in fresh-cut pineapple after packaging

The evolution of *E. coli* previously inoculated on the surface of pineapple pieces is presented in Figure 9. The *L. innocua* evolution was depicted in Figure S3. Passive packaged pineapple showed a slow and gradual decrease in bacterial population. After nine days of storage pathogen population was reduced 50 and 70%, for *E. coli* and *L. innocua*. Similarly, others studies reported a reduction of population of *E. coli* on fresh-cut pineapple (Strawn & Danyluk, 2010). Regarding *L. innocua*, previous studies reported that pineapple resulted largely unsuitable for its growth (Ziegler, Rüeg, Stephan, & Guldimann, 2018). The low pH of pineapple could inhibit bacterial growth. Depending
on pineapple variety, fruit acidity, nutrient composition and pH are different, and these parameters affect the growth of microorganisms (Abadias et al., 2012). Responsive packages incorporating CSHX and CSSL films showed antimicrobial activity after 6 days of storage against *E. coli*, exerting bactericidal activity after nine days of refrigerated storage. However, no significant reduction in the *L. innocua* growth was observed, which could be related to the greater MIC and MMC values measured for *L. innocua* than *E. coli*.

![Figure 9](image)

**Figure 9.** Evolution of *E. coli* inoculated in pineapple pieces stored at 4 °C for 9 days under passive and responsive packaging.

### 4. Conclusions

Trans-2-hexenal and salicylaldehyde were successfully grafted to the surface of chitosan films by means of imine bonds. The films were pH-responsive and after immersion in aqueous solution buffered at pH ranging 3-7, imines suffered hydrolysis and consequently, the aldehydes were released to the surrounding atmosphere. The amount of aldehyde liberated depended on the pH of the medium and the physico-chemical features
of the molecule. *In vitro* assays showed films that presented a significant inhibitory effect against tested microorganisms when they were activated at pH 3.6. The responsive films were incorporated in a double-bottom tray used to package refrigerated fresh-cut pineapple. Juice leaked from pineapple pieces entered in contact with the responsive films placed in the bottom of the container, and thus, triggered the release of the aldehydes to the surrounding atmosphere. CSHX and CSSL films considerably reduced microbial load in packaged fruit, and also delayed browning. However, CSSL films led to a marked decrease in fruit firmness and an increase in juice leakage, that could be due to an excessive release of SL from the films at the pH of pineapple exudate. Moreover, responsive packages showed bactericidal activity against inoculated *E. coli*. Future studies are needed to optimize the amount of CSSL film incorporated into the double-bottom package in order to avoid the observed negative effects of SL on the texture of pineapple pieces without compromising antimicrobial effectiveness. Moreover, a sensory evaluation should be also conducted to determine how the aldehydes affect the flavour of pineapple pieces.

**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.

**Acknowledgments**

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Development of antimicrobial pH-responsive food packaging based on reversible covalent bond to extend shelf life of fresh-cut pineapple

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**Figure S1.** ATR-FTIR spectra in the range of 4000 to 600 cm$^{-1}$ of chitosan and imine-chitosan films.

**Table S1.** Antimicrobial activity of trans-2-hexenal (HX) and salicylaldehyde (SL) tested as vapour phase against *E. coli* and *L. innocua* incubated at 37 ºC for 24 h and *S. cerevisiae* and PWY incubated at 26 ºC for 48 h.

<table>
<thead>
<tr>
<th></th>
<th>Trans-2-Hexenal (HX)</th>
<th>Salicylaldehyde (SL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC  (μL/plate)</td>
<td>MMC  (μL/plate)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>2.5</td>
<td>7.5</td>
</tr>
<tr>
<td><em>L. innocua</em></td>
<td>2.5</td>
<td>10</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>1</td>
<td>2.5</td>
</tr>
<tr>
<td>PWY</td>
<td>1</td>
<td>2.5</td>
</tr>
</tbody>
</table>
Table S2. Elemental analysis of chitosan and imine-chitosan films.

<table>
<thead>
<tr>
<th>Films</th>
<th>N (%)</th>
<th>C (%)</th>
<th>C/N</th>
<th>Substitution degree (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS</td>
<td>7.8 ± 0.2 b</td>
<td>42.1 ± 0.4 a</td>
<td>5.4 ± 0.1 a</td>
<td>-</td>
</tr>
<tr>
<td>CSHX</td>
<td>5.6 ± 0.1 a</td>
<td>50.6 ± 0.1 b</td>
<td>9 ± 0.1 b</td>
<td>71.6 ± 2.7</td>
</tr>
<tr>
<td>CSSL</td>
<td>5.6 ± 0.1 a</td>
<td>51.6 ± 0.1 c</td>
<td>9.2 ± 0.2 b</td>
<td>64.3 ± 2.6</td>
</tr>
</tbody>
</table>

Different letters (a-c) in the same column indicate a statistically significant difference between samples.

Figure S2. Antimicrobial effect of untreated chitosan and imine-chitosan films of trans-2-hexenal (CSHX) and salicylaldehyde (CSSL) at several pH against E. coli, L. innocua S. cerevisae and isolated yeast (PWY) after incubation time.
Figure S3. Evolution of *L. innocua* inoculated in pineapple pieces stored at 4 °C for 9 days under passive and responsive packaging.