



Development and optimization of antifungal packaging for sliced pan loaf based on garlic as active agent and bread aroma as aroma corrector



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ABSTRACT

The aim of the present work was the development of antimicrobial films containing garlic extract to be applied as active packaging for preservative-free sliced pan loaf, with the goal of extending its shelf-life. First, the antimicrobial capacity of garlic extract, a compound used as active agent, was tested against *Penicillium expansum* by the disc diffusion method. The extract showed high antimicrobial activity, 0.1 µL per Petri dish being the minimum inhibitory amount, and 0.25 µL the minimum fungicidal amount. Bread aroma was also used to mask the pungent odour of garlic and it was confirmed to have no antimicrobial activity. Subsequently, polyethylene (PE) aqueous emulsion and ethylene-vinyl alcohol copolymer (EVOH) and zein hydroalcoholic solutions containing 0.25 and 0.5% (w/w per dry polymer) of garlic extract and bread aroma were used to coat PE films, producing PE/PE, PE/EVOH and PE/zein active films. The antimicrobial capacity of the films was studied *in vitro* against *Penicillium expansum*, and *in vivo* with natural sliced bread. The results showed that all the films presented some antimicrobial activity, PE film coated with zein containing 0.5% of garlic extract and bread aroma being the film presenting the best results, maintaining bread free of mould infection for 30 days. Sensory tests showed that the addition of 1% of bread aroma improved the sensory experience of consumers and also revealed good purchase intention.

1. Introduction

Bread is an essential food product in the traditional diet in Europe, the Middle East, India, America and Oceania. According to the Codex Alimentarius, bread is the product resulting from baking dough obtained by mixing flour and water, with or without addition of edible salt, fermented by baker's yeast, *Saccharomyces cerevisiae*. Bread slowly deteriorates after baking, owing to a combination of chemical and physical processes called staling and owing to microbiological spoilage.

Pan loaf is a type of bread baked in a pan, with a soft texture and high water activity ($0.94 < a_w < 0.97$) (Legan, 1993), and commonly sold sliced. To delay staling and dehydration, the sliced bread is packaged in bags, keeping a humid headspace and, consequently, great conditions for fungal spoilage.

Fungal spores are very resistant to thermal treatments employed by the food industry, including baking, which explains their prevalence in a wide range of products. Under favourable conditions they develop and disperse the fungi, colonizing not only the bread slice surface but also the inside of the crumb. This phenomenon causes deterioration of

the sensory properties of the product, discoloration, decomposition and generation of mycotoxins, which can pose a health risk (Filtenborg et al., 1996). Mould growth causes great economic losses to the industry.

Aspergillus, *Eurotium* and *Penicillium* species in general are the main fungi implicated in the deterioration of bakery products and pan loaf (Dagnas et al., 2014). *Penicillium expansum* is a widespread fungal pathogen in many foods, which causes great economic losses and also health issues, since it produces toxic secondary metabolites (Andersen et al., 2004; Sadok et al., 2018; Tannous et al., 2017). To reduce fungal growth and increase shelf-life, the bakery industry adds low amounts of authorized preservatives to the dough, mainly sodium propionate (E 281), sorbic acid (E 200), sodium sorbate (E 201), potassium sorbate (E 202), calcium acetate (E 263) and ascorbic acid (E 300) (Guynot et al., 2005). Owing to the inclusion of these chemicals, and the slicing and packaging, sliced pan loaves are associated with artificial bread (compared to freshly baked baguettes).

Consumers are concerned about the presence of chemical additives and preservatives in food, and are demanding their elimination and the

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provision of more natural and healthy products. Consequently, companies are looking for alternative ways to inhibit microbiological growth and maintain both sensory and nutritional quality of the product, including the development of active packaging incorporating antimicrobial agents.

Active packaging is a food/packaging/environment system that acts in a coordinated way to improve food safety and quality and increase its shelf-life. The purpose of antimicrobial packaging is to impede or inhibit the growth of microorganisms in food. The great advantage of antimicrobial packaging over the direct addition of preservatives is that the active agent is gradually released on the food surface during storage and distribution, producing an inhibitory or lethal effect against pathogens that affect food products (Balaguer et al., 2013). The current demand for natural products also affects the selection of antimicrobial agents, and there is a preference for the incorporation of natural compounds in packaging materials, such as herbs, spices and, especially, essential oils (Ribeiro-Santos et al., 2017). Numerous studies have demonstrated the inhibitory activity of various essential oils and extracts from plants such as tea, cloves, mustard, oregano, anise, cinnamon or garlic against various microorganisms in food (Giteru et al., 2017; Higuera et al., 2014; Johnson et al., 2016; Ju et al., 2018; Matan et al., 2012; Teixeira et al., 2014).

In this work, garlic (*Allium sativum*) extract was selected as a natural antimicrobial agent against *Penicillium expansum*, and it was used to develop active packaging for preservative-free sliced pan loaf. In addition to having a great antioxidant capacity (Fratianni et al., 2016), garlic extract is a broad-spectrum antimicrobial agent, fungi being highly susceptible to its action (Ross et al., 2001). The antimicrobial capacity of garlic is mainly due to the presence of sulfur compounds, also responsible for its characteristic flavour and aroma, as well as its therapeutic properties (Agarwal, 1996; Chen et al., 1998; Fratianni et al., 2016).

Alliin is an organosulfur compound containing the thiosulfinate group generated by the enzymatic conversion of alliin, the active substance responsible for the antimicrobial activity in garlic (Borlinghaus et al., 2014; Kyung, 2012). Alliin decomposes when garlic is processed at high pressures and temperatures, producing volatile sulfur compounds that include diallyl sulfide (DAS) and diallyl disulfide (DADS), which maintain the biological activity (Corzo-Martínez et al., 2007).

A limiting factor in the use of an extract or essential oil as antimicrobial packaging is the potential organoleptic impact on the packaged product. Owing to the pungent aroma of garlic extract, in this work we also incorporated an industrial essence of bread aroma which could hide the sensory impact of garlic. One of the compounds in the bread crust used is 2-acetyl-1-pyrroline, responsible for the smell of freshly baked bread (Cho and Peterson, 2010).

Most common packaging systems for sliced pan loaf are low-density polyethylene (LDPE) bags. LDPE is a plastic polymer that is converted by extrusion into flexible, transparent films with a good barrier to water vapour but highly permeable to oxygen and apolar compounds, and with easy heat sealability (Catala and Gavara, 2001). In this work, these LDPE bags are used as the substrate for the development of the active packaging system because they provide excellent functional properties (mechanical, barrier, optical, thermal, ...) at low cost. The activity is incorporated by adding an internal coating with the active agents. As potential polymers for the coatings, polyethylene, ethylene-vinyl alcohol copolymer (EVOH) with a molar ethylene content of 29% (EVOH-29) and zein were selected.

EVOH is a hydrophilic material that provides a good barrier to gases and aromas at low relative humidity, but the barrier declines severely in wet conditions (Cerisuelo et al., 2014; Muriel-Galet et al., 2014). EVOH has been used in previous works for the development of active coatings (Catala et al., 2015).

Zein is the protein fraction from corn, a biopolymer insoluble in water but soluble in alcohol, owing to its high content of proteins rich in hydrophobic amino acids, glutamic acid, leucine, alanine and

prolamin (Bisharat et al., 2018; Shukla and Cheryan, 2001). Zein has good film formability, although these films are rather fragile, so the use of plasticizing agents to impart flexibility and facilitate handling is necessary (Liang et al., 2015). Zein films have been used previously in the preparation of active packaging (Kashiri et al., 2016, 2017).

The aim of the present work was to develop active antifungal packaging by incorporating garlic extract and bread aroma in a polymeric coating on a PE bag for the preservation of natural sliced pan loaf without chemical preservatives.

2. Materials and methods

2.1. Chemicals and reagents

Linear low-density polyethylene (LLDPE) aqueous emulsion, Aquaseal™ 2200, was kindly provided by Paramelt (the Netherlands). A poly(ethylene-co-vinyl alcohol) with 29% ethylene molar content, Soarnol 2908, was kindly provided by The Nippon Synthetic Chemical Company (Osaka, Japan). Odourless zein was obtained from the Kobayashi Perfumery Co., Ltd. (Japan). 1-Propanol and ethanol 100% were acquired from Sigma (Madrid, Spain). Garlic extract (GE) was kindly provided by DOMCA (Granada, Spain) and bread aroma containing 2-acetyl-1-pyrroline by Dulcesol (Gandia, Spain). Glycerol was supplied by WVR International S.A.S. (France). Water was obtained from a Milli-Q Plus purification system (Millipore, Molsheim, France).

2.2. Preparation of fungal cultures

Penicillium expansum, previously isolated and identified from natural sliced bread, was grown on potato dextrose agar (PDA) in polystyrene Petri dishes for 7 days at 28 °C. The inoculum was collected by flooding the surface of the plates with sterile peptone water with Tween 80 (0.05% v/v) and then scraping the surface with a spatula. A 5 mL sample of the mould culture suspension was transferred to sterile polypropylene tubes and shaken to obtain a homogeneous suspension. Several dilutions were made to obtain 10⁶ spores/mL. The spore count was determined using the improved Neubauer method (Bright-Line Hemacytometer, Hausser Scientific, Horsham, PA).

2.3. Garlic extract and bread aroma inhibition assay

The antimicrobial activity of garlic extract (GE) and bread aroma (BA) was analysed by determining the minimum inhibitory amount and the minimum fungicidal amount (volume) against *P. expansum*. Samples of 3 µL of the mould culture suspension with 10⁶ spores/mL were inoculated at three equidistant points on PDA. Different concentrations of each compound (GE and BA) were added to 25 mm sterile blank filter discs and placed on top of a Petri dish. The plates were sealed with Parafilm® to prevent leakage of the volatile agent. Blanks were also prepared. The plates were incubated at 28 °C for 7 days. The minimum amount of agent spread in the paper disk, is considered the smallest amount that causes a reduction in fungal growth (compared to the control sample). The minimum fungicidal amount is the smallest amount that yields a no-growth effect.

2.4. Film preparation

A polyethylene (PE) dispersion was stirred for 15 min using a magnetic stirrer. Ethylene-vinyl alcohol copolymer (EVOH) was dissolved in a 1:1 (v:v) 1-propanol:Milli-Q water mixture at 75 °C. The solution was stirred for 30 min using a magnetic stirrer hotplate. Zein was dissolved in an 80:20 (v:v) of ethanol:Milli-Q water mixture at 80 °C. The solution was stirred for 60 min using a magnetic stirrer hotplate. After complete dissolution, the solution was cooled down to 37 °C and the plasticizer (15% of glycerol with respect to polymer content) was added and stirred for 5 min. Once the film solutions had

been prepared, GE and BA were added to achieve a final concentration with respect to the dry polymer of 0.5% GE and 0.5% BA or 0.25% GE and 0.25% BA and stirred again for about 10 min. Control films were obtained without GE or BA.

PE films used to pack wheat bread were fixed to a glass plate. A corona treatment was applied with a BD-20 AC high frequency corona surface treater (Electro-Technic Products, Inc., Chicago, IL, USA) for 2 min to improve the adhesion of the various coatings. The coating-forming solutions were spread on PE film using an extension bar with a 50 µm deep thread (Lin-Lab Rioja, Logroño, Spain) and immediately placed in a drying tunnel equipped with 3250-W halogen lamps for 5 min. Finally, the films were stored in glass desiccators at 22 °C in dry conditions (silica gel) prior to use. For the zein coating, a Teflon threaded bar was used for solution extension. PE/PE, PE/EVOH and PE/zein films were employed in the manufacture of 18 × 22 cm bags.

2.5. Antifungal activity of films

The antifungal activity of the PE/PE, PE/EVOH and PE/zein films with GE and BA was tested against *P. expansum*. Film discs (9 cm diameter) were stuck to Petri dish lids, and 3 µL samples of the mould culture suspension containing 10⁶ spores/mL were inoculated at three equidistant points on PDA. The plates were incubated at 28 °C for 12 days and growth was monitored by measuring the mould diameter on the 3rd, 5th, 7th and 9th days of storage. After the 9th day the films were removed from the lids of the Petri dishes, and at day 12 the colony diameter was measured to observe any fungicidal effect. The antifungal activity was also tested on control samples without film. All these experiments were performed in triplicate.

2.6. Bread production

White bread was produced according to the traditional method using a Moulinex bread-making machine (Balaguer et al., 2013) in program 8 with browning medium (level 2). Leavening time was 43 min and baking time, 45 min at 175 min. The ingredients, purchased from a local supermarket, were 500 g of white wheat, 10 g of dehydrated yeast, 340 mL of water, 6 g of salt and 12.5 g of sugar. After baking, the bread was cooled at ambient temperature for 90 min and cut into 1 cm thick slices with a standard bread slicer (Model TP 60/2, E. Gabarró, Barcelona, Spain). The dimensions of the bread slices were approximately 12 cm × 12 cm and the weight was around 45 g.

2.7. Bread packaging, storage and shelf-life study

Bread slices were cut in two, one half of each sample was inoculated at three points on the surface with 8 µL of the *P. expansum* conidial suspension, and the other half of each slice was not inoculated. Each sample was placed in a plastic bag (18 cm × 22 cm) made of PE/PE, PE/EVOH or PE/zein with 0.5% GE and 0.5% BA. Two different types of control samples were also prepared, one with an uncoated bag and the other with polymer coatings without active compound. The plastic bags were closed using a manual impulse sealing machine (Sealboy 420 SBM, Rovebloc S.A., Barcelona, Spain) and stored at room temperature. The antifungal effect was evaluated over time by visual inspection of the fungal growth. Analyses were performed on the 5th, 9th, 12th, 20th and 30th days after packaging. The tests were performed in duplicate.

2.8. Sensory analyses

To assess the acceptability of the aroma and taste of bread samples packaged in an active PE/zein bag, two sensory tests were carried out in a standardized test room (ISO, 2007). Slices were presented on plates covered with aluminium film and identified by three-digit codes. All sensory tests were carried out on the 1st, 5th and 10th days after packaging.

The first test was a ranking method (ISO, 2006), a method that assesses the differences between several samples based on the intensity of a single attribute. This method was used to determine the amount of BA required to hide the pungent aroma of garlic. Loaf slices were packaged in 5 sample bags of PE/zein films containing 0.5% GE and increasing amounts of BA (0, 0.5%, 1% and 1.5%) and stored at room temperature. Also, bread was packaged in plain PE bags (without coating). On the 5th day of storage, samples were opened and immediately analysed. The panel was composed of 10 untrained members and they were asked to order the 5 samples (5 the best sample, 1 the worst).

Once the active agent content had been optimized with the first sensory test, new samples of bread were packaged in PE/zein with 0.5% GE and 1% BA, and evaluated using a hedonic scale (1 = dislike extremely, 5 = neither like nor dislike, 9 = like extremely) to determine the sensory properties on the 1st, 5th and 10th days of storage at room temperature (MacFie et al., 1989). The panel consisted of 46 untrained members. In this test, members were also asked for their purchase intention on a 1–5 scale (1 = I would never buy it, 5 = I would certainly buy it).

2.9. Statistical analysis

All the microbiological experiments were conducted at least in triplicate. Statistical analysis of the results was performed using a one-way analysis of variance (ANOVA). Means were separated using the Tukey test ($P < 0.05$) (SPSS commercial software, SPSS Inc., Chicago, IL).

Statistical analysis of the sensory data was performed by a one-way ANOVA study. P-values of 0.05 or less were considered significant. The design of the sensory evaluation and the data analysis were carried out with Compusense Five software (release 5.0, Ontario, Canada).

3. Results and discussion

As stated above, the aim of this work was the development of an antifungal packaging material to be applied in the packaging of wheat pan loaf, incorporating garlic extract as an antifungal agent and bread aroma to reduce any potential sensory effect on the bread. A chromatographic analysis of the commercial garlic extract revealed the presence of many sulfur compounds, including: *n*-propanethiol, 2-propen-1-thiol, dipropyl sulfide, methyl propyl disulfide, isopropyl sulfide, sec-butyl sulfide, dipropyl disulfide, *S,S'*-diacetyl-1,3-benzenedithiol, propyl hydrodisulfide and dipropyl trisulfide. Several sulfide, disulfide and trisulfide compounds have been suggested as being responsible for the antimicrobial effect of garlic extract (Casella et al., 2013). A similar analysis of bread aroma showed the presence of 2-3-pentanedione, 3-methyl-1-butanol, benzyl methyl carbonate, methyl-pyrazine, acetyl pyrazine, 2-acetylpyridine or 2-acetyl-1-pyrroline, among others, all of them related to toasted flavours (Cho and Peterson, 2010). Both garlic extract and bread aroma were used as supplied.

To analyse the effectiveness of this development, the two compounds were added to different polymer matrixes, PE, zein and EVOH, and they were coated on PE. The antifungal activity of the agents was tested against *P. expansum*. The effect of the package on the bread was analysed from microbiological and sensory points of view.

3.1. Garlic extract and bread aroma inhibition assay

The antifungal effect of GE and BA in vapour phase against *P. expansum* was determined by the disc diffusion method, and the results are presented in Fig. S1 (supplementary file) and Table 1. As can be seen, 0.1 µL of GE resulted in growth inhibition of *P. expansum*, whilst 0.25 µL proved lethal for this mould. Earlier works have reported that bacteria and fungi are highly sensitive to the effect of GE (Casella et al., 2013; Pranoto et al., 2005). Although the mechanism of action is not clear, in general it is considered that essential oils and plant extracts can

Table 1

Diameter (average and standard deviation) of *P. expansum* colonies after exposure to diverse amounts of garlic extract (GE) or bread aroma (BA) using the disc diffusion method and incubated for 7 days at 28 °C.

GE (µL)	Diameter (cm)	BA (µL)	Diameter (cm)
4	5.00 ± 0.11	0	4.88 ± 0.04
0.1	3.10 ± 0.19	10	4.98 ± 0.15
0.25	0	20	4.79 ± 0.25
0.5	0	50	4.79 ± 0.25
1.0	0	100	4.79 ± 0.25

penetrate the plasma membrane because of their lipophilic character (Nogueira et al., 2010; Rasooli and Owlia, 2005). Nielsen and Rios (2000) analysed the antifungal effectiveness of various essential oils on the fungi that usually colonize bread and concluded that mustard and garlic presented the highest activity against the microorganisms tested, including *Penicillium*. Indeed, Li et al. (2014) reported that analysis by transmission and scanning electron microscopy showed that garlic components could penetrate into hyphae cells and even their organelles, and then destroy the cellular structure, leading to cytoplasm leakage. They also reported that garlic could penetrate the cellular and organelle membranes of *Candida albicans*, altering gene expression and finally affecting normal growth of the moulds (Li et al., 2016).

With respect to bread aroma, as expected and shown in Table 1, no antifungal effect against *P. expansum* was observed. 2-Acetyl-1-pyrroline has been suggested to be the key odorant of bread crust and responsible for the cracker-like odour properties (Cho and Peterson, 2010), but, to our knowledge, no antifungal properties have been reported for the bread aroma compounds.

3.2. Film preparation

PE, zein and EVOH coatings containing 0.5% GE and 0.5% BA, 0.25% GE and 0.25% BA, and without agents (as control) were prepared on PE films as described in the experimental section. The PE coating was slightly whitish with an average thickness of $20 \pm 1.0 \mu\text{m}$. The heat treatment performed to remove water from the emulsion was probably not sufficient to produce a homogeneous film, and partial particle sintering or coalescence was obtained during the manufacturing process (Cerisuelo et al., 2015), responsible for light scattering. The EVOH coatings were transparent and colourless, with an average thickness of $6.0 \pm 1.0 \mu\text{m}$. The zein coating was also transparent, with a visible yellowish colour and without discontinuities, and a thickness of $25 \pm 1.0 \mu\text{m}$. The thickness values were obtained by subtracting the thickness of the uncoated film (PE) from that of the coated one.

3.3. Antifungal activity of films

The antifungal activity of the films against *P. expansum* was evaluated by exposing the mould to the atmosphere created by the various active film (PE/PE, PE/zein and PE/EVOH) within the Petri dishes. Table 2 shows the evolution with time of the colony diameter for each type of coating and the concentration of the agents (control, 0.25% GE and 0.25% BA, and 0.50% GE and 0.50% BA). Fig. S2 (supplementary file) contains photos of the differential growth of *P. expansum* observed for each material after 12 days of storage at 28 °C.

Table 2 shows the growth of the colony diameters over time. The films containing 0.50% GE and 0.50% BA presented total inhibition of fungal growth during the storage period, irrespective of the coating polymer. Also, the moulds did not grow when exposed to films with zein coatings containing 0.25% GE and 0.25% BA throughout the 12-day storage. In contrast, films coated with PE or EVOH containing 0.25% GE exerted only partial inhibition during the first 5 days, but no differences with respect to control films were found after 7 days of

storage.

Notably, after removal of the film from the lid, no growth was observed in the Petri dishes stored for 3 more days at 28 °C in the samples where no growth was detected at day 12, suggesting that GE produced a fungicidal effect on the microorganism.

The differences between coatings might be due to the amount of antimicrobial compounds present in the film and the ability of the film to release the compounds. PE is a hydrophobic/lipophilic material and strongly retains apolar compounds (Kwapong and Hotchkiss, 1987). Thus, a slow release could be expected that would reduce the antifungal effect of the film coated with PE. In the case of the EVOH coatings, the polymer is very hydrophilic and therefore a fast release could be expected upon hydration of the film. The reason for a low effect on *Penicillium* could be related to the thinness of the coating, which means a small amount of agent in the Petri dish, not enough to maintain inhibition of the mould up to the end of the storage test.

The use of GE as an antibacterial agent for active food packaging has been reported previously. Sung et al. (2014) found a reduction in *L. monocytogenes* growth in low-density polyethylene (LDPE) film containing between 2 and 8% (w/w) of garlic oil, and Llana-Ruiz-Cabello et al. (2017) observed that polypropylene containing 4% of garlic extract inhibited growth of *B. thermosphacta*. Seydim and Sarikus (2006) observed that whey protein isolate (WPI) films containing at least 3% (w/w) reduced growth of bacteria, Gram-positive bacteria being more sensitive than Gram-negative bacteria. However, the incorporation of much lower concentrations of GE in the coatings developed in this work appears to produce a significant effect against *Penicillium expansum*, indicating the effectiveness of this agent against moulds, and its potential to reduce food spoilage in active packaging applications.

3.4. Bread packaging, storage and shelf-life study

The films that showed total inhibition in the *in vitro* tests were also used to pack preservative-free pan loaf slices. Bags were sealed with an impulse thermosealer. The coatings did not affect the sealing properties of the films. Packages were stored at room temperature, and samples were opened and analysed at 5, 9, 12, 20 and 30 days. Two controls were used, the standard PE film used in conventional packaging of pan loaf, and this film coated with PE, zein or EVOH without agents. Bread slices inoculated with *P. expansum* were also packaged with all the packaging films.

No differences in the infection results were observed between inoculated and non-inoculated samples for the same treatment and storage time, so in this work we are only reporting the results of non-inoculated bread samples. Since spores survive the baking process, the absence of antifungal compounds results in food spoilage at nearly the same period than by spore inoculation in slice surface. All control samples (uncoated PE and coated ones without agents) presented the same behaviour. To avoid repetition, only samples packaged with uncoated film are presented as controls. The results are presented in Table 3, and representative images taken during storage are included in Fig. 1 for zein-coated films and in Fig. 2 for films coated with EVOH and PE.

Control samples showed first symptoms of mould growth at day 5. From that day the infections increased with time, covering the whole surface of the slice at day 30. In the case of film coated with EVOH or PE, mould growth began at days 9 and 12, respectively. Samples of PE-coated film presented increasing infection with time, although the infected surface was below 50% at the end of storage. Films with zein at both concentrations of GE successfully inhibited *P. expansum* growth throughout one month of storage time, although signs of infection were observed in some samples stored in PE/zein with 0.25% GE and 0.25% BA. As occurred in the *in vitro* tests, zein-coated PE bags presented the highest potential for the production of active packaging for pan loaf bread. Antifungal activity of PE and EVOH coatings on natural bread resulted in lower efficiency than in the *in vitro* studies, in which the

Table 2

Diameter of *P. expansum* colony after exposure to the PE/PE, PE/zein and PE/EVOH films with GE and BA after 12 days of storage at 28 °C.

Diameter of <i>P. expansum</i> colony (cm)					
	Day 3	Day 5	Day 7	Day 9	Day 12
PE					
PE control	1.62 ± 0.12 ^c	3.45 ± 0.02 ^c	4.65 ± 0.15 ^b	4.50 ± 0.04 ^b	5.0 ± 0.04 ^b
PE/PE control	1.74 ± 0.12 ^c	3.37 ± 0.21 ^c	4.38 ± 0.56 ^b	4.47 ± 0.54 ^b	4.51 ± 0.76 ^b
0.25%GE + 0.25%BA	0.41 ± 0.21 ^b	2.97 ± 0.48 ^b	4.29 ± 0.63 ^b	4.43 ± 0.44 ^b	4.46 ± 1.01 ^b
0.50%GE + 0.50%BA	0 ^a				
EVOH					
PE control	1.62 ± 0.05 ^b	3.37 ± 0.21 ^c	4.38 ± 0.07 ^b	4.52 ± 0.38 ^b	5.07 ± 0.12 ^b
PE/EVOH control	1.41 ± 0.12 ^b	3.52 ± 0.11 ^c	4.42 ± 0.20 ^b	4.82 ± 0.27 ^b	5.26 ± 0.07 ^b
0.25%GE + 0.25%BA	0 ^a	2.44 ± 0.13 ^b	4.04 ± 0.58 ^b	4.75 ± 0.55 ^b	4.74 ± 0.69 ^b
0.50%GE + 0.50%BA	0 ^a				
ZEIN					
PE control	1.59 ± 0.16 ^b	3.66 ± 0.08 ^b	4.28 ± 0.51 ^b	4.5 ± 0.30 ^b	5.38 ± 0.15 ^b
PE/Zein control	1.62 ± 0.13 ^b	3.64 ± 0.15 ^b	3.92 ± 0.54 ^b	4.70 ± 0.19 ^b	5.40 ± 0.22 ^b
0.25%GE + 0.25%BA	0 ^a				
0.50%GE + 0.50%BA	0 ^a				

a, b, c Different letters for the same column indicate significant differences between treatments (P ≤ 0.05).

Table 3

Evaluation of visual observation of *P. expansum* growth in packaged sliced bread with different coatings stored at room temperature.

Intensity of fungal growth on packed sliced bread					
	Day 5	Day 9	Day 12	Day 20	Day 30
Control	+	+	++	+++	++++
PE/PE 0.50%GE + 0.50%BA	-	-	+	++	++++
PE/EVOH 0.50%GE + 0.50%BA	-	+	+	++	++
PE/zein 0.25%GE + 0.25%BA	-	-	-	-	+
PE/zein 0.50%GE + 0.50%BA	-	-	-	-	-

No fungal growth, + fungal growth < 25% of sliced bread, ++ fungal growth > 25% and < 50%, +++ fungal growth > 50% and < 90%, ++++ fungal growth around > 90% of sliced bread.

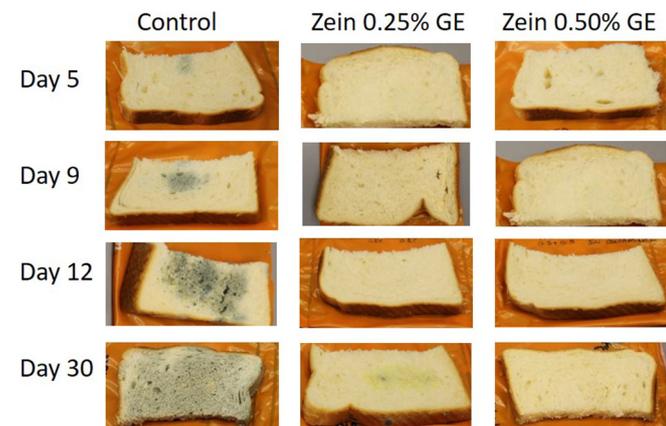


Fig. 1. Pan loaf slices packaged in control bags (uncoated PE film) and in active bags of zein-coated PE film at two concentrations. Images show the effect of package type on growth of *P. expansum* after various periods of storage.

films showed a fungicidal effect. This difference between *in vitro* and *in vivo* tests has often been observed and is commonly related to the food matrix effect, which can interfere with the antimicrobial compound and require a higher concentration to have the same effect (Gutiérrez et al., 2011).

Several authors have reported that the use of active packaging or active materials can reduce the incidence of mould infections in bread. In these papers, oregano essential oil or cinnamon essential oil were used at high concentrations, and some detrimental effects on sensory

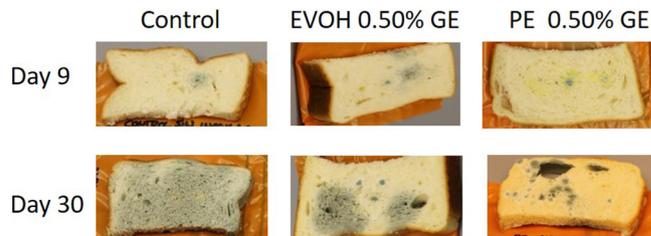


Fig. 2. Pan loaf slices packaged in control bags (uncoated PE film) and in active bags of EVOH-coated PE film and PE-coated PE film. Images show the effect of package type on growth of *P. expansum* after various periods of storage.

Table 4

Results of the ranking test on the aroma and taste of pan loaf slices packaged with the various bags analysed. Numbers are the sum of judges punctuation (10 judges from 1 to 5).

Packaging type	Aroma	Taste
Uncoated PE film	50 ^a	49 ^a
PE/zein 0.5% GE & 0% BA	12 ^d	12 ^d
PE/zein 0.5% GE & 0.5% BA	24 ^c	26 ^c
PE/zein 0.5% GE & 1.0% BA	35 ^b	36 ^b
PE/zein 0.5% GE & 1.5% BA	29 ^{bc}	27 ^c

a, b, c, d Different letters for the same column indicate significant differences between treatments (P ≤ 0.05).

Table 5

Average values of the hedonic test (46 judges) on the aroma and taste of pan loaf slices packaged in a bag composed of PE/zein with 0.5% of GE and 1.0% of BA (ranked from 1 to 9), and of the product purchase intention (ranked from 1 to 5).

Time of storage	Acceptability		Purchase intention
	Aroma	Taste	
Day 1	6.37 ^a	5.80 ^a	3.20 ^a
Day 5	6.07 ^a	5.80 ^a	3.13 ^a
Day 10	5.98 ^a	5.70 ^a	3.11 ^a

a, b Different letters for the same column indicate significant differences between treatments (P ≤ 0.05).

properties were also reported owing to the intense aroma of these substances (Balaguer et al., 2013; Passarinho et al., 2014). To reduce the potential gain of inappropriate aromas, some authors have prepared



Fig. 3. Loaf slices packaged with passive and active bags after 12 days of storage.

active materials containing non-volatile agents. Although they do not present any activity with solid products, sometimes they indirectly reduce infections by increasing the barrier properties against water and oxygen (Noshirvani et al., 2017). In the present work we adopted a different strategy, to partially hide the garlic aroma retained in the bread slices by the use of bread crust aroma.

3.5. Sensory analyses

After the good results observed with the PE films coated with zein containing 0.5% of garlic extract, two sensory tests were carried out on this material. First, the efficiency of bread aroma to mask the pungent aroma of garlic extract was analysed. For this study, a ranking test was planned to assess the differences in the intensity of garlic aroma between 4 samples packaged with films containing 0.5% of GE and 0, 0.5, 1 and 1.5% of BA. A sample packaged in uncoated PE was also included as control.

The results of the ranking test, included in Table 4, clearly showed that the control sample was the best evaluated, as it was ranked first by all judges. With respect to the samples packaged in the active bags, the test showed significant differences between samples. The sample in bags without bread aroma was ranked the worst as the garlic smell was unpleasant. In contrast, the bread in bags containing 0.50% GE and 1% BA was ranked the best. Comments of the judges on this sample were that the bread presented a slight but acceptable garlic scent and taste. Therefore this sample was selected for the second sensory test.

New bread samples were baked, sliced and packaged with 0.5% GE and 1% BA and submitted to a hedonic test at 1, 5 and 10 days of storage to check for consumer acceptance (1 to 9 scale) and purchase intention (1 to 5 scale). The results of this test are listed in Table 5. As can be seen, the judges qualified the product positively, with values above 5 in aroma and taste throughout the 10 days of storage. In addition, no significant differences were found for aroma and taste with storage time. With respect to purchase intention (1 to 5 scale), the test showed that the judges would possibly buy the preservative-free pan loaf in active packages with GE. No significant differences were found in purchase intention related to storage time. This result indicates that consumers would accept a slight garlic scent to avoid the addition of preservatives in the product and to provide an adequate shelf-life.

Finally, a last test was prepared to determine whether the use of a bag made of PE/zein with 0.5% of GE and 1.0% of BA was effective in reducing the growth of moulds. For this purpose, new loaves were baked and sliced, and half of the loaves were in uncoated PE and half in active PE. Loaves in passive bags were spoiled by moulds in < 9 days, whilst loaves in active bags were free from infection for the 15 days of test. Fig. 3 shows representative examples of the results.

Therefore, the use of antifungal packaging based on active coatings on standard bags can provide a suitable way of increasing the shelf-life of additive-free sliced pan loaf. Among possible options, zein-based coatings incorporating garlic extract were shown to significantly delay infection by moulds, especially *P. expansum*. An aroma corrector, bread crust aroma, was proved to be suitable for partially masking the pungent aroma of garlic, reducing sensory impact. After optimization, a

panel of judges considered the final product as acceptable for consumption and with good purchase intention.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijfoodmicro.2018.09.024>.

References

- Agarwal, K.C., 1996. Therapeutic actions of garlic constituents. *Med. Res. Rev.* 16, 111–124.
- Andersen, B., Smedsgaard, J., Frisvad, J.C., 2004. *Penicillium expansum*: consistent production of patulin, chaetoglobosins, and other secondary metabolites in culture and their natural occurrence in fruit products. *J. Agric. Food Chem.* 52, 2421–2428.
- Balaguer, M.P., Lopez-Carballo, G., Catala, R., Gavara, R., Hernandez-Munoz, P., 2013. Antifungal properties of gliadin films incorporating cinnamaldehyde and application in active food packaging of bread and cheese spread foodstuffs. *Int. J. Food Microbiol.* 166, 369–377.
- Bisharat, L., Berardi, A., Perinelli, D.R., Bonacucina, G., Casettari, L., Cespi, M., Alkhatib, H.S., Palmieri, G.F., 2018. Aggregation of zein in aqueous ethanol dispersions: effect on cast film properties. *Int. J. Biol. Macromol.* 106, 360–368.
- Borlinghaus, J., Albrecht, F., Gruhlke, M., Nwachukwu, I., Slusarenko, A., 2014. Allicin: chemistry and biological properties. *Molecules* 19, 12591.
- Casella, S., Leonardi, M., Melai, B., Fratini, F., Pistelli, L., 2013. The role of diallyl sulfides and dipropyl sulfides in the in vitro antimicrobial activity of the essential oil of garlic, *Allium sativum* L., and Leek, *Allium porrum* L. *Phytother. Res.* 27, 380–383.
- Catala, R., Gavara, R., 2001. Developments in active food packaging. *Arbor-Ciencia Pensamiento Y Cultura* 168, 109–127.
- Catala, R., Lopez-de-Castillo, C., Cerisuelo, J.P., Muriel Galet, V., Lopez Carballo, G., Hernandez Munoz, P., Gavara, R., 2015. Novel EVOH developments for active/bioactive food packaging applications. In: Kontominas, M. (Ed.), *Bioactive Food Packaging: Strategies, Quality, Safety*. DesTech Publications Inc., Lancaster, PA, pp. 421–442.
- Cerisuelo, J.P., Gavara, R., Hernandez-Munoz, P., 2014. Natural antimicrobial - containing EVOH coatings on PP and PET films: functional and active property characterization. *Packag. Technol. Sci.* 27, 901–920.
- Cerisuelo, J.P., Gavara, R., Hernández-Muñoz, P., 2015. Antimicrobial-releasing films and coatings for food packaging based on carvacrol and ethylene copolymers. *Polym. Int.* 16, 1747–1753.
- Chen, G.W., Chung, J.G., Hsieh, C.L., Lin, J.G., 1998. Effects of the garlic components diallyl sulfide and diallyl disulfide on arylamine *N*-acetyltransferase activity in human colon tumour cells. *Food Chem. Toxicol.* 36, 761–770.
- Cho, I.H., Peterson, D.G., 2010. Chemistry of bread aroma: a review. *Food Sci. Biotechnol.* 19, 575–582.
- Corzo-Martínez, M., Corzo, N., Villamiel, M., 2007. Biological properties of onions and garlic. *Trends Food Sci. Technol.* 18, 609–625.
- Dagnas, S., Onno, B., Membré, J.-M., 2014. Modeling growth of three bakery product spoilage molds as a function of water activity, temperature and pH. *Int. J. Food Microbiol.* 186, 95–104.
- Filtenborg, O., Frisvad, J.C., Thrane, U., 1996. Moulds in food spoilage. *Int. J. Food Microbiol.* 33, 85–102.
- Fратиanni, F., Ombra, M.N., Cozzolino, A., Riccardi, R., Spigno, P., Tremonte, P., Coppola, R., Nazzaro, F., 2016. Phenolic constituents, antioxidant, antimicrobial and anti-proliferative activities of different endemic Italian varieties of garlic (*Allium sativum* L.). *J. Funct. Foods* 21, 240–248.
- Giteru, S.G., Oey, I., Ali, M.A., Johnson, S.K., Fang, Z., 2017. Effect of kafirin-based films incorporating citral and quercetin on storage of fresh chicken fillets. *Food Control* 80, 37–44.
- Gutiérrez, L., Batlle, R., Andújar, S., Sánchez, C., Nerín, C., 2011. Evaluation of antimicrobial active packaging to increase shelf life of gluten-free sliced bread. *Packag. Technol. Sci.* 24, 485–494.
- Guynot, M.E., Ramos, A.J., Sanchis, V., Marín, S., 2005. Study of benzoate, propionate, and sorbate salts as mould spoilage inhibitors on intermediate moisture bakery products of low pH (4.5–5.5). *Int. J. Food Microbiol.* 101, 161–168.
- Higuera, L., Lopez-Carballo, G., Hernandez-Munoz, P., Catala, R., Gavara, R., 2014. Antimicrobial packaging of chicken fillets based on the release of carvacrol from chitosan/cyclodextrin films. *Int. J. Food Microbiol.* 188, 53–59.
- ISO, 2006. *Sensory Analysis – Methodology – Ranking*.
- ISO, 2007. *Sensory Analysis – General Guidance for the Design of Test Rooms*.
- Johnson, M., Olaleye, O.N., Kolawole, O.S., 2016. Antimicrobial and antioxidant properties of aqueous garlic (*Allium sativum*) extract against *staphylococcus aureus* and *pseudomonas aeruginosa*. *Br. Microbiol. Res. J.* 14, 1–11.

- Ju, J., Xu, X., Xie, Y., Guo, Y., Cheng, Y., Qian, H., Yao, W., 2018. Inhibitory effects of cinnamon and clove essential oils on mold growth on baked foods. *Food Chem.* 240, 850–855.
- Kashiri, M., Cerisuelo, J.P., Dominguez, I., Lopez-Carballo, G., Hernandez-Munoz, P., Gavara, R., 2016. Novel antimicrobial zein film for controlled release of lauroyl arginate (LAE). *Food Hydrocoll.* 61, 547–554.
- Kashiri, M., Cerisuelo, J.P., Dominguez, I., Lopez-Carballo, G., Muriel-Gallet, V., Gavara, R., Hernandez-Munoz, P., 2017. Zein films and coatings as carriers and release systems of *zataria multiflora* boiss. essential oil for antimicrobial food packaging. *Food Hydrocoll.* 70, 260–268.
- Kwapong, O.Y., Hotchkiss, J.H., 1987. Comparative sorption of aroma compounds by polyethylene and ionomer food-contact plastics. *J. Food Sci.* 52, 761–763.
- Kyung, K.H., 2012. Antimicrobial properties of allium species. *Curr. Opin. Biotechnol.* 23, 142–147.
- Legan, J.D., 1993. Mould spoilage of bread: the problem and some solutions. *Int. Biodeterior. Biodegrad.* 32, 33–53.
- Li, W.-R., Shi, Q.-S., Liang, Q., Huang, X.-M., Chen, Y.-B., 2014. Antifungal effect and mechanism of garlic oil on *Penicillium funiculosum*. *Appl. Microbiol. Biotechnol.* 98, 8337–8346.
- Li, W.-R., Shi, Q.-S., Dai, H.-Q., Liang, Q., Xie, X.-B., Huang, X.-M., Zhao, G.-Z., Zhang, L.-X., 2016. Antifungal activity, kinetics and molecular mechanism of action of garlic oil against *Candida albicans*. *Sci. Rep.* 6, 22805.
- Liang, J., Xia, Q., Wang, S., Li, J., Huang, Q., Ludescher, R.D., 2015. Influence of glycerol on the molecular mobility, oxygen permeability and microstructure of amorphous zein films. *Food Hydrocoll.* 44, 94–100.
- Llana-Ruiz-Cabello, M., Pichardo, S., Bermudez, J.M., Baños, A., Ariza, J.J., Guillamón, E., Aucejo, S., Cameán, A.M., 2017. Characterisation and antimicrobial activity of active polypropylene films containing oregano essential oil and Allium extract to be used in packaging for meat products. *Food Addit. Contam. Part A* 1–10.
- MacFie, H.J., Bratchell, N., Greenhoff, K., Vallis, L.V., 1989. Designs to balance the effect of order of presentation and first-order carry-over effects in hall tests. *J. Sens. Stud.* 4, 129–148.
- Matan, N., Matan, N., Ketsa, S., 2012. Effect of heat curing on antifungal activities of anise oil and garlic oil against *Aspergillus niger* on rubberwood. *Int. Biodeterior. Biodegrad.* 75, 150–157.
- Muriel-Galet, V., López-Carballo, G., Hernández-Muñoz, P., Gavara, R., 2014. Characterization of ethylene-vinyl alcohol copolymer containing lauril arginate (LAE) as material for active antimicrobial food packaging. *Food Packag. Shelf Life* 1, 10–18.
- Nielsen, P.V., Rios, R., 2000. Inhibition of fungal growth on bread by volatile components from spices and herbs, and the possible application in active packaging, with special emphasis on mustard essential oil. *Int. J. Food Microbiol.* 60, 219–229.
- Nogueira, J.H.C., Gonzalez, E., Galleti, S.R., Facanali, R., Marques, M.O.M., Felício, J.D., 2010. *Ageratum conyzoides* essential oil as aflatoxin suppressor of *Aspergillus flavus*. *Int. J. Food Microbiol.* 137, 55–60.
- Noshirvani, N., Ghanbarzadeh, B., Rezaei Mokarram, R., Hashemi, M., 2017. Novel active packaging based on carboxymethyl cellulose-chitosan-ZnO NPs nanocomposite for increasing the shelf life of bread. *Food Packag. Shelf Life* 11, 106–114.
- Passarinho, A.T.P., Dias, N.F., Camilloto, G.P., Cruz, R.S., Otoni, C.G., Moraes, A.R.F., Soares, N.d.F.F., 2014. Sliced bread preservation through oregano essential oil-containing sachet. *J. Food Process Eng.* 37, 53–62.
- Pranoto, Y., Salokhe, V.M., Rakshit, S.K., 2005. Physical and antibacterial properties of alginate-based edible film incorporated with garlic oil. *Food Res. Int.* 38, 267–272.
- Rasooli, L., Owlia, P., 2005. Chemoprevention by thyme oils of *Aspergillus parasiticus* growth and aflatoxin production. *Phytochemistry* 66, 2851–2856.
- Ribeiro-Santos, R., Andrade, M., Melo, N.R.d., Sanches-Silva, A., 2017. Use of essential oils in active food packaging: recent advances and future trends. *Trends Food Sci. Technol.* 61, 132–140.
- Ross, Z.M., O'Gara, E.A., Hill, D.J., Sleightholme, H.V., Maslin, D.J., 2001. Antimicrobial properties of garlic oil against human enteric bacteria: evaluation of methodologies and comparisons with garlic oil sulfides and garlic powder. *Appl. Environ. Microbiol.* 67, 475–480.
- Sadok, I., Szmagara, A., Staniszewska, M.M., 2018. The validated and sensitive HPLC-DAD method for determination of patulin in strawberries. *Food Chem.* 245, 364–370.
- Seydim, A.C., Sarikus, G., 2006. Antimicrobial activity of whey protein based edible films incorporated with oregano, rosemary and garlic essential oils. *Food Res. Int.* 39, 639–644.
- Shukla, R., Cheryan, M., 2001. Zein: the industrial protein from corn. *Ind. Crop. Prod.* 13, 171–192.
- Sung, S.-Y., Sin, L.T., Tee, T.-T., Bee, S.-T., Rahmat, A.R., Rahman, W.A.W.A., 2014. Control of bacteria growth on ready-to-eat beef loaves by antimicrobial plastic packaging incorporated with garlic oil. *Food Control* 39, 214–221.
- Tannous, J., Keller, N.P., Atoui, A., El Khoury, A., Lteif, R., Oswald, I.P., Puel, O., 2017. Secondary metabolism in *Penicillium expansum*: emphasis on recent advances in patulin research. *Crit. Rev. Food Sci. Nutr.* 1–17.
- Teixeira, B., Marques, A., Pires, C., Ramos, C., Batista, I., Saraiva, J.A., Nunes, M.L., 2014. Characterization of fish protein films incorporated with essential oils of clove, garlic and origanum: physical, antioxidant and antibacterial properties. *LWT Food Sci. Technol.* 59, 533–539.