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Synergistic properties of mustard and cinnamon essential oils for the inactivation of foodborne moulds in vitro and on Spanish bread

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ABSTRACT

This work was performed to evaluate the antifungal effect of cinnamon and mustard essential oil (EO) alone and in combination against a range of mould strains. A wide range of resistance levels was observed among different mould species, being *R. stolonifer* the most resistant one. Mustard EO showed the biggest antifungal effect, and for this reason it was selected to study its effect in vapour phase on bread. The shelf life of bread inoculated with *R. stolonifer* and non- inoculated increased by 3–4 days at 25 °C, while at 4 °C the shelf-life increased beyond 50 days. However, the results from the acceptability test were negative. Based on this, the antifungal effect of cinnamon/mustard EO in combination was evaluated, with results mostly additive and synergistic. The ratio of the combination was defined taking into account the most resistant strain (100:8, cinnamon and mustard respectively) and their application was performed in vapour phase. This combination maintained the antifungal activity presented by mustard EO by itself and masked the mustard flavour, providing positive results in the acceptability tests. It was therefore suggested to be used at industrial scale.

1. Introduction

Food safety is a global priority and one of the major objectives of the food industry, due to the high incidence of spoilage microorganisms that cause a risk after consumption. The food industry continues to examine ways to reduce the use of traditional chemical preservatives, some of which have been more linked with microbial resistance and health risks, while improving quality and food safety by controlling the growth of pathogens and spoilage microorganisms (Pisoschi et al., 2018). The use of essential oils (EOs) as an alternative to traditional chemical preservatives has increased in recent years (Burt, 2004) and have been categorised as GRAS (generally recognised as safe) in the Code of Federal Regulation (CFR) by the FDA (US Food and Drug Administration) (FDA, 2017). Several studies demonstrated the preservative action of spices and their EOs through the effective inhibition of antimicrobial growth (Goni et al., 2009; Suhr and Nielsen, 2005). The antimicrobial effect of EOs, such as cinnamon and mustard, on moulds has been evaluated previously (Dimic et al., 2015; Horvath et al., 2016). The combination of EOs with synergistic properties is an effective way to obtain similar antimicrobial effect reducing the EO volumes (Clemente et al., 2016a, b).

Bakery products, especially bread, are intermediate moisture content products and highly perishable. The most common forms of bread deterioration are moisture loss and microbiological spoilage. Owing to the low water activity of bread, the main spoilage agents are moulds, particularly *Rhizopus stolonifer* and *Nerospora sitophila*, and yeasts, which can cause the defect known as “chalky bread” (Guizani and Mothershaw, 2007). Spoilage of wheat bread and other bakery products by colonization and growth of moulds represents > 90% of the total microbial contamination (Saranraj and Sivasakthivelan, 2016). Traditional Spanish bread is a hand-made product widely consumed without preservatives or stabilizers being the increase of its safety a great target of study.

The study of foodborne moulds has gained importance over the last years. This is mainly due not only to the huge economic losses that food industry must face every year, but also to the potential risk of mycotoxin production, derived from mould contamination (Clemente et al., 2016a). Moulds can be found in a wide range of food products such as fruits, vegetables, cereals and legumes. In addition, some species are able to produce secondary metabolites which can pose a serious health risk (Amezqueta et al., 2012).

Hence, the main goal of this work was to assess the antifungal activity of mustard and cinnamon EOs and their combination in vitro (using ten different strains of moulds considered as common food contaminants), and its effectiveness as preservatives for Spanish bread non-inoculated and inoculated with *R. stolonifer*.

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2. Materials and methods

2.1. Antimicrobial substances

Cinnamon essential oil (CIN EO), (*Cinnamomum zeylanicum* CAS 8015-91-6) was provided by Argolide (Barcelona, Spain). Cinnamon EO corresponded to cinnamon from the bark fortified with cinnamaldehyde, its composition had been previously analysed (Becerril et al., 2007). Mustard essential oil (CAS 57-06-7) was supplied by Sigma (Sigma-Aldrich Química S.A, Madrid, Spain) containing mainly allyl isothiocyanate (> 95% pure AITC).

2.2. Mould strain and culture media

Penicillium roqueforti CECT 2905, *Penicillium verrucosum* CECT 2096, *Fusarium oxysporum* CECT 20201, *Penicillium expansum* CECT 2278, *Aspergillus niger* CECT 2088, *Botryotinia fuckeliana* CECT 2100, *Aspergillus flavus* CECT 2949, *Geotrichum* spp. CECT 1102, *Aspergillus ochraceus* CECT 2093 and *Rhizopus stolonifer* CECT 2344, were provided by Spanish Type Culture Collection (CECT). Potato Dextrose Agar (PDA) was used as solid media and Yeast Extract Broth (YEB) as liquid media, both supplied by Scharlau (Spain).

2.3. Antimicrobial agent selection

2.3.1. Antifungal activity in liquid medium

The antifungal susceptibility of foodborne moulds to both EOs under study was evaluated using a broth macrodilution method previously described (Clemente et al., 2017) to determine the minimal inhibitory concentration (MIC).The minimal fungicidal concentration (MFC) was determined inoculating 100 µL of the non-growth suspensions (from MIC determination experiments) in PDA medium and the lowest concentration with no growth was defined as MFC (Matan et al., 2009). All assays were performed at least in triplicate in different working days.

2.3.2. Antifungal activity in diffusion

The susceptibility of the moulds strains to cinnamon and mustard EOs was evaluated using the disc diffusion test, according to the standard M2-A8 from the Clinical Laboratory Standards Institute (CLSI, 2003). PDA plates were inoculated with 100 µL of the fungal suspension containing approximately 10^6 CFU/mL and dried at room temperature. Then, 10 µL of pure cinnamon EO and different volumes (10 µL, 5 µL, 3 µL, 1 µL and 0.5 µL) of pure mustard EO were added to a 9 mm Whatman sterile filter disk and placed in the middle of the Petri dish lid (vapour diffusion assays) or placed on the top of the agar (agar diffusion). Plates were sealed with two Parafilm® tapes and incubated for 21 days at 25 °C. After incubation filter disks and lids were changed, and both Parafilm® tapes were renewed (Nielsen and Rios, 2000) and plates were checked for signs of growth every day during the next 21 days. After 42 days the diameter of the inhibition area was measured with a digital calliper (COMECTA S.A.). The antimicrobial activity was evaluated in terms of inhibition halo formed (Manso et al., 2015) and the results were expressed as percentages (Clemente et al., 2016a, b).

2.4. Combinatorial assays

2.4.1. Checkerboard assay

This study was performed with 200 µL of 10^6 CFU/mL of the different mould suspensions in physiological saline solution, 20 µL of each EO concentration or EtOH (in concentration 0), and 1760 µL of YEB were added in each microtube to obtain a final volume of 2 mL. The incubation conditions were the same above mentioned. EOs concentrations were selected taking into account the MIC values previously obtained (0, 1/16, 1/8, 1/4, 1/2, 1 and 2 times the MIC). Two different controls were added, without EO (positive control) and without inoculation (negative control) (Mulyaningsih et al., 2010). The interaction between both compounds was expressed as FIC index (fractional inhibitory concentration) (Odds, 2003).

The values obtained for this index determined the combined effect of both compounds: FIC > 4 (antagonism), 1 < FIC < 4 (indifferent), 0.5 < FIC ≤ 1 (additive) or FIC ≤ 0.5 (synergy) (Hyldgaard et al., 2012). To determine the minimal fungicidal combined concentration of EOs, a method described by Mosquera et al. was used to calculate the FFC index (fractional fungicidal concentration) (Mosquera et al., 2002).

2.4.2. Combinatorial vapour diffusion assay

PDA plates were inoculated with 100 µL of spores mould suspension containing approximately 10^6 CFU/mL. Sterile filter discs (10-mm Whatman®) were loaded with 10 µL of cinnamon EO (10,560 µg of cinnamon) that was afterwards spiked with 1 µL of increasing concentrations of mustard EO (50, 100, 200, 400, 800, 900 and 1000 µg). Based on Lopez et al. and considering 57.3 cm^3 as the volume of the atmosphere inside the Petri dish, these volumes corresponds to 184.29 µg/mL of cinnamon EO and 0.87, 1.74, 3.49, 6.98, 13.96, 15.71 and 17.68 µg/mL for mustard EO (Lopez et al., 2005). The Petri dishes were sealed using two Parafilm® tapes and incubated as previously mentioned. The antimicrobial activity was evaluated in terms of inhibition halo formed (Clemente et al., 2016a) and results were presented as inhibition percentages.

2.5. Antifungal activity of mustard essential oil on traditional bread

The antifungal activity of mustard EO in vapour phase was evaluated on commercial traditional Spanish bread, obtained in a traditional bakery shop in Zaragoza (Spain). This bread had been manufactured without preservatives. For this purpose, a slice of bread was placed at the bottom part of a Petri dish, and then the system was closed. In order to generate a similar headspace as in the previous experiment (see Section 2.3.2), the system was sealed by using adhesive tape in two points of the petri dish. Finally, each Petri dish containing the slice of traditional bread was placed inside an LDPE (low-density polyethylene) bag in order to simulate the real storage condition.

The antifungal evaluation was performed as follows: slices of bread were inoculated with 100 µL of a 10^4, 10^5 or 10^6 CFU/mL suspension of *R. stolonifer,* applying 1 µL of mustard EO on a filter disc placed over the top of the Petri dish. Plates were incubated at two temperatures, 25 °C and 4 °C. After this, the experiment was repeated in the same way, but with non-inoculated slices of bread.

Finally, the slices of bread showing a total mould inhibition were analysed by dilution plating in order to quantify mould concentration. For this, the FDA official method was followed (Tournas et al., 2001). Samples were weighed and diluted with 0.1% peptone water and homogenize in a stomacher for 2 min at 300 rpm. After appropriate 1/10 dilutions in SFE, 100 µL of each suspension were inoculated in PDA medium. Plates were counted after 5 days of incubation at 25 °C, expressing the results as colony forming units (CFU)/g.

2.6. Antifungal activity of mustard and cinnamon essential oil in combination on traditional bread

The antifungal activity of the combination of cinnamon and mustard EO in vapour phase was evaluated on traditional bread. For this purpose, experiments were performed as it was described in Section 2.5. In this occasion, with the aim to emulate a real situation, the study was only carried out with non-inoculated slices of bread. In test samples, 1 µL of the combination of cinnamon-mustard EOs in a ratio 100:8 was added to the filter disk. Both, control and test samples, were incubated at 25 °C.

2.7. Evaluation of aroma quality on packaged traditional bread

In order to assess the acceptability of bread packed in an active packaging based on a combination of cinnamon and mustard EOs, a hedonic test was performed (Juyun, 2011). The quality of the bread aroma was evaluated on a 5-point scale: 1-Dislike very much, 2-Dislike moderately, 3-Neither like nor dislike, 4-Like moderately and 5-Like very much. The test was performed by 15 qualified tasters in bread packaged with two different treatments, one without any compound and another one with a combination of cinnamon and mustard EOs (100:8) at day 2 of storage. No longer evaluation was performed since bread without any added compound showed saprophyte mould at day 3 of the experiment. The aroma of the bread exposed to the combination was also evaluated at day 7, and the panelists did not detect any unpleasant aroma in these samples (data not shown).

2.8. Statistical analysis

The data were evaluated using GraphPad PRISM 7.0 software (GraphPad Software, Inc., San Diego, CA, USA). Both, *t*-test and ANOVA followed by Tuckey's test were applied. Differences were considered significant for $p \leq 0.05$. Error bars in the figures correspond to the standard error of the mean. All experiments were repeated at least in triplicate in different working days.

3. Results and discussion

3.1. Antimicrobial agent selection

3.1.1. Antimicrobial activity in liquid medium

The antifungal activity of mustard and cinnamon EO was determined against ten foodborne moulds strains. The MICs and MFCs obtained are shown in Table 1. Both compounds were active against all strain tested, but with very different MIC and MFC values. Mustard EO showed higher antifungal activity against all selected moulds, with MIC values ranging from 0.8 to 50 ppm (μg/mL) depending on the strain; whereas for cinnamon EO MIC range was obtained from 25 to 200 μg/mL. *R. stolonifer* was the most resistant strain, while *P. roqueforti* was the most sensitive to both EOs. The results obtained for cinnamon are in agreement with those described by Manso et al. for *A. niger* and *P. roqueforti* (Manso et al., 2015) and with Horvath et al. for *Aspergillus*, *Fusarium* and *Rhizopus* (Horvath et al., 2016).

In addition, a great variability on the MFC values was observed between EOs, with lower values for mustard EO (between 6.25 and 100 μg/mL depending on the strain tested) compared to cinnamon EO (between 25 and 200 μg/mL). As it can be observed, the results highlight the strong antifungal activity of mustard EO compared to cinnamon EO. This higher activity of the mustard EO is in agreement with the experiments performed in foodborne bacteria (Clemente, Aznar, Silva, et al., 2016).

Table 1
Antifungal susceptibility of ten mould strains to a mustard and cinnamon EO as measured by minimal inhibitory concentration (MIC) (ppm (μg/mL)) and minimal fungicidal concentration (MFC) (μg/mL).

Mould strain	Mustard EO		Cinnamon EO	
	MIC	MFC	MIC	MFC
P. roqueforti	0.8	12.5	25	200
P. verrucosum	6.25	12.5	25	200
F. oxysporum	1.6	12.5	50	100
P. expansum	1.6	12.5	100	200
A. niger	3.125	6.25	100	100
B. fuckeliana	6.25	12.5	100	100
A. flavus	6.25	6.25	100	100
Geotrichum spp.	3.125	12.5	200	400
A. ochraceus	6.25	25	200	200
R. stolonifer	50	100	200	400

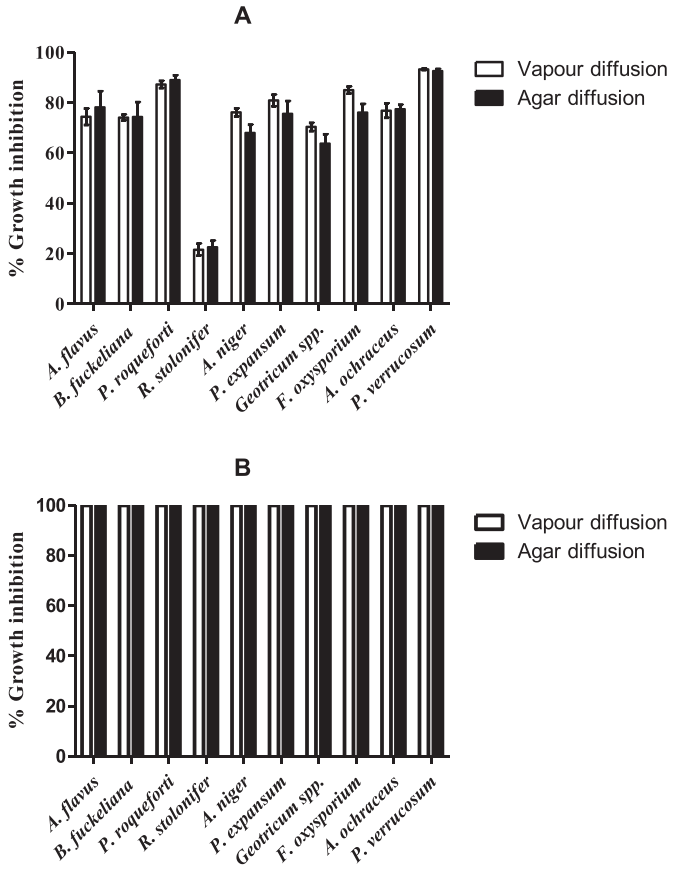

Fig. 1. Agar and vapour phase activity of A) 10 μL of cinnamon essential oil, and B) 10 μL of mustard essential oil against ten mould strains during 21 days at 25 °C.

3.1.2. Antifungal activity in diffusion

The susceptibility of the foodborne mould strains to the EOs was evaluated using different diffusion methods (vapour and agar). The aim was to determine the activity of each EO and to prove if the type of diffusion showed different antifungal activities. Mustard and cinnamon EO were found to be active against each mould strain, but with inhibition values ranging from 21.5 to 100% (Fig. 1). Fig. 1a shows the antifungal activity of cinnamon EO in both types of diffusions. As Fig. 1a shows, inhibitory activity of cinnamon was in the range of 21–93% depending on the strain tested. *R. stolonifer* showed the highest resistance (21% of inhibition) while *P. verrucosum* was the most sensitive to the growth inhibitory activity of cinnamon (93% of inhibition). These results were in agreement with those published by other authors with values ranging from 19.2% to 87.5% for different mould strains (Angelini et al., 2006; Dimic et al., 2015; Tzortzakis, 2009). In most of the strains tested no statistically significant differences in growth inhibition were observed regardless of diffusion type ($p < 0.05$), with the exception of *F. oxysporum* and *A. niger* in which vapour diffusion showed significantly better inhibition results.

On the other hand, the antifungal activity of cinnamon EO in diffusion showed different behaviour compared to the activity achieved in liquid media, being *R. stolonifer* the most outstanding one. *Geotricum* spp. and *R. stolonifer* showed the same MIC/MFC values in liquid media. However, the inhibitory effect observed in agar or vapour diffusion was very different. It has been reported that *Rhizopus* species are resistant to most of the common antifungal compounds because their numerous hydrolyzing enzymes can break down the active molecules (Tzortzakis,

2009). This could explain the differences observed between the different methodologies used.

Fig. 1b shows the antifungal activity of mustard EO in both types of diffusions. In all cases mustard produced the complete inhibition of all mould strain tested. These results are in agreement with those obtained in liquid phase, and in agreement with those described by Mejia-Garibay et al. for *A. ochraceus* (Mejia-Garibay et al., 2015) and Nielsen et al. for *P. roqueforti* and *A. flavus* (Nielsen and Rios, 2000). These results open the possibility for the successful use of mustard EO as antifungal compound for active packaging.

Due to the strong antifungal activity observed for mustard EO, lower volumes of this compound were further evaluated. At volumes ≥1 μL a complete inhibition of fungal growth was observed after 21 days of incubation (Data not shown). After this, the atmosphere of the plates was renewed and further incubated for 21 additional days, keeping in all cases a total mould inhibition. These results can be attributed to a great fungicidal effect of this compound instead of fungistatic. On the other side, the use of 0.5 μL of mustard EO produced the complete inhibition of all mould strains except for *R. stolonifer*, which showed a decrease on its growth but a homogeneous distribution without a remarkable inhibition area. The absence of inhibition halo has been reported due to a convection model of the transfer mechanism (Clemente, Aznar, Silva, et al., 2016). These results together with those obtained for cinnamon EO proved the relevance of *R. stolonifer* for further experimentation due to its high resistance against both EOs.

3.2. Antimicrobial activity of mustard essential oil on traditional bread

Based on the results obtained in in-vitro conditions, mustard EO was selected as antimicrobial active agent to be tested as preservative for traditional Spanish bread. Its use as active agent has recently been investigated to control microbial growth in packaged foods (Kwon et al., 2017). Mustard EO is permitted in food packaging by European Food Safety Authority, either in the vapour phase or in direct contact with food (EFSA, 2010) as well as all the compounds from isothiocyanate's family (EFSA, 2008). Regarding the type of diffusion to be applied, most of the antimicrobial activity is produced by the volatilized compounds (Becerril et al., 2007), and for this reason and based on previous results, vapour diffusion exposure was used for the experiments with Spanish bread. Table 2/Fig. S-1 and Table 3/Fig. S-2 show the antifungal activity of mustard EO in vapour phase on Spanish bread slices, inoculated with *R. stolonifer* and non-inoculated, stored at 25 °C and at 4 °C respectively.

As shown in Table 2, mould started to growth in all control slices at day 3 of incubation. This was in agreement with Sahan et al., who reported that the unpreserved bread may have a shelf life of 3–4 days (Sahan, 2011). Samples exposed to 1 μL of mustard EO inoculated with a mould concentration of 10^6 CFU/mL and 10^5 CFU/mL showed a growth reduction, whereas samples inoculated with a mould concentration of 10^4 CFU/mL showed a total growth inhibition of *R. stolonifer* up day 7 of storage. After this point an increase of mould growth was observed. In the case of non-inoculated samples exposed to the action of mustard EO, a complete inhibition of saprophytic mould growth was observed until the last day of storage (day 10). In the non-inoculated slices (Fig. S-1) there was no growth in exposed samples until day 10, increasing the Spanish bread shelf life beyond 3–4 days. After 10 days of the experiment, the control slices were fully covered by saprophyte species, whereas the slices exposed to mustard EO showed only a slight growth, showing a very significant decrease in mould growth and pointing out the preservative effect of mustard EO against foodborne mould. Other authors have studied the effect of EO to prevent the mould growth on bread. Rodriguez et al. developed an active paper package based on the incorporation of cinnamon EO to wax paraffin as an active coating, in which after 3 days of storage, a reduction of the growth of *R. stolonifer*, was obtained (Rodriguez et al., 2008). However, the results obtained in the present work showed a more fungistatic effectiveness of mustard EO over storage time, achieving a total growth inhibition in non-inoculated bread slices, using a lower amount of EO.

In the bread samples stored at 4 °C (Table 3) after 40 days of storage, mould growth was completely inhibited in all exposed samples while all control slices, started to show saprophyte mould growth different to *R. stolonifer* species. This could be because the refrigeration temperature avoided the development of *R. stolonifer*. On the other side, the samples exposed to 1 μL of mustard EO were free from mould growth until day 90 of storage. So mustard EO was able to increase more than two fold the shelf-life of traditional Spanish bread stored at 4 °C. To assess the mustard EO activity on bread slices stored at 4 °C, mould counts were also evaluated after 90 days of storage. The counts observed were 1.56 ± 0.59, 2.14 ± 0.56, 1.72 ± 0.68 and 1.57 ± 0.41 CFU/g for bread slices inoculated with 10^6 CFU/mL, 10^5 CFU/mL, 10^4 CFU/mL and non-inoculated respectively. In all cases counts in the slices exposed to mustard EO were below 3 log CFU/g, fulfilling the maximum limit of microorganism for bread according to OECD norm (OECD, 2012).

The fungicidal activity of mustard EO in Spanish bread was lower than that observed in the in-vitro results above presented. This could be due to the fact that in the bread trials Petri dishes were not sealed as in the case of in-vitro experiments. In addition the plates were stored inside a LDPE bag, which had a bigger inner volume leading to a final lower concentration of mustard EO in the vapour phase. In any case, differences between in vitro and in vivo tests had been previously reported by other authors (Luz et al., 2018; Rodriguez et al., 2008).

Although, satisfactory results were obtained in terms of mould growth inhibition for mustard EO, a preliminary sensory test revealed the presence of off-odours that could produce the unacceptability of the bread. For this reason, a combination of both EO, cinnamon and mustard, was studied, in order to determine if the presence of cinnamon EO could mask mustard EO off-flavours maintaining the antimicrobial capacity (Gutierrez et al., 2009). In addition, the existence of possible synergies between them would allow reducing the quantity of EOs necessary to avoid microbial growth and food spoilage. The results are

Table 2
Evaluation of vapour phase activity of 1 μL of mustard EO or cinnamon-mustard EO (100:8) on traditional bread slices, non-inoculated and inoculated with *R. stolonifer* at 10^6 CFU/mL, 10^5 CFU/mL, 10^4 CFU/mL, incubated at 25 °C during the time.

Time (day)	Mustard EO								Cinnamon-mustard EO (100:8)	
	Inoculated 10^6 CFU/mL		Inoculated 10^5 CFU/mL		Inoculated 10^4 CFU/mL		Non inoculated			
	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed
1	−	−	−	−	−	−	−	−	−	−
3	+ +	+	+ +	+	+ +	−	+	−	+	−
7	+ + +	+ +	+ + +	+ +	+ + +	+	+ +	−	+ +	−
10	+ + +	+ +	+ + +	+ +	+ + +	+	+ + +	+	+ + +	+

(+ + +) intense growth, (+ +) moderate growth, (+) slight growth, (−) no growth.

Table 3
Evaluation of vapour phase activity of 1 μL of mustard EO on traditional bread slices, non-inoculated and inoculated with *R. stolonifer* at 10^6 CFU/mL, 10^5 CFU/mL, 10^4 CFU/mL, incubated at 4 °C during the time.

Time (day)	Mustard EO							
	Inoculated 10^6 CFU/mL		Inoculated 10^5 CFU/mL		Inoculated 10^4 CFU/mL		Non inoculated	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
0	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−	−
10	−	−	−	−	−	−	−	−
40	+	−	+	−	+	−	+	−
60	+ +	−	+ +	−	+ +	−	+ +	−
80	+ + +	−	+ + +	−	+ + +	−	+ + +	−
90	+ + +	−	+ + +	−	+ + +	−	+ + +	−

(+ + +) intense growth, (+ +) moderate growth, (+) slight growth, (−) no growth.

shown in the next section.

3.3. Essential oils combination assay

3.3.1. Checkerboard assay

A checkerboard assay was performed to study the effects of the combination of mustard and cinnamon EOs. The checkerboard method remains the most popular approach for evaluating drug interactions between antifungals (Mukherjee et al., 2005). Table 4 shows the indices of fractional inhibitory concentration (FIC) and fractional fungicidal concentration (FFC). None combination showed antagonistic effects; indifferent effect was observed for *P. roqueforti* in both FIC and FFC indices and for *Geotricum* spp. but only in FFC index. However, a synergistic effect was observed for *A. ochraceus* in FIC index, and for *R. stolonifer* and *F. oxysporum* for FFC index, while the rest of combinations and moulds studied showed an additive effect. These results constitute a first evaluation of the potential use of the combination of mustard and cinnamon EOs on foodborne moulds. A previous work performed with bacteria obtained similar effectiveness using the combination of these two EOs (i.e. cinnamon and mustard) (Clemente, Aznar, Silva, et al., 2016). However, to the best authors' knowledge this is the first research in which the effectiveness of the combination of mustard and cinnamon EOs is evaluated in moulds.

Different combinations have been reported by other authors. Pinto et al. reported that the combination of methyl-eugenol/fluconazole on *A. flavus* and *A. niger* did not show interactions while the combination limonene/fluconazole showed synergistic behaviour. Nevertheless, higher concentration of the active compounds, compared to the concentrations used in this work, were necessary (Pinto et al., 2017). Horvath et al. evaluated the possible combinations of six EOs on five mould strains obtaining additive, indifferent or not interactions between them (Horvath et al., 2016). So, the results achieved in this work highlight the efficacy of the combination of mustard and cinnamon EOs, showing mostly additive and synergistic effect while antagonistic effect was not detected.

Table 4
Checkerboard assay using values ranging from 0 to 2 times (0, 1/16, 1/8, 1/4, 1/2, 1 and 2) the MIC values obtained for cinnamon and mustard essential oils. The interaction between both compounds was expressed in terms of a fractional inhibitory concentration (FIC) index and fractional fungicidal concentration (FFC). Synergy (FIC ≤ 0.5); additive (0.5 < FIC ≤ 1); indifferent (1 < FIC < 4); and antagonism (FIC > 4).

Moulds	Fungistatic combination (FIC)	Fungicidal combination (FFC)
A. flavus	1	1
B. fuckeliana	0.62	0.62
P. roqueforti	1 < FIC ≤ 4	1 < FIC ≤ 4
R. stolonifer	0.75	0.5
A. niger	1	1
P. expansum	0.56	0.56
Geotrichum spp.	0.56	1 < FFC ≤ 4
F. oxysporum	0.56	0.5
A. ochraceus	0.5	0.53

3.3.2. Combinatorial vapour phase activity

The results obtained whit these experiments (Fig. 2) confirmed a great interaction between cinnamon and mustard EO in vapour phase. The growth inhibitory potential of cinnamon EO at a concentration of 184.29 μg/mL itself and its combination with mustard EO at concentrations of 0.87 and 1.74 μg/mL, was evaluated for the 10 different moulds used in this research. As Fig. 2 shows, when the different moulds were exposed to the action of cinnamon EO, a partial inhibition of mould growth was observed. In this scenario, *P. verrucosum* was the most sensitive while *R. stolonifer* showed the highest resistance. On the other side, when cinnamon EO was added together with mustard EO, a higher growth inhibition was observed in all moulds used. When mustard EO was added in a concentration of 0.87 μg/mL (ratio 100:0.5) to the system, the complete inhibition of the three *Penicillium* species and *F. oxisporium* was observed. However, for *A. flavus*, *B. fuckeliana*, *A. niger*, *Geotricum* spp., and *A. ochraceus* it was necessary to increase the mustard concentration up to 1.74 μg/mL (ratio 100:1) in the system to produce their total inhibition. In the case of *R. stolonifer*, slightly increases of the inhibitory effect were observed when mustard EO was added, and the maximum inhibition achieved using the ratio 100:1 cinnamon/mustard was 29%, showing the highest resistance among all moulds tested.

Since *R. stolonifer* showed the highest resistance against EO, increasing concentrations of mustard EO added to 184.29 μg/mL of cinnamon were evaluated. Fig. 3 shows the inhibition profile of *R. stolonifer* exposed to increasing concentrations of mustard EO (0.87, 1.74, 3.49, 6.98, 13.96, 15.71 and 17.68 μg/mL) combined with cinnamon. As Fig. 3 shows, higher concentrations of mustard EO involved higher inhibition of *R. stolonifer* up to 13.96 μg/mL, where the complete inhibition was detected. These results showed that a combination of cinnamon/mustard EOs in a ratio of 100:8 is required in order to achieve the complete inhibition of this mould.

In a previous work performed with bacteria using the same cinnamon/mustard combinations, a lower inhibition effect was observed, being necessary to increase up to 13.96 μg/mL of mustard EO to obtain between 75 and 100% of inhibition depending on the bacteria studied (Clemente, Aznar, Silva, et al., 2016). In the present work, the proposed combination of mustard and cinnamon EO in a ratio 100:1 was efficient to achieve the total inhibition of all tested mould, except *R. stolonifer* where a ratio 100:8 was necessary. These ratios were in all cases lower than the ones observed by other authors. Tunc et al. reported the combination of mustard EO and cinnamaldehyde (ratio 2.1:3.1), on the

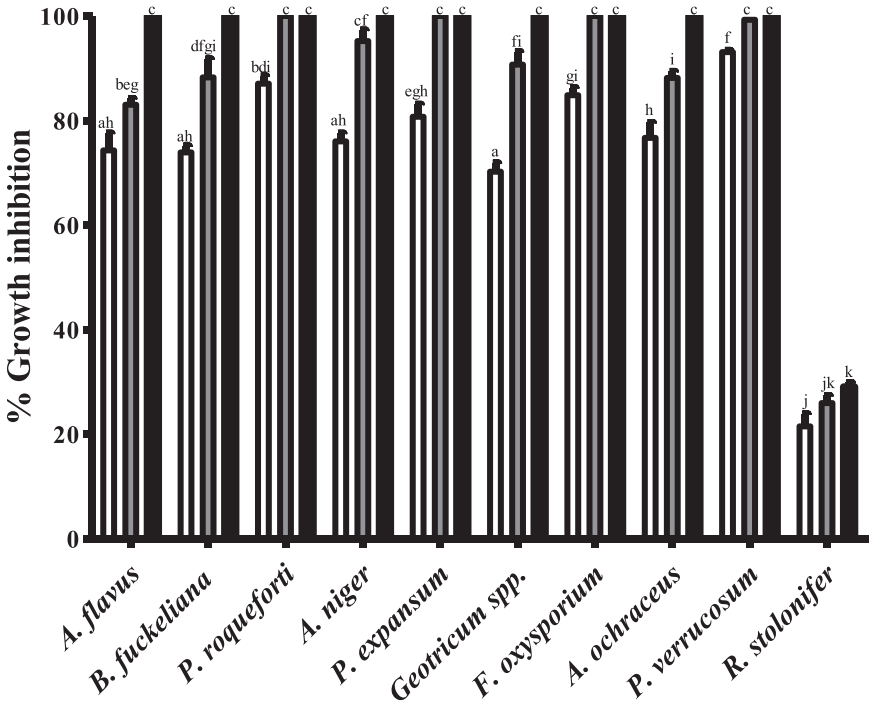

Fig. 2. Combinatorial vapour phase activity assay using disks loaded with 10 µL of cinnamon essential oil (184.29 µg/mL) (white bars) and spiked with 1 µL of increasing concentrations (0.87 (grey bars) and 1.74 µg/mL (black bars)) of mustard essential oil EO against ten mould strains. Letters refer to significant differences ($p \leq 0.05$) between samples.

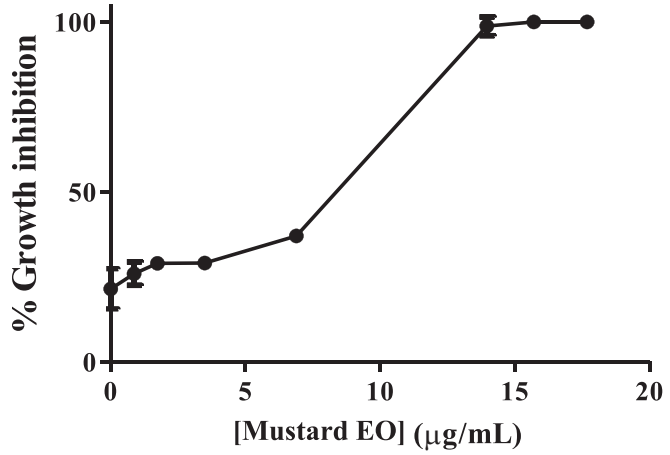

Fig. 3. Combinatorial vapour phase activity assay using disks loaded with 10 µL of cinnamon essential oil (184.29 µg/mL) and doped with 1 µL of increasing concentrations (0.87, 1.74, 3.49, 6.98, 13.96, 15.71 and 17.68 µg/mL) of mustard EO against *R. stolonifer*.

mould *P. notatum* where a total growth inhibition was not achieved (Tunc et al., 2007). Wu et al. performed combinations between mustard EO and EITC (ethyl isothiocyanate) in the ratio 3:1, which showed a significant synergistic effect that was more efficient controlling spore germination than controlling the growth (Wu et al., 2011).

3.4. Antimicrobial activity of mustard and cinnamon essential oil in combination on traditional bread

The results obtained in the previous section proved the synergistic activity between cinnamon and mustard EO in a ratio 100:8 to achieve the complete inhibition of the most resistant mould tested (*R. stolonifer*). The advantage of these combinations would be the reduction of the concentration of mustard EO required, which in turn would reduce the relative impact on the organoleptic properties of bread.

Once the organoleptic suitability of the combination cinnamon/mustard in a ratio 100:8 was proved, its potential as preservative of Spanish bread in regular storage conditions was evaluated. Table 2 and Fig. S-1 show the results obtained on sliced bread stored at 25 °C and the conditions described in Section 2.5. As shown in Table 2, at day 3 of incubation, control samples showed mould growth but samples exposed to cinnamon/mustard combination did not show any mould. After 7 days of incubation an increase of mould growth was observed in the control samples while exposed samples did not show any mould growth (Fig. S-1/Table 2). After 10 days of storage, control bread slices were fully covered by saprophyte species whereas slices exposed to the combination of cinnamon EO and mustard EO (100:8) showed a significant decrease in mould growth, indicating the potential as bread preservative. On the other side, the use of EOs combination showed similar inhibitory efficiency than mustard only (Section 3.2) endorsing the synergistic effect observed between cinnamon and mustard, which in turn open the possibility of reduce the quantity of mustard (e.g. > 10-fold in the present work) while maintaining similar shelf-life extension of bread slices). Therefore, this combination of EOs maintains the antimicrobial properties and presents a pleasant aroma for bakery products.

Other authors have reported extensions between 1 and 3 for the shelf-life of bread using different active packaging (Luz et al., 2018; Rodriguez et al., 2008). In this study showed shelf-life extensions between 3 and 4 days (i.e. 10 days of storage at 25 °C) when the combination of mustard and cinnamon EO was applied while at the same time the negative impact on the bread aroma produced by mustard only disappeared.

3.5. Acceptability of bread packed in active packaging

Mustard EO has a great antimicrobial activity, however, its odour is strong and penetrating and it could affect negatively to food sensory properties, especially in pastries and bakery, as it has been already mentioned in Section 3.2. For this reason, a combination of mustard EO with cinnamon, that would present a more pleasant aroma, was tested.

For this purpose a triangular test was performed in order to determine if the combination cinnamon/mustard would be acceptable from a sensory perspective. The results of the triangular test showed that there were no significant differences between the aroma of cinnamon EO itself and the aroma of cinnamon EO combined with mustard

EO (α of 0.05) in a ratio 100:8. Thus, the consumers' acceptability of bread packaged with cinnamon EO or with the mustard/cinnamon EOs combination was evaluated. Both breads obtained a similar acceptability value, 3.25 for cinnamon EO and 3.17 for EOs combination, without significant differences ($p < 0.05$). In both cases, the values were above 3, which means that there was not a lack of acceptability by the tasters. For this reason, this combination of EOs would be an efficient and natural alternative to extend bakery products shelf-life without the use of synthetic preservatives. The results observed open the possibility of using a mixture of EOs with a very high antimicrobial activity and suitable organoleptic properties for bakery products.

4. Conclusion

Mustard EO showed higher antifungal activity than cinnamon EO. This activity was especially remarkable in vapour phase. Mustard EO proved to be efficient to reduce the growth of moulds in non-inoculated and inoculated bread, extending its shelf-life more than two fold at 25 and 4 °C. The use of mustard EO alone as preservative was refused in the sensory study, although the combination of cinnamon and mustard in a ratio 100:8 showed suitable organoleptic properties for packaging bakery products and it was able to inhibit the growth of all moulds tested.

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