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Validation of *in-vitro* antifungal activity of thyme essential oil on *Aspergillus niger* and *Penicillium paneum* through application in par-baked wheat and sourdough bread

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ABSTRACT

The main objectives of this study were to investigate the antifungal activity of thyme essential oil (*Thymus zygis*) *in-vitro* and to validate its activity in par-baked wheat and sourdough bread (*in-vivo*) using the fungal strains *Aspergillus niger* and *Penicillium paneum*. The impact of thyme oil was evaluated *in-vitro* with the macro-dilution method with modified pH (4.8, 5.0, 5.5 & 6.0), a_w (0.95 & 0.97) and temperature (22 & 30 °C) to mimic the conditions of wheat and sourdough par-baked bread stored at room temperature. Furthermore, the oil was applied in par-baked bread (0, 15 and 30 g sourdough/100 g dough). Challenge and shelf-life tests were performed to investigate the biopreservative potential of thyme oil in par-baked wheat and sourdough bread. Despite the promising *in-vitro* potential of thyme oil, no clear shelf-life extension was observed for par-baked bread which could be attributed to the oil. More research on food applications of essential oils is necessary to give the abundance of *in-vitro* studies industrial relevance. The use of sourdough, on the contrary, resulted in much more promising shelf-life extending properties. In both *in-vitro* as *in-vivo* assays, pH and a_w showed no significant effect on fungal growth.

1. Introduction

Par-baking is a technology designed to enable consumers and food stores to provide freshly baked bread at any moment of the day after fully baking. Par-baked bread is characterized by a light crust color and soft/non-crispy crust (Debonne, Van Bockstaele, Philips, De Leyn, & Eeckhout, 2017) and a high water activity (a_w) (Hamdami, Monteau, & Bail, 2006). Therefore, they are highly sensitive to post-baking microbial contamination. Mould growth is by far the most important shelf-life limiting factor of par-baked bread, with *Penicillium* spp. and *Aspergillus* spp. being the most dominant species (Legan, 1993). However, yeast spoilage can also occur on par-baked modified atmosphere packed breads (Deschuyffeleer et al., 2011). Microbiological shelf-life of bread is generally prolonged by the use of the chemical preservative calcium propionate (Belz et al., 2012). In Europe, maximum limits for propionic acid and propionate (expressed as propionic acid) have been established: 3000 mg/kg in pre-packed sliced bread and rye bread, 2000 mg/kg in energy reduced bread, partially/pre-baked bread, pre-packed rolls and buns and 1000 mg/kg in pre-packed bread (EU, 2011). However, as a result of increasing signs of negative effects on consumer health (Dengate & Ruben, 2002; Priftis et al., 2007) and the changing consumer perception towards chemical food preservatives (Shim et al., 2011), biopreservation strategies are given further attention. Biopreservation can be divided into two subcategories. First of all, an antimicrobial active ingredient can be added. In this group essential oils and plant extracts with antifungal activity are situated which can be used as an ingredient of the bread dough (Doudi, Setorki, & Rezayatmand,

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2016; Rizzello, Lavecchia, Gramaglia, & Gobbetti, 2015; Saladino et al., 2017; Teodoro, Fernandes, Botrel, Borges, & de Souza, 2014), or as part of the packaging atmosphere (Jideani & Vogt, 2016; Otoni, Espitia, Avena-Bustillos, & McHugh, 2016; Nguyen Van Long, Joly, & Dantigny, 2016). The antifungal activity of thyme essential oil has been widely described in *in-vitro* assays. However, validation of *in-vivo* assays in food products, more particularly in bakery products, is rarely found in literature. The components of thyme essential oil, thymol and carvacrol, show no potential risk for animal nor human safety at normal ingestion levels (Domarackỳ, Rehák, Juhás, & Koppel, 2007; Jenner, Hagan, Taylor, Cook, & Fitzhugh, 1964). When thyme essential oil is used as a food preservative, it should be labeled on the food package as a food additive. However, in case essential oils are only used to modify flavor or taste, it is not be considered as a food additive (EU, 2008). Secondly, an ingredient with *in-situ* production of antifungal active compounds can be added to the dough recipe. Within this group, the use of lactic acid or propionic acid bacteria through cultures or sourdough fermentation are of particular interest (Axel et al., 2016; Demirbaş, İspirli, Kurnaz, Yilmaz, & Dertli, 2017; Denkova et al., 2014; Gerez, Torino, Rollan, & de Valdez, 2009; Russo et al., 2017). The main antifungal activity of lactic or propionic acid bacteria cultures is hypothesized to be due to the formation of organic acids, lactic and acetic acid (Axel et al., 2016; C.; Le Lay et al., 2016). Thirdly, fermentation products can also be used as a conservation strategy (Samapundo, Devlieghere, Vroman, & Eeckhout, 2016; Samapundo, Devlieghere, Vroman, & Eeckhout, 2017).

This study had the major objective of evaluating the antifungal activity of thyme essential oil (*Thymus zygis*, ch. Thymol) through both *in-vitro* as *in-vivo* assays. Thyme essential oil was used as a model essential oil with well-described *in-vitro* antifungal activity. The *in-vitro* influences of a_w, pH and incubation temperature on the growth of *A. niger* and *P. paneum* were examined as well. The *in-vivo* assays were conducted by challenge and shelf-life tests of par-baked wheat and sourdough bread. Additionally, the overall quality of the par-baked breads was evaluated. The influence of thyme oil on sensory characteristics was set aside for this research as the main goal was to compare *in-vitro* results with par-baked bread results of a well-described antifungal active essential oil to make recommendations concerning the technological and antifungal properties for further essential oil testing in bread.

2. Materials and methods

2.1. Fungal isolates and spore preparation

Aspergillus niger (P1118) and *Penicillium paneum* Frisvad (IHEM 6652) were kept active on malt extract agar (MEA) (Oxoid). These two moulds are maintained in the culture collection of the Laboratory of Applied Mycology MYCOLAB, Department of Food Science and Technology, Ghent University (Ghent, Belgium). These were chosen for this study as they have been determined to be the main spoilage agents of wheat bread (Legan, 1993). The spore preparation was similar to the method described in Deschuyffeleer et al. (2011), with slight modifications. One week prior to use, fungal spores were transferred to fresh MEA plates (3x) and incubated for 7 days at 26 °C. Sterile Tween 80 (polyoxyethyleensorbitan mono oleaat, Merck) - water solution (1 g Tween 80 per liter distilled water) (5 mL) was added to a full-grown petridish. All the fungal material was scraped loose from the petridish. The Tween-solution was filtered in a sterile falcon tube using a sterile cotton filter (3x). The filter was removed and the falcon tube was centrifuged for 15 min at 8000 rpm and 4 °C. After removal of the supernatant, the pellet was resuspended in 25 mL Tween-PBS (1 g Tween 80 and 10 tablets of PBS per liter of distilled water) (Phosphate buffered saline, Oxoid). The centrifugation step was repeated and the supernatant was removed. Furthermore, the pellet was resuspended in 25 mL of PBS and the latter centrifugation step was repeated a second time. The concentration of spores present was determined by a microscopic evaluation using a Thoma counting chamber.

2.2. Media preparation

A_w and pH of malt extract agar (MEA, Oxoid) were adjusted using respectively glycerol and a 0.1 M citric acid/citrate buffer. The MEA medium of different a_w/pH conditions was prepared and autoclaved during 15 min at 121 °C. Thyme oil was emulsified at a ratio of 1 over 9 (1:9) with a 0.1 m/V% agar solution (Agar Bacteriological no. 1, Oxoid). After autoclaving and cooling down to 60 °C, the content of a test tube was homogenized with a vast amount of sterilized malt extract agar. The concentrations of thyme oil tested were 0, 0.2, 0.5, 1 and 2 μL/mL MEA. Then, agar (15 mL) was poured into each Petri dish and the a_w of the medium was checked with a LabMaster-A_w (Novasina) whereas the pH was measured with a portable pH meter (Hanna Instruments).

2.3. Inoculation and incubation of media

A spore solution (20 μL) of 5×10^3; spores/ml was spotted in the middle of each petridish, in order to inoculate each plate with 10^2 spores of *A. niger* or *P. paneum*. The plates were incubated at two different incubation temperatures for 20 days (22 and 30 °C) and colony diameters were measured using a digital caliper (Taurus impact GmbH). Each combination of a_w, pH, incubation temperature and concentration of thyme essential oil was performed in triplicate.

2.4. Data processing of radial growth on MEA

A non-linear regression in *SPSS statistics version 24* (SPSS Inc., Chicago, Illinois, U.S.A.) was performed on the whole of the data concerning the colony diameters. The maximal growth rate (μ_{max}, mm/day) and the lag phase (λ, days) were determined according to the Baranyi model (equations [1–3]) (Baranyi & Roberts, 1994) as previously reported by Samapundo, Deschuyffeleer, Van Laere, De Leyn, and Devlieghere (2010). The goodness-of-fit of the Baranyi model was determined through the measure of the correlation coefficient R^2 between the predicted diameter values by the model and the values of colony diameter actually observed ($R^2 > 0.99$). Differences in growth parameters, μ_{max} and λ, based on the different growth conditions were evaluated on a 95% significance level.

$$N = N_0 + \mu_{MAX} * B - \ln\left(1 + \left(\frac{exp^{\mu_{MAX} * B} - 1}{exp^{N_{MAX} - N_0}}\right)\right) \quad [1]$$

$$B = t + \frac{1}{\mu_{MAX}} * \ln\left(\frac{exp^{-\mu_{MAX} * t} + A}{1 + A}\right) \quad [2]$$

$$A = \frac{1}{exp^{\lambda * \mu_{MAX}} - 1} \quad [3]$$

with N being the diameter of the fungal colony (mm), N_0 the initial inoculation diameter in mm (= 0), N_{max} the maximal colony diameter reached during incubation in mm, μ_{max} maximal growth rate in mm/day, λ: lag phase time (days), t: time in days and exp the exponential equal to 2.718.