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Growth/no-growth models of *in-vitro* growth of *Penicillium paneum* as a function of thyme essential oil, pH, a_w, temperature

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ABSTRACT

The aims of this study were (i) screening of antifungal activity of thyme essential oil on *Penicillium paneum*; (ii) development of growth/no-growth models (G/NG); and (iii) validation of the G/NG models by performing bread baking trials. The screening method was based on the measurement of fungal growth in a semi-solid medium through optical density. The combined influence of a_w (0.88–0.97), pH (4.8–7.0), temperature (22 and 30 °C), time (0–144 h) and varying concentrations of thyme oil (0–2 µL/mL YES) were assessed. Growth of *P. paneum* at a_w 0.88 was significantly reduced compared to a_w 0.93–0.97. A slight pH effect was observed at a_w 0.93; growth was delayed at pH 6 compared to pH 4.8. The lowest concentration of thyme oil preventing growth during 144 h of incubation was 1 µL/mL medium. According to the results of the shelf-life test of par-baked bread, fungal growth was inhibited for more than 45 days using 0.3 mL thyme oil/100 g dough. To conclude, this study recognized the potential of using G/NG models to develop better product formulations and to facilitate product innovation.

1. Introduction

Annually, at least 5–10% of the global food supply is lost due to the presence of fungi and mycotoxins (Cook and Johnson, 2009). More specifically, mould spoilage is a serious problem in the bakery industry, mainly due to growth of species of *Penicillium* spp. and *Aspergillus* spp. (Legan, 1993) such as *Penicillium paneum*, *P. polonicum*, *P. roqueforti*, *P. commune*, *Paecilomyces variotti, Aspergillus flavus and A. sydowii* (Santos et al., 2016; Suhr and Nielsen, 2005). Besides, there is a growing interest in replacing chemical bread preservatives such as propionate and sorbate by natural preservatives in order to meet up with the consumers' demand for *clean label* products (Samapundo et al., 2017).

One of the largest groups of natural preservatives are plant extracts and essential oils (EOs) (Debonne et al., 2018b). EOs are natural and volatile liquids, extracted from various aromatic plants which have antimicrobial activity (Burt, 2004; Hyldgaard et al., 2012; Leite de Souza et al., 2005). Their antimicrobial activity depends not only on the presence of volatile molecules such as terpenes and terpenoids (Hyldgaard et al., 2012), but also on the intrinsic and extrinsic factors of the food, such as pH, water activity (a_w) and storage temperature (Burt, 2004). An example of an EO with antifungal activity is thyme EO (Askarne et al., 2012; Debonne et al., 2018a; Kumar et al., 2008). The antifungal activity of thyme EO is mainly due to the presence of thymol which can induce cell lysis and alter the cell structure of proliferating cells (Hyldgaard et al., 2012). However, the antifungal activity is influenced by the exact composition, concentration, structure and functional groups of the constituents present (Burt, 2004) and the intrinsic and extrinsic parameters of the growth medium. In Europe, ingredients that are used as food preservatives, should be labeled on the food package as a food additive. The positive list for food additives in Europe is restricting the use of thyme oil as food additive. However, in case EOs are used to modify flavor or taste, they are not being considered as food additives (EU, 2008) but as food flavorings (EU, 2012). The use of flavoring substances is permitted in accordance with good

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manufacturing practices. This regulation is also based on a positive list; for thymol no restrictive use in food is listed.

To determine the required amount of the active component in food, the minimal inhibitory concentration (MIC) is assessed. It is a widely used and valuable parameter for screening of antimicrobial activity and is defined as the lowest concentration of the compound that prevents growth at optimal growing conditions (Van Long et al., 2016). Having knowledge of the MIC is very important for the food industry in order to optimize the required concentration of the component and as such reduce potential physico-chemical and sensorial effects, while maintaining the antimicrobial power of the agent. There is a wide range of MIC-values of thyme oil and thymol on fungal species reported between 2.5 and 512 ppm (Abbaszadeh et al., 2014; Guarda et al., 2011; Jersek et al., 2014; Mota et al., 2012; Sokolic-Mihalak et al., 2012). The main reasons for the MIC variations are differences in screening methods, intrinsic factors of the media, incubation time, temperature, inoculum size and fungal species. The screening method exerts a very important influence on data interpretation, e.g. diffusion versus dilution test (Debonne et al., 2018b; Tullio et al., 2007). Due to insufficient standardization and reporting of a_w, pH and media composition, comparison of these values is often impossible. Moreover, essential oils are not water-soluble, posing a problem for *in-vitro* antifungal screening. Most of the screening techniques generally consist of preparing a water-based agar or broth (Abbaszadeh et al., 2014; Guarda et al., 2011; Mota et al., 2012; Sokolic-Mihalak et al., 2012). To investigate the effect of low water-soluble substances, very often solvents or detergents are added to increase the stability of the emulsion (Remmal et al., 1993) which might decrease (Bennis et al., 2004) or increase (Cheng et al., 2012) its antimicrobial activity. Additionally, most articles do not report on the difficulty of using an oily substance in a water-based agar/broth for micro-dilution screening. Therefore, it seems this does not pose any difficulty for screening. However, phase separation can clearly lead to inconsistent results.

Food companies are interested in defining the conditions at which food products are microbial stable, in other words in the no-growth region. Therefore, G/NG probabilistic models are very useful in the context of microbial stability studies and are often more suitable compared to kinetic growth models (Vermeulen et al., 2012). With this study, we aim to provide a clear overview of the antifungal activity of thyme oil on *P. paneum* which is a common bread mould (Debonne et al., 2018a), in combination with pH, a_w and temperature using an *in-vitro* method based on the screening method of Medina et al. (2012). The data obtained were used to develop G/NG models. Furthermore, these results were validated on an actual bread matrix by performing shelf-life and challenge tests of par-baked wheat breads.

2. Materials and methods

2.1. Fungal isolate

The methodology for inoculum preparation was based on Deschuyffeleer et al. (2011) with slight modifications. *P. paneum* Frisvad (IHEM 6652) is part of the culture collection of the Laboratory of Applied Mycology MYCOLAB, Department of Food Technology, Safety and Health, Ghent University (Belgium) and is stored under freeze-dried condition at - 80 °C. During the experiments, the mould was kept active by maintaining the species on malt extract agar (MEA) (Oxoid). One week prior to use, fungal spores were transferred to fresh MEA plates (3 ×) 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 *Petri dish*. All fungal material was scraped loose from the *Petri dish*. The Tween-solution was filtered in a sterile falcon tube by the aid of a sterile cotton filter. This step was repeated three times. 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. Further, the pellet was resuspended in 25 mL of PBS (10 tablets of PBS per liter of distilled water). The latter centrifugation step was repeated a second time. The concentration of spores present was determined by a microscopic evaluation using a Thoma cell counting chamber.

2.2. Media preparation

The method for preparing the semi-solid yeast extract sucrose (YES) medium was based on Medina et al. (2012), with a slight modification for setting the pH and a_w. The YES medium was prepared with 20 g/L yeast extract (Oxoid), 150 g/L sucrose, 0.5 g/L magnesium sulphate (Ucb) (viscosity of the medium was 6.2 mPa s at 22 °C). The pH of the water phase was adjusted with 0.1 M citric acid/citrate buffer [citric acid-1-hydrate gritty, chem. pure (Lamers and Pleuger BV); tri-sodium citrate dehydrate (Merck)] (pH 4.8, 5.0, 5.5, 6.0 and 7.0); a_w was adjusted with glycerol bidistilled 99.5% (VWR chemicals) (a_w 0.88, 0.93, 0.95 and 0.97). Thyme EO [Physalis Aromatherapy, *Thymus zygis* (chemotype thymol)] was added in the appropriate concentration in test tubes with screw caps (0–2 μL/mL). These test tubes also contained 12 mg technological agar (Agar Technological no. 3, Oxoid) and 10 mL YES medium, with adjusted pH and a_w, and were autoclaved for 15 min at 121 °C. The pH and a_w were always measured after autoclaving and cooling. The a_w of the medium was checked with a Lab-Master-a_w (Novasina) and pH with a portable pH meter (model HI 83141, Hanna Instruments). An overview of all growth conditions tested on *P. paneum* is presented in Table 1. This resulted in a total set of media of 340 different combinations of pH, a_w, temperature and concentration of thyme oil.

2.3. Data generation for the model development

The YES medium in the test tubes was inoculated to 3.3×10^4 spores/mL. The wells in columns 1, 2, 4, 6, 8, 10, 12 of the sterile 96-well plate (96-well polystyrene microplate, clear, flat bottom, 382 μL/w, Greiner bio-one bvba) were filled with 150 μL of the inoculated YES medium without thyme oil (Fig. 1). The wells in the uneven columns (3, 5, 7, 9, 11) were filled with 300 μL of the corresponding thyme oil solutions (2.0, 1.5, 1.2, 1.0 and 0.4 μL/mL YES). Further, 150 μL of the wells in these uneven columns was pipetted and transferred to the well right of it, homogenized and again 150 μL of this new well was pipetted and discarded. This resulted in serial dilutions (1:2) of thyme oil from the uneven wells. All 96-well plates contained 8 replicates of 9 different thyme oil concentrations (0.2, 0.4, 0.5, 0.6, 0.75, 1.0 (× 2), 1.2, 1.5 and 2.0 μL/mL total product basis) and 16 control wells (0 μL/mL). The spore concentration in the wells was 5×10^3 spores (per well or per 150 μL). The plate was covered with a Breath easy film (Sigma-Aldrich) to avoid evaporation of volatile compounds. During incubation, no

Table 1
Conditions of the experimental set-up for *in-vitro* growth of *Penicillium paneum.*

Water activity (a_w)	pH	Concentration (μL/mL)	Temperature
0.88	4.8	0	22 °C
0.93	5.0	0.20	30 °C
0.95	5.5	0.40	
0.97	6.0	0.50	
	7.0[a]	0.60	
		0.75	
		1.00	
		1.20	
		1.50	
		2.00	

[a] Only for a_w 0.97.

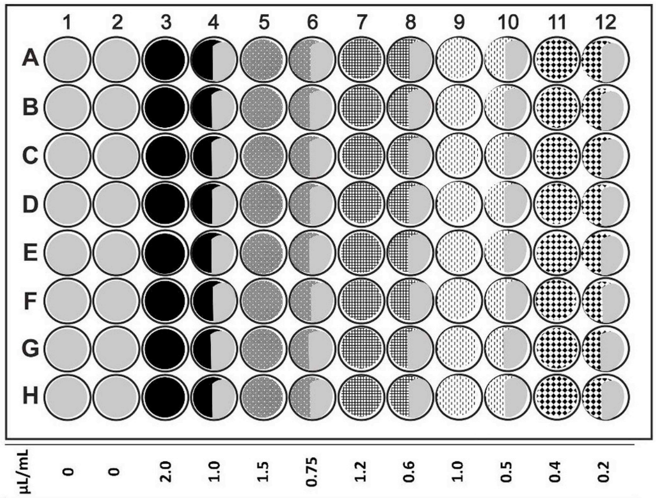

Fig. 1. Schematic overview of a 96-well plate containing YES medium and thyme oil (0–2 µL/mL YES) (Color legend: plain grey: inoculated YES medium; black: 2.0 µL/mL YES; grey with dots: 1.5 µL/mL YES; grey with stripes: 1.2 µL/mL YES; white with dots: 1.0 µL/mL YES; white with black boxes: 0.4 µL/mL YES).

shaking steps were acquired. Each 96-well microplate was placed in a Multiskan Ascent 96/384 Plate Reader. Optical density (OD) was measured every 20 min at 595 nm during 6–7 days (> 144 h). Incubation temperature was set at 22 or 30 °C. The YES medium gave a base OD signal of 0.12. To define growth, the difference was made between the OD of the wells and the base OD signal at the start of incubation. If this difference was higher than 0.1, this well was considered showing growth. This theory was based on Medina et al. (2012), where conidia of *Aspergillus flavus* were considered germinated when germination tubes were at least three times as long as the conidia diameter, matching with the OD increase of 0.1. This was validated for *P. paneum* (initial spore diameter of *P. paneum* spores was 5 µm; OD increase of 0.1 matched with spore diameters of 17.1 ± 3.1 µm (n = 68)). The growth probability at each condition was computed by considering the number of observations with respect to the number of replicates tested, e.g., if 2 out of the 8 wells showed growth, the probability of growth was 25% (Vermeulen et al., 2012).

2.4. Development of growth/no-growth models

The G/NG data were used to develop two models for *P. paneum* with pH, a_w, concentration of thyme oil and incubation time as explanatory variables, incubated at 22 and 30 °C (Eqs. (1a) and (1b)). Additionally, an extra set of two models were developed with pH, concentration and time as variables, at fixed a_w 0.97, incubated at 22 and 30 °C (Eqs. (2a) and (2b)). Similar to Vermeulen et al. (2012), an ordinary logistic regression model was used to describe the data. The equation of the models consists of a polynomial (right-hand side) and logit(p) = ln (p/(1-p)) (left-hand side) with p the probability of growth. The two sets of models are represented (Eq. [1] and [2]).

In these equations, b_i (i = 0, …, 14) are the parameters to be estimated, and pH, a_w, *conc* (concentration of thyme oil expressed in µL/mL total product basis) and *time* (incubation time expressed in h) the variables. The models were fitted in *SPSS Statistics* 25 (SPSS, Inc., Chicago, IL, USA) using linear logistic regression according to the procedure described in Vermeulen et al. (2007) which implies that the main effects were forced to stay in the final model equation irrespective of their *p*-value. The quadratic and interaction terms were selected by the backward stepwise procedure, based on the significance of the likelihood-ratio criterion (p = 0.001). Model building stopped when no more variables met entry or removal criteria. The predicted G/NG interfaces were plotted in *Matlab* 9.3 (The Mathworks, Inc., Natick, MA, USA). Goodness-of-fit statistics considered were: (1) Nagelkerke R^2, (2) receiver operating curve (ROC-curve) c-value statistics and (3) the degree of agreement (%) between observations and predictions (Santos et al., 2018).

2.5. Validation of antifungal activity on par-baked wheat bread

2.5.1. Bread making procedure

All experiments were performed using a single batch of commercial wheat flour (Epi B type 55) supplied by Brabomills NV (Merksem, Belgium). The flour had the following product specifications: 15 g water/100 g flour, 10.6 g protein/100 g dry matter, 1.2 g fat/100 g dry matter, 0.5 g ash/100 g dry matter. The production of bread dough was similar to the method described in Debonne et al. (2018a) and dough was prepared on a flour weight basis. For 100 g flour, 57.8 g water [water absorption was determined by a Farinograph (Farinograph-E, Brabender)], 1.5 g table salt, 1 g of instant dry baker's yeast (Algist Bruggeman, Belgium), 0.1 g malt flour and 5 mg ascorbic acid/100 g flour. The following breads were prepared: a control (= without any preservatives), a reference containing 200 mg propionic acid/100 g dough under the form of calcium propionate, CaP (= maximum allowed in the EU (EU, 2011), in pre-baked bread), and bread containing 0.1, 0.2, 0.3 or 0.4 mL thyme EO/100 g dough. These concentrations were selected based on previous work described in Debonne et al. (2018a). The dough was mixed for 7 min in a De Danieli spiral mixer (Verhoest Machinery). After a 10 min rise at 30 °C and 80–90% relative humidity (proofing cabinet Panimatic), dough was divided into 15 pieces of 65 g and rounded manually. The dough pieces were then placed back into the rise cabinet for a fermentation time of 90 min. The breads were par-baked for 10 min (2 min at 170 °C (200 mL steam) and 8 min at 150 °C) (MIWE aeromat FB12 (oven type 4.64)). Each baking test was performed in duplicate. Eight out of the 15 breads per test were characterized according to the following methods described: bread volume was measured by a Volscan Profiler 600 (Stable Micro Systems); breads were weighed using a KERN balance (± 0.01 g); specific volume was determined by dividing bread volume by bread weight (mL/g); pH and a_w were measured. Moreover, the moisture content of the breads was determined using the AACCI Method 44–15.02 whereby moisture content is determined as weight loss of a sample when heated under specified conditions. The a_w of the bread was measured with a LabMaster-a_w (Novasina) (a_w 0.96–0.97) and pH with a portable pH meter (model HI 83141, Hanna Instruments) (pH 5.7–6.0).

$$logit(p) = b_0 + b_1 * pH + b_2 * a_w + b_3 * conc + b_4 * time + b_5 * pH^2 + b_6 * a_w^2 + b_7 * conc^2 + b_8 * time^2 + b_9 * pH * a_w + b_{10} * pH * conc + b_{11} * pH * time + b_{12} * aw * conc + b_{13} * aw * time + b_{14} * conc * time \quad [1\ (a,b)]$$

$$logit(p) = b_0 + b_1 * pH + b_2 * conc + b_3 * time + b_4 * pH^2 + b_5 * conc^2 + b_6 * time^2 + b_7 * pH * conc + b_8 * pH * time + b_9 * conc * time \quad [2\ (a,b)]$$

2.5.2. Challenge test of par-baked bread

Six out of the 15 breads were used for the challenge test. Each bread was sliced in two pieces, resulting in 12 replicates per baking test. Bread crumbs (inner part of bread) were challenged with ± 20 spores of *P. paneum* (20 µL) through spot-inoculation as previously described in Debonne et al. (2018a). All bread pieces were individually packed under air atmosphere and placed at 22 °C till visible mould spoilage. As baking tests were performed in duplicate, 24 data points per condition were collected.

2.5.3. Shelf-life test of MA-packed par-baked bread

Because par-baked breads are usually packed under modified atmosphere (MA) and exposed to a natural environmental post-baking contamination, a shelf-life test with MA-packaging and without artificial inoculation was performed. Wheat breads containing thyme EO (0, 0.1, 0.2, 0.3 and 0.4 mL/100 g bread dough) were par-baked and cooled to room temperature in the bakery environment. They were packed with a Tray Sealer (DECA Packaging Group, Herentals, Belgium) using a gas composition of 70% CO_2 and 30% N_2. The breads were packed per two in a tray (632 mL) made out of PP/EVOH/PP (PP: polypropylene; EVOH: ethylene vinyl alcohol) and sealed with a cover film OPALEN HB 65 AF PP PL (OPA/PE/EVOH/PE/PP (OPA: orientated polyamide; PE: polyethylene; EVOH: ethylene vinyl alcohol; PP: polypropylene)). The oxygen transmission rate of the film was 5 cm^3 x m^{-2} x day^{-1} and water vapor transmission rate was 12 g x m^{-2} x day^{-1}. The breads were stored at 22 °C and the samples were checked daily during 45 days for the development of visible mould colonies. Concentration of oxygen inside the package was recorded and ranged from 0 to 1% at the beginning to 0–3.5% at the end of the shelf-life (Checkmate 3, Dansensor, Denmark).

2.6. Statistical analysis

To assess significant differences among samples (e.g. pH of bread), a multiple comparison analysis of samples was performed using *SPSS Statistics 25*. In case the results were normally distributed, either a Tukey test (homoscedasticity) or a Dunnett T3 test was used to describe the means with 95% confidence (p = 0.05). A Dunn test for multiple comparison was applied, preceded by a non-parametric Kruskal-Wallis 1-way ANOVA, for non-normally distributed data.

3. Results and discussion

3.1. Growth/no-growth models

A G/NG model was developed for each of the two different data sets (22 and 30 °C). Two separate models including a_w, pH, thyme oil concentration and time as independent variable and growth probability as dependent variable were developed (Eq. (1)). The estimated parameters with their standard deviations of the models and the goodness-of-fit statistics are summarized in Table 2. The statistics show that both models are able to predict the data well (≥96.5%). Some cross-sections are selected showing the most important outcomes of the 5-dimensional models (Figs. 2 and 4).

Among the tested conditions, a_w exerted the largest influence on the growth of *P. paneum* (Fig. 2). In this figure, the effect of pH as a function of time on the growth of *P. paneum* at 22 °C for 0.2 µL thyme oil/mL YES is presented at several a_w levels. At a_w 0.88, no growth of *P. paneum* was observed. This study showed that the minimal a_w for growth must be between 0.88 and 0.93. At a_w 0.93, 0.2 µL thyme oil/mL YES medium and 22 °C, growth occurred after 50 h. The influence of pH on the growth of *Penicillium* is inferior to the a_w effect and corresponds to findings reported in literature (Dantigny, 2016; Van Long et al., 2017). The effect of pH on *P. paneum* was low, corresponding to results of Sautour et al. (2001) for *Penicillium chrysogenum*. Mould growth is indeed not or slightly affected by pH within the range of 3–8 (Dantigny, 2016; Van Long et al., 2016). Dagnas et al. (2014), on the other hand, stated that the role of pH in mould growth limitation has never been clearly established. In this study, there is a pH effect observed at a_w 0.93, the models show that there is a growth delay at pH 6 compared to pH 4.8. At higher a_w 0.95–0.97, this pH effect reduces strongly. The effect of pH on growth of *P. paneum* was further investigated at a_w 0.97 in order to simulate the activity in bread with this water activity. Data of pH 7.0 – a_w 0.97 was added and a new model at a_w 0.97 was developed. Parameters for these two extra models are presented in Table 3. In Fig. 3 the results of the model cross-sections without thyme oil are shown for 22 and 30 °C. At 22 °C, 98% of the data could be predicted correctly, compared to 91.5% at 30 °C. Incubation temperature is generally the second most important growth factor after a_w (Plaza et al., 2003). In this study, however, the temperature range was too small to see significant effects. In Fig. 4, the effect of thyme oil concentration as function of time on the growth of *P. paneum* at 22 and 30 °C is represented for pH 6.0. The onset of growth of *P. paneum* in YES medium without thyme oil at 22 °C is observed after 55–75 h, 40–50 h and 30–40 h for respectively a_w of 0.93, 0.95 and 0.97. Modelling showed that the lowest concentrations of thyme oil necessary to prevent growth of *P. paneum* depend strongly on the a_w of the medium (Table 4). From these results, it could be concluded that a concentration of 1.0 µL thyme oil/mL YES medium with a_w 0.97 is sufficient to prevent growth during 144 h.

Table 2
Model factors with their estimates and standard deviations and goodness-of-fit statistics of the growth/no-growth models of *Penicillium paneum* at 22 and 30 °C (Eq. (1a) (22 °C) and 1b (30 °C)).

Factors	22 °C		30 °C	
	Estimate	p-value	Estimate	p-value
a_w	2217.6 ± 387.4	< 0.001	5493.6 ± 848.7	< 0.001
pH	- 72.9 ± 14.3	< 0.001	−62.1 ± 13.8	< 0.001
conc	206.1 ± 21.5	< 0.001	34.4 ± 16.5	0.030
time	- 0.5 ± 0.2	0.044	−0.5 ± 0.2	0.003
a_w^2	- 1310.9 ± 215.8	< 0.001	−3173.5 ± 452.5	< 0.001
pH^2	ns		−4.2 ± 0.6	< 0.001
$conc^2$	ns		2.9 ± 0.4	< 0.001
$time^2$	−0.0 ± 0.0	< 0.001	−0.0 ± 0.0	< 0.001
a_w * pH	74.3 ± 15.0	< 0.001	112.8 ± 13.6	< 0.001
a_w * conc	- 234.9 ± 23.4	< 0.001	−41.5 ± 17.3	0.047
a_w * time	1.0 ± 0.2	< 0.001	1.0 ± 0.2	< 0.001
pH * conc	ns		ns	
pH * time	ns		−0.0 ± 0.0	0.001
Conc * time	ns		−0.1 ± 0.0	< 0.001
constant	- 928.1 ± 180.6	< 0.001	−2479.9 ± 403.8	< 0.001
Statistics				
Nagelkerke R^2	0.952		0.913	
c-value (ROC-curve)	0.998 ± 0.000		0.994 ± 0.001	
% correct predicted	98.1		96.5	

ns: not significant.

3.2. Validation of in-vitro antifungal activity of thyme oil on par-baked bread

Par-baked wheat breads were produced and subjected to challenge and shelf-life tests. The effects of thyme EO (0–0.4 mL/100 g dough) and calcium propionate (200 mg propionic acid/100 g dough) were tested. Addition of 0.2 mL thyme oil/100 g dough or higher led to an increased water retention capacity of the dough which can be seen by an increase in bread weight (Table 5). This was linked to the effect of thyme oil on bread volume. Bread volume was significantly reduced with more than 50% with 0.4 mL thyme oil/100 g dough, as compared to the control bread. Specific volume reduced from 2.8 (control) to 1.8 and 1.2 for respectively 0.2 mL and 0.4 mL thyme oil/100 g dough. Thyme oil is also known to interact with baker's yeast *Saccharomyces cerevisiae*, hereby reducing its leavening activity in dough (Debonne

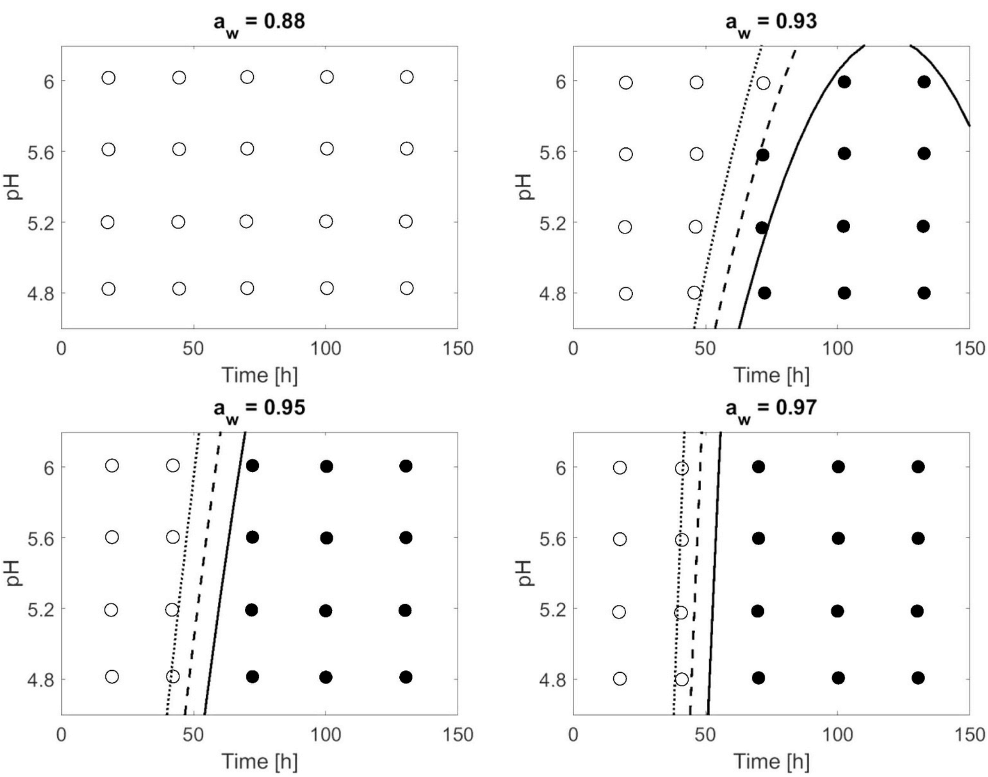

Fig. 2. *In-vitro* growth/no-growth model for *Penicillium paneum* in YES medium, incubated at 22 °C and containing 0.2 µL thyme oil/mL YES. Lines represent the ordinary logistic regression model predictions: p = 0.9 (solid line), p = 0.5 (dashed line), and p = 0.1 (dotted line). Solid symbols indicate fungal growth; open symbols indicate no growth (graphic representation of models expressed in Table 2).

Table 3
Model factors with their estimates and standard deviations and goodness-of-fit statistics of the different growth/no growth models of *Penicillium paneum* at fixed a_w 0.97 (Eq. (2a) (22 °C) and 2b (30 °C)).

Parameters	22 °C		30 °C	
	Estimate	p-value	Estimate	p-value
pH	−1.5 ± 0.3	< 0.001	−0.1 ± 0.3	0.765
conc	−33.9 ± 7.4	< 0.001	−22.3 ± 2.6	< 0.001
time	0.7 ± 0.1	< 0.001	0.3 ± 0.0	< 0.001
conc^2	−8.7 ± 3.3	0.008	2.3 ± 0.5	< 0.001
time^2	−0.0 ± 0.0	< 0.001	−0.0 ± 0.0	< 0.001
pH * conc	ns		1.9 ± 0.4	< 0.001
pH * time	ns		−0.0 ± 0.0	< 0.001
Conc * time	0.2 ± 0.1	< 0.001	ns	
constant	−11.1 ± 2.9	< 0.001	−3.4 ± 1.5	0.023
Statistics				
Nagelkerke R^2	0.965		0.868	
c-value (ROC-curve)	0.998 ± 0.000		0.984 ± 0.002	
% correct predicted	98.0		91.5	

ns: not significant.

et al., 2018c). Due to the lower dough rise, volumes of the par-baked breads were lower, resulting in less surface for water evaporation and therefore higher water retention. From this study it could be concluded that concentrations of thyme oil higher than 0.2 mL/100 g dough negatively impacted bread quality. Moreover, Debonne et al. (2018a) found that negative effects upon thyme oil addition already occur from 0.15 mL/100 g dough. Next to the physico-chemical effects, essential oils such as thyme oil can also affect the sensorial quality of the bread when used in high concentrations (Debonne et al., 2018b). Sensorial quality however, was not taken up in this study but must be given further attention when bringing essential oils to the market.

The concentration range of thyme oil was based on data derived from the models for conditions of pH and a_w matching those of the breads: pH 6.0 and a_w 0.97 (Table 5). The lowest concentration of thyme oil preventing *in-vitro* growth of *P. paneum* was 1 µL/mL (Fig. 4; Table 4). In order to validate the results of the *in-vitro* models, it is important to convert the concentration of thyme oil expressed in µL/mL YES medium to mL/100 g dough to be able to compare both results. From a microbiological point of view only the phase in which microorganisms are active is important, the water phase. Besides the water phase, there are three other phases that can be distinguished in bread:

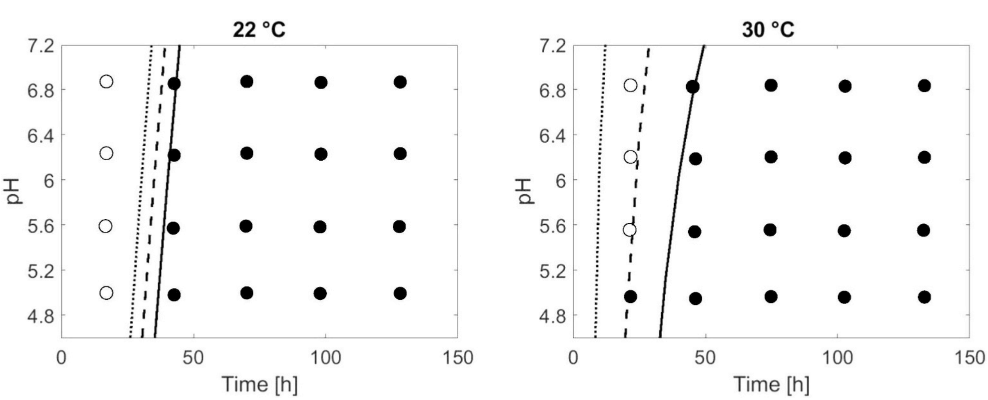

Fig. 3. *In-vitro* growth/no growth model for *Penicillium paneum* in YES medium with a_w 0.97, incubated at 22 or 30 °C, without thyme oil. Lines represent the ordinary logistic regression model predictions: p = 0.9 (solid line), p = 0.5 (dashed line), and p = 0.1 (dotted line). Solid symbols indicate fungal growth; open symbols indicate no growth (graphic representation of models expressed in Table 3).

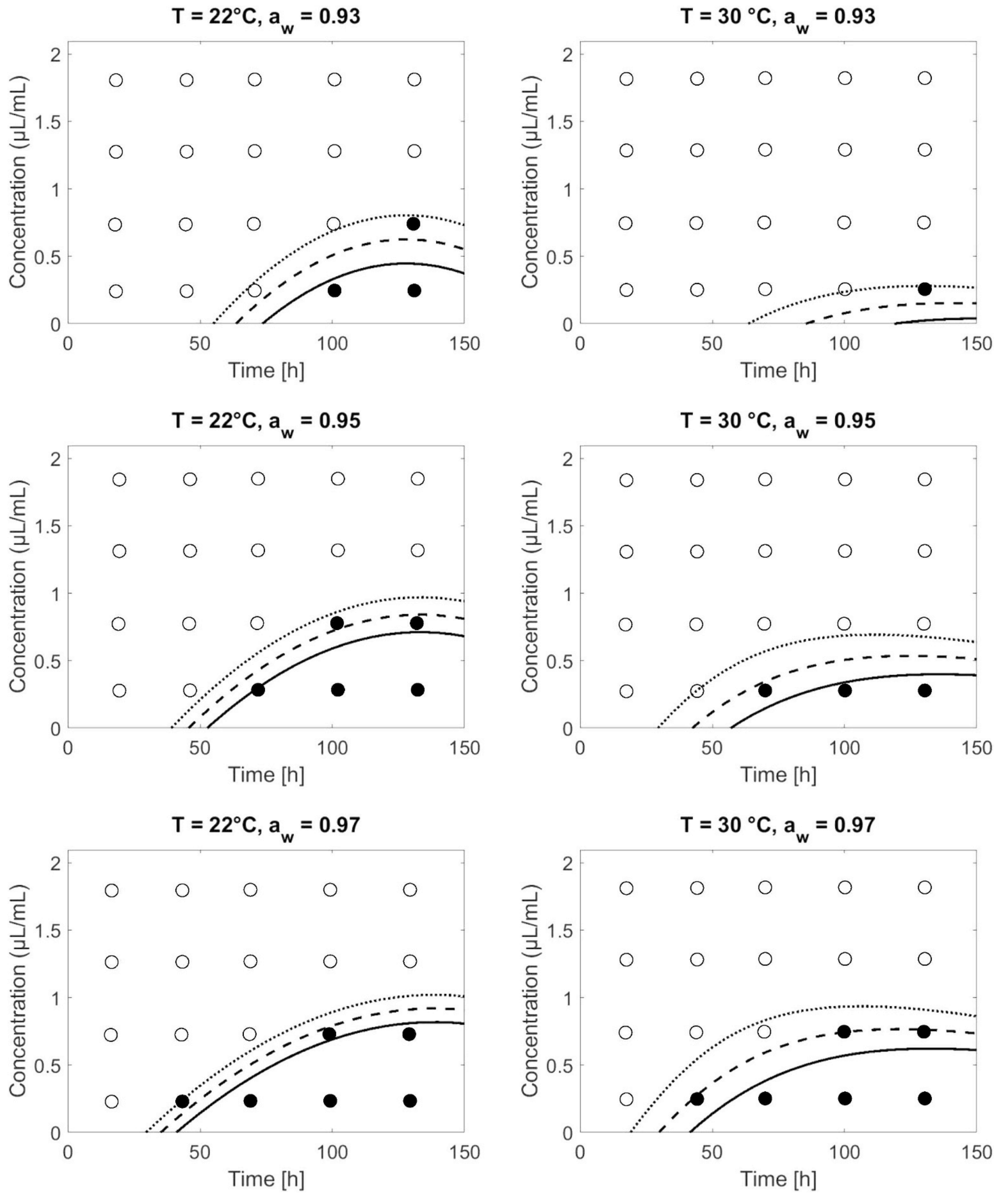

Fig. 4. *In-vitro* growth/no growth model for *Penicillium paneum* in YES medium with pH 6.0, varying *aw* (0.93, 0.95 and 0.97), incubated at 22 or 30 °C, containing thyme oil (μL/mL YES medium). Lines represent the ordinary logistic regression model predictions: p = 0.9 (solid line), p = 0.5 (dashed line), and p = 0.1 (dotted line). Solid symbols indicate fungal growth; open symbols indicate no growth (graphic representation of models expressed in Table 2).

Table 4
Minimal inhibitory concentrations of thyme oil in YES medium with pH 6.0 after 144 h incubation at either 22 or 30 °C, derived manually from the growth/no-growth models presented in Table 2 and Fig. 4, expressed in function of the limits of prediction (p = 0.9 and 0.1).

	22 °C		30 °C	
a_w **0.93**	p = 0.9	0.40 μL/mL	p = 0.9	0.10 μL/mL
	p = 0.1	0.70 μL/mL	p = 0.1	0.25 μL/mL
a_w **0.95**	p = 0.9	0.70 μL/mL	p = 0.9	0.35 μL/mL
	p = 0.1	0.90 μL/mL	p = 0.1	0.65 μL/mL
a_w **0.97**	p = 0.9	0.80 μL/mL	p = 0.9	0.60 μL/mL
	p = 0.1	1.00 μL/mL	p = 0.1	0.90 μL/mL

the oil phase, the solid fat phase and the inert organic matter phase. Thyme oil will not dissolve in the latter two phases. Additionally, only the free lipids in the oil phase will compete with water for dissolving thyme oil, not the bound lipid fraction. In this study no additional oil or fat was added to the bread recipe. The oil originated from the Epi B flour with 1.2% lipid content (0.81% in PB-bread). Flour lipids contain approximately 57% free lipids (Pomeranz, 1988), resulting in 0.46% free lipids in PB-bread in this study (1.2% lipids in flour x 57% free lipids). Thyme oil will preferentially bind to the free oil fraction in bread, resulting in loss of active concentration in the water phase. Additionally, the moisture content of the par-baked breads was determined (Table 5). A significant effect in moisture content was observed between the groups 0–0.1 mL thyme oil/100 g dough (35.9 ± 0.4%, n = 8) and 0.2–0.4 mL/100 g dough (37.5 ± 0.2%, n = 12) ($p < 0.05$). Overall, the moisture content of the par-baked breads was 37 ± 1%.

In a study of Oszagyan et al. (1996), values for the partitioning coefficients of the major volatile components of thyme oil are reported (including thymol, carvacrol, *p*-cymene and γ-terpinene). The partitioning coefficient of thymol is $10^{3.34}$. This means that $10^{3.34}$ parts of thymol go to the oil phase and 1 part to the water phase. However, research within the Department of Food Technology, Safety and Health (Ghent University, Belgium) has shown that partitioning is highly correlated with the oil/water ratio of the product (Unpublished results).

Table 5
Quality characteristics of par-baked wheat bread supplemented with thyme essential oil or calcium propionate: pH (n = 6); water activity, a_w (n = 6); bread weight (W; n = 16); moisture content (M; n = 4); volume (V; n = 16) and specific volume (SV; n = 16).

	pH	a_w	W (g)	M (%)	V (mL)	SV (mL/g)
0 mL/100 g dough	5.69 ± 0.02^{a}	0.97 ± 0.01^{a}	59.1 ± 0.5^{a}	35.8 ± 0.5^{a}	165 ± 9^{a}	2.8^{a}
0.1 mL/100 g dough	5.72 ± 0.04$^{a\ c}$	0.97 ± 0.01^{a}	59.4 ± 0.4^{a}	36.0 ± 0.2^{a}	139 ± 7^{a}	2.3^{a}
0.2 mL/100 g dough	5.82 ± 0.03$^{a\ c\ d}$	0.97 ± 0.00^{a}	60.9 ± 0.5^{b}	37.5 ± 0.2^{b}	107 ± 2^{b}	1.8$^{b\ d}$
0.3 mL/100 g dough	5.87 ± 0.02$^{b\ c}$	0.96 ± 0.01^{a}	61.1 ± 0.6^{b}	37.6 ± 0.2^{b}	91 ± 3$^{b\ c}$	1.5$^{b\ c}$
0.4 mL/100 g dough	5.95 ± 0.02$^{b\ d}$	0.96 ± 0.00^{a}	61.0 ± 0.6^{b}	37.5 ± 0.3^{b}	71 ± 4^{c}	1.2^{c}
CaP*	5.72 ± 0.06$^{a\ c}$	0.97 ± 0.01^{a}	60.4 ± 0.5^{b}	36.4 ± 0.5^{a}	141 ± 5$^{a\ b}$	2.3$^{a\ d}$

*CaP: calcium propionate, 200 mg propionic acid/100 g dough.
[a-d]Values in the same column followed by different superscripts are significantly different ($p < 0.05$).

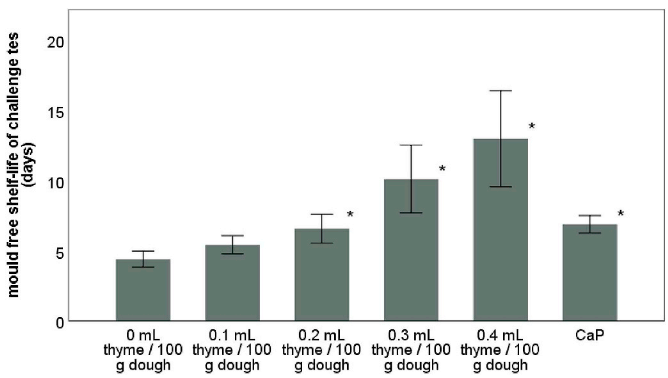

Fig. 5. Mould free shelf-life of challenge test (days) of par-baked wheat breads with thyme essential oil (0–0.4 mL/100 g dough) or calcium propionate (CaP), followed up for 45 days. The challenge test was performed with spores of *Penicillium paneum* and breads were packaged under air atmosphere (n = 24) (* indicate significant differences compared to the control (0 mL thyme/100 g dough ($p < 0.05$))).

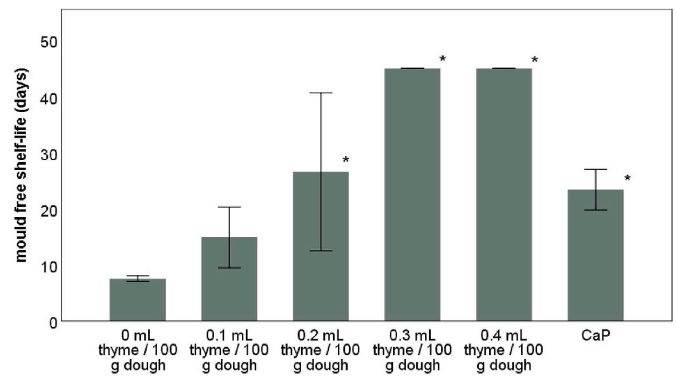

Fig. 6. Mould free shelf-life (days) of par-baked wheat breads with thyme essential oil (0–0.4 mL/100 g dough) or calcium propionate (CaP), followed up for 45 days. Breads were exposed to natural post-baking contamination and packaged under modified atmosphere (% of spoilage after 45 days: 67% for 0.2 mL thyme/100 g dough and 0% for 0.3 and 0.4 mL/100 g dough (n = 12) (* indicate significant differences compared to the control (0 mL thyme/100 g dough ($p < 0.05$))).

The ratio free oil/water in PB bread was 1/80 (0.46% oil/37% water). Within our Department, researchers optimized the Henderson-Hasselbalch equation as reported in Wilson et al. (2000) with dimensional volumes rather than masses which is of great importance in the emulsion industry (see Supplementary material). The optimized equation could be used in this study, assuming that thyme oil consists only out of thymol and that pH has no influence on thymol protonation (100% undissociated). Resulting from this equation, concentrations of thymol in the water phase of bread could be derived. Concentrations of 0.1; 0.2; 0.3 and 0.4 mL thyme oil/100 g dough were equal to respectively 2.4; 4.8; 7.2 and 9.7 μL thymol/mL water phase.

The results of the *in-vitro* tests were validated in bread. The challenge test of par-baked breads showed a significant increase in mould free shelf-life for concentrations of thyme oil of 0.2 mL/100 g dough and higher (Fig. 5). The shelf-lives of respectively 0, 0.2, 0.3 and 0.4 mL/100 g were 4.4 ± 0.6 days; 6.6 ± 0.7 days; 10.1 ± 2.4 days and 13.0 ± 3.4 days (n = 24). Complete growth inhibition could not be achieved with the challenge test. Next to the challenge test, a shelf-life test with modified atmosphere packaging was performed (Fig. 6). The antifungal effect of thyme EO was enhanced by MAP as complete growth inhibition during the incubation period of 45 days was achieved for 0.3 and 0.4 mL thyme oil/100 g dough and at 0.2 mL/100 g dough, only 67% of the samples showed spoilage. The control had a shelf-life of 7.6 ± 0.5 days and CaP addition resulted in a shelf-life of 22.7 ± 3.7 days. The MIC of thyme oil in combination with MAP of the par-baked breads was situated between 0.2 and 0.3 mL/100 g dough.

Based on these results, It could be concluded that the *in-vitro* method overestimates the antifungal activity of thyme oil in bread (modelled value: 1 μL/mL water phase (YES medium) versus actual value: 4.8–7.2 μL/mL water phase in bread). The concentration needed in bread is factor 5–7 times higher than the modelled value. Several other authors have already acknowledged the fact that in food often higher concentrations of essential oils are needed to achieve the same level of preservation compared to *in-vitro* tests (Burt, 2004). This can be explained by several mechanisms. First, thyme oil preferentially binds to the fat phase in bread, resulting in a lower concentration of thyme oil in the water phase when the fat content is increased. Similar conclusion was found by Gutierrez et al. (2008) who investigated the influence of sunflower oil on the antimicrobial efficacy of thyme oil in a model system. High concentrations of oil negatively impacted the antimicrobial power of the essential oil. Second, in the same study of Gutierrez et al. (2008) the influence of starch on the antimicrobial efficacy of thyme oil was investigated. They discovered that 5% starch is already enough to reduce the antimicrobial activity. Third, Watanabe et al. (2003) revealed that liquid oil is adsorbed in the gluten structure of dough, causing the aggregation of the gluten. This phenomenon can also result in a reduced antimicrobial efficacy of thyme oil. Fourth, it is possible that due to the volatile behavior of essential oil, there is a loss of activity in bread upon baking and storage. Karoui et al. (2011) investigated the effect of heating temperature on the total phenolic content of corn oil flavored with *Thymus capitatus*. A gradual decrease in phenolic content was observed with increasing heating temperatures between 25 and 200 °C. The time-temperature combination applied on the YES medium was 15 min 121 °C compared to par-baking of 12 min 150–170 °C (heating and cooling time not included). Another factor is loss of antimicrobial activity during storage. However, Turek and Stintzing (2012) found that during 72 weeks of storage at 23 °C thyme essential oil composition remained stable. Additionally, thyme oil showed more resistance towards degradation compared to rosemary oil. A possible reason for this remarkable resistance might lie in the high amount of phenolic compounds such as thymol and carvacrol, as hypothesized by Turek and Stintzing (2012).

4. Conclusion

Growth/no-growth models were developed for growth of *P. paneum* in synthetic medium with different conditions of pH, a_w, temperature and concentration of thyme essential oil. Based on these models, a minimal inhibitory concentration in synthetic medium with conditions similar to bread could be predicted (1 µL thyme oil/mL water phase). Further, this result was validated in bread by performing bread baking trials and consecutive microbiological shelf-life tests. Despite the good predictive power of the G/NG models in synthetic medium, several other mechanisms in bread proved to be responsible for a loss of antimicrobial efficacy of thyme oil in bread. Fungal growth on par-baked MAP bread was inhibited for more than 45 days in bread formulated with 0.3 mL thyme oil/100 g dough. Based on our results, we could conclude that the antifungal active concentration of thyme oil in bread must lie between 0.2 and 0.3 mL/100 g dough. Modelling of the migration of thymol in the bread system has resulted in calculated concentrations of 4.8–7.2 µL thyme oil/mL water phase in bread. This study has also found that dosage of concentrations of thyme oil equal to or higher than 0.2 mL/100 g dough negatively affected the physico-chemical quality characteristics of the bread. Nevertheless, this study showed the potential applicability of G/NG models to assess the microbiological stability of par-baked breads enriched with natural antifungal compounds such as essential oils. The future challenge is to identify the key mechanisms in the bread matrix responsible for important losses of antimicrobial efficacy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.04.003.

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