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# Effect of weak acid preservatives on growth of bakery product spoilage fungi at different water activities and pH values

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**Abstract**

Inhibition of spoilage organisms from bakery products by weak acid preservatives in concentrations of 0%, 0.003%, 0.03% and 0.3% (w/v) was investigated experimentally on a substrate media with water activity ($a_w$) and pH ranging from sourdough-fermented acidic rye bread to alkaline intermediate moisture sponge cake types ($a_w$ 0.80–0.95, pH 4.7–7.4). Initially, rye bread conditions ($a_w$ 0.94–0.97 and pH 4.4–4.8) in combination with calcium propionate were investigated. Results showed that the highest concentration of propionate (0.3%) at all conditions apart from high $a_w$ (0.97) and high pH (4.8) totally inhibited fungal growth for a 2-week period, with the exception of *Penicillium roqueforti*, *Penicillium commune* and *Eurotium rubrum*. Characteristically for the major spoiler of rye bread, *P. roqueforti*, all three isolates tested were stimulated by propionate and the stimulation was significantly enhanced at high water activity levels. The effect of propionate on production of secondary metabolites (mycophenolic acid, rugulovasine, echinulin, flavoglaucin) was also studied, and variable or isolate dependent results were found. Subsequently, a screening experiment representing a wider range of bakery products was conducted using calcium propionate, potassium sorbate and sodium benzoate. The obtained data was modelled using survival analysis to determine 'spoilage-free time' for the fungi. At the low $a_w$ level (0.80) only *Eurotium* species grew within the test period of 30 days. Higher water activity levels as well as higher pH values decreased spoilage-free times of the fungi. The preservative calcium propionate was less effective than potassium sorbate and sodium benzoate.


*Keywords:* Weak acid preservatives; Calcium propionate; Potassium sorbate; Sodium benzoate; Spoilage fungi; Bakery products; Survival analysis

## 1. Introduction

Mould spoilage is a serious and costly problem for bakeries and use of preservatives is therefore an attractive means to diminish spoilage and insure food safety. However, consumers today are not in favour of additives as preservatives and an urge to reduce the quantities used exists within the bakery industry (Membre et al., 2001). Reduction of preservatives to sub-inhibitory levels has nevertheless been shown to stimulate growth of spoilage fungi in some cases (Magan and Lacey, 1986; Marin et al., 1999) or/and stimulate mycotoxin production (Yousef and Marth, 1981; Gareis et al., 1984; Bullerman, 1985).

Spoilage of bakery products is caused mainly by moulds and yeasts and occasionally by bacteria such as the rope-causing heat-resistant endospore-forming *Ba-*

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*cillus subtilis* (Earle and Putt, 1984). Mould spores are killed in the baking process (Knight and Menlove, 1961), leaving after contamination to be the source of spoilage problems. Contaminants of wheat bread are mainly *Penicillium* species (90–100%), but *Clodosporium* and *Aspergillus* species also occur (Legan and Voysey, 1991), the latter especially in warmer climates. The most important mould species on bread are *Penicillium commune*, *Penicillium crustosum*, *Penicillium brevicompactum*, *Penicillium chrysogenum*, *Penicillium roqueforti*, *Aspergillus versicolor* and *Aspergillus sydowii* (Northolt et al., 1995). On rye bread *P. roqueforti* is the major contaminant (Hartog and Kuik, 1984; Spicher, 1985; Lund et al., 1996). In a four-year investigation of rye bread in Denmark *P. roqueforti* (27%), *Penicillium corylophilum* (20%) and *Eurotium* sp. (15%) (*Eurotium repens*, *Eurotium rubrum*) were identified as the most important species (Lund et al., 1996). Varieties of *P. roqueforti* have later been elevated to species; *Penicillium paneum* and *Penicillium carneum* (Boysen et al., 1996). On cakes with low water activity *Eurotium* sp., *Aspergillus* sp. and *Wallemia sebi* are expected spoilage organisms (Northolt et al., 1995). Yeast contaminants—also known as 'chalk moulds'—are most common on sliced bread, and on rye bread *Endomyces fibuliger* and *Hyphopichia burtonii* have been reported as dominant species (Spicher, 1984; Lund et al., 1996).

Propionic, sorbic and benzoic acids are among the most commonly used food preservatives. Propionic acid inhibits moulds and *Bacillus* spores, but not yeasts to the same extent, and has therefore been the traditional choice for bread preservation (Ponte and Tsen, 1987). Sorbic acid is considered to be more effective than propionic acid. It inhibits both moulds and yeasts, and is used in a broad variety of food products (Sofos and Busta, 1981), including fine bakery products, confectionary and bread. According to the European Parliament and Council Directive No. 95/2/EC, propionic and sorbic acid may be added to bakery wares in concentrations up to 3000 and 2000 ppm, respectively (European Union, 1995). Benzoic acid is used in many types of acidic food products, although it is mainly associated with fruit preservation. It is also used in combination with sorbic acid for confectionary and other types of products. Benzoic acid is allowed in concentrations of up to 1500 ppm (European Union, 1995).

The preservatives are often added as a salt of the acid because salts are more soluble in aqueous solution. The effectiveness of the preservatives is dependent on the pH of the product, as the antimicrobial effect of the undissociated acid is much stronger than the dissociated acid. The $pK_a$ values of propionic acid, sorbic acid and benzoic acid are 4.88, 4.76 and 4.18, respectively (Lück and Jager, 1995). Maximum pH for activity is around 6.0–6.5 for sorbate, 5.0–5.5 for propionate and 4.0–4.5 for benzoate (Liewen and Marth, 1985c).

The objective of this study was to investigate inhibition of spoilage organisms by preservatives (concentration 0–0.3%) at a range of water activity and pH levels representative of bakery products from sourdough-fermented rye bread to alkaline, relatively dry sponge cake types. Initially, rye bread conditions and propionate were examined more profoundly, and the effect on secondary metabolite production was also assessed. Subsequently, a screening experiment covering a wider range of bakery goods and weak acid preservatives was conducted and used for statistical modelling of 'spoilage-free time'.

## 2. Materials and methods

### 2.1. Organisms and preparation of inoculum

Three isolates of *P. roqueforti* (IBT 5309, IBT 5426, IBT 1887), one isolate of *P. brevicompactum* (IBT 13995), *P. corylophilum* (IBT 6978), *P. commune* (IBT 18708), *Eu. repens* (IBT 18000), *Eu. rubrum* (3.228) and the chalk mould/yeasts *H. burtonii* (IBT 604) and *En. fibuliger* (IBT 605) were used for the experiments. All originated from bakery products (bread and cakes) and were obtained from the culture collection at Biocentrum-DTU, Technical University of Denmark, except *Eu. rubrum*, which was kindly provided by the Food Technology Department of Lleida University, Spain.

Conidiated cultures were prepared by spreading silica-dried spores on Czapek Yeast autolysate extract Agar (Pitt, 1979) with modifications (Samson et al., 1995). Plates were incubated for 7 days at 25 °C in the dark, after which colonies were transferred to fresh media and reincubated for 7 days under the same conditions. Suspensions of spores for inoculation

were made as $10^6$–$10^7$ spores $ml^{-1}$ in double-distilled water with 0.5 agar and 0.5% Tween-80.

### 2.2. Media preparation

#### 2.2.1. Rye bread experiment

The 'rye bread medium' contained 2% (w/v) dried granulated rye bread (Q-Brød, Smørum, Denmark), 2% (w/v) agar (Oxoid, Basingstoke, UK) and calcium propionate (Merck, Damstadt, Germany) in concentrations of 0%, 0.003%, 0.03% and 0.3% (w/v) in double-distilled water. Water activity was adjusted to 0.94 and 0.97 with glycerol (Mallinckrodt J.T. Baker, Deventer, Holland). Additionally, 1% (w/v) $KH_2PO_4$, 0.3% (w/v) $(NH_4)_2SO_4$, 0.1% (v/v) trace metal solution (according to Samson et al., 2000) and 1% mineral solution (Samson, R.A., Hoekstra, E.S., Frisvad, J.C. and Filtenborg, O., 00) were added. The pH levels of 4.4 and 4.8 were adjusted with HCl and NaOH. All chemicals used were from Merck, unless otherwise stated in the text. Agar fractions of the media were autoclaved separately to avoid acid hydrolysis of the agar prior to mixing and pouring into petri dishes.

#### 2.2.2. Screening experiment

Media for the screening experiment was made by combining 2% (w/v) wheat flour (Campden, UK), 2% (w/v) agar (Oxoid) and preservatives in concentrations from 0.003% to 0.3% (w/v) according to the design (Table 1). The preservative added was either calcium propionate, potassium sorbate or sodium benzoate (Merck). pH ranged from 4.7 to 7.4 in the media (Table 1) and was set by McIlvaines citrate-phosphate buffer (Dawson et al., 1969) of 0.1 M citrate (Bie and Berntsen, Rødovre, Denmark) and 0.2 M $Na_2HPO_4$ (Merck) solutions. Water activity was adjusted from 0.80 to 0.95 with glycerol (Mallinckrodt J.T. Baker). For media with pH below 5, agar and a fraction of the phosphate solution were autoclaved in a separate mixture to avoid acid hydrolysis of the agar. The media were poured into petri dishes after autoclavation.

### 2.3. Inoculation and incubation

The prepared media were inoculated with spore suspension in three points with a needle, in replicates. The petri dishes were stored in micro-perforated polyethylene bags at 25 °C in the dark for up to 30 days. The micro-perforation of the bags allowed freely exchange of gasses while hindering drying out of the media.

### 2.4. Experimental design

#### 2.4.1. Rye bread experiment

The rye bread media experiment was designed as a full factorial design with 16 different media; two levels of pH (4.4; 4.8) and $a_w$ (0.94; 0.97); four levels of propionate (0%; 0.003%; 0.03%; 0.3%) and one centre point media at mean pH (4.6) and $a_w$ (0.95) with no propionate addition.

#### 2.4.2. Screening experiment

A factional factorial experimental design with water activity, pH, preservative concentration and preservative type was designed in the software programme for modelling and design MODDE 4.0 (Umetri, Umeå, Sweden). This expanded the wide area of different bakery products, ranging from dry alkaline sponge cakes to sourdough fermented moist acid rye bread. Thus, the resulting design was composed of two 'cubes'; one in low $a_w$ and high pH area, and one in high $a_w$ and low pH area. The preservatives were varied across the whole of this area. Thirty-six different media compositions, including one centre point medium in triplicate, formed the design (Table 1).

### 2.5. Colony measurements

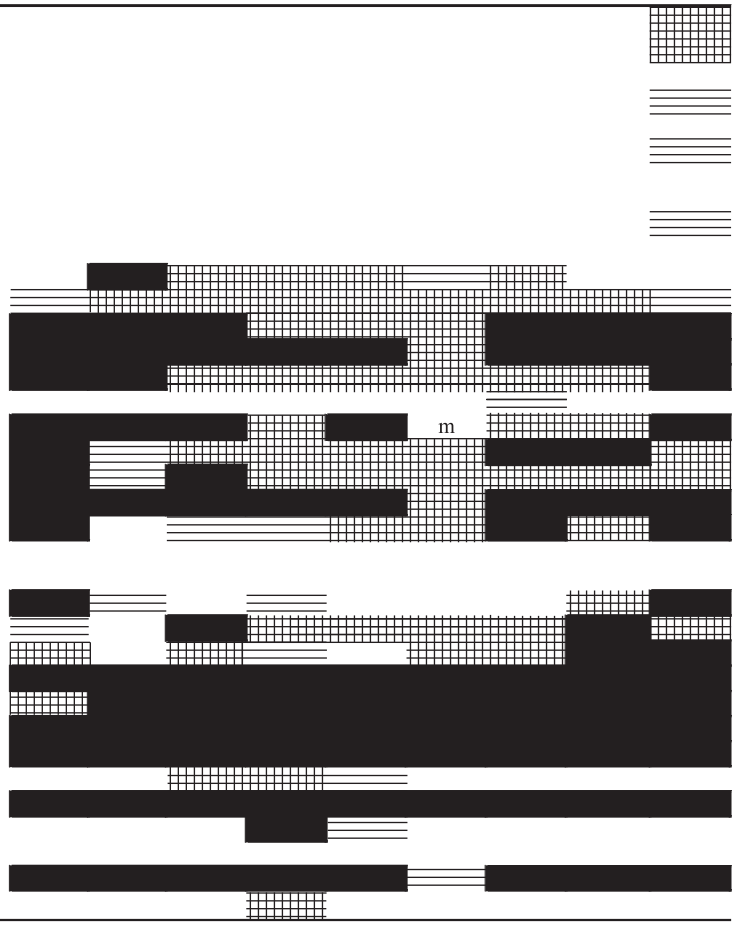
The colony diameter was recorded (in millimetres) and the mean of six colony measurements was used for data analysis. For the full factorial rye bread experiment, colonies were measured after 1, 2, 3, 4, 7 and 15 days of incubation. For the screening experiment, measurements were taken at 2, 3, 5, 6, 7, 9, 11, 13, 16, 20, 25 and 30 days.

### 2.6. Extraction and analysis of metabolites

Extracts were prepared according to the methodology of Smedsgaard (1997). Three plugs of 6 mm diameter per fungus (= approximately 85 $mm^2$ of surface) were extracted ultrasonically with 500 μl ethylacetate added 0.5% formic acid. The superna-

Table 1
Design of screening experiment and quantitative responses

| Media | $a_w$ | Conc.% | pH | Preservative | Censoring/growth[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | *E.fib* | *H.bur* | *P.ro1* | *P.ro2* | *P.ro3* | *P.bre* | *P.cor* | *P.com* | *E.rep* |
| N5 | 0.80 | 0.003 | 6.0 | Propionate | | | | | | | | | |
| N14 | 0.80 | 0.003 | 6.0 | Sorbate | | | | | | | | | |
| N2 | 0.80 | 0.003 | 7.4 | Propionate | | | | | | | | | |
| N25 | 0.80 | 0.003 | 7.4 | Benzoate | | | | | | | | | |
| N32 | 0.80 | 0.03 | 6.0 | Benzoate | | | | | | | | | |
| N21 | 0.80 | 0.03 | 7.4 | Sorbate | | | | | | | | | |
| N6 | 0.80 | 0.3 | 6.0 | Propionate | | | | | | | | | |
| N16 | 0.80 | 0.3 | 6.0 | Sorbate | | | | | | | | | |
| N4 | 0.80 | 0.3 | 7.4 | Propionate | | | | | | | | | |
| N27 | 0.80 | 0.3 | 7.4 | Benzoate | | | | | | | | | |
| N13 | 0.88 | 0.003 | 4.8 | Sorbate | | | | | | | | | |
| N28 | 0.88 | 0.003 | 4.8 | Benzoate | | | | | | | | | |
| N11 | 0.88 | 0.003 | 6.0 | Propionate | | | | | | | | | |
| N18 | 0.88 | 0.003 | 7.4 | Sorbate | | | | | | | | | |
| N30 | 0.88 | 0.003 | 7.4 | Benzoate | | | | | | | | | |
| N9 | 0.88 | 0.03 | 4.8 | Propionate | | | | | | | | | |
| N23 | 0.88 | 0.03 | 6.0 | Sorbate | | | | | | m | | | |
| N34[b] | 0.88 | 0.03 | 6.0 | Benzoate | | | | | | | | | |
| N35[b] | 0.88 | 0.03 | 6.0 | Benzoate | | | | | | | | | |
| N36[b] | 0.88 | 0.03 | 6.0 | Benzoate | | | | | | | | | |
| N10 | 0.88 | 0.03 | 7.4 | Propionate | | | | | | | | | |
| N15 | 0.88 | 0.3 | 4.8 | Sorbate | | | | | | | | | |
| N29 | 0.88 | 0.3 | 4.8 | Benzoate | | | | | | | | | |
| N12 | 0.88 | 0.3 | 6.0 | Propionate | | | | | | | | | |
| N20 | 0.88 | 0.3 | 7.4 | Sorbate | | | | | | | | | |
| N31 | 0.88 | 0.3 | 7.4 | Benzoate | | | | | | | | | |
| N1 | 0.95 | 0.003 | 4.8 | Propionate | | | | | | | | | |
| N24 | 0.95 | 0.003 | 4.8 | Benzoate | | | | | | | | | |
| N7 | 0.95 | 0.003 | 6.0 | Propionate | | | | | | | | | |
| N17 | 0.95 | 0.003 | 6.0 | Sorbate | | | | | | | | | |
| N22 | 0.95 | 0.03 | 4.8 | Sorbate | | | | | | | | | |
| N33 | 0.95 | 0.03 | 6.0 | Benzoate | | | | | | | | | |
| N3 | 0.95 | 0.3 | 4.8 | Propionate | | | | | | | | | |
| N26 | 0.95 | 0.3 | 4.8 | Benzoate | | | | | | | | | |
| N8 | 0.95 | 0.3 | 6.0 | Propionate | | | | | | | | | |
| N19 | 0.95 | 0.3 | 6.0 | Sorbate | | | | | | | | | |



Abbreviations: Conc., Concentration; *E.fib, En. fibuliger; H.bur, H. burtonii; P.rol, P. roqueforti* IBT 5309; *P.ro2, P. roqueforti* IBT 5426; *P.ro3, P. roqueforti* IBT 18687; *P.bre, P. brevicompactum; P.cor, P. corylophilum; P.com, P. commune; E.rep, Eu. repens.*

[a] Censoring/growth according to: : Censored value (no visible growth at day 30).
: Last day with no visible growth day 16 – 30.
: Last day with no visible growth day 7 – 15.
: Last day with no visible growth day 0 – 6.
**m** : Missing data.

[b] Centre points of the design. (*E. rubrum* showed no growth on two of the centre points and was subsequently removed from the analysis – data not shown).

tant was removed and dried on a rotary vacuum concentrator until dryness. Extraction of the plugs was repeated with iso-propanol as solvent, and this supernatant was united with the first extract and evaporated until dry. The residues were re-dissolved ultrasonically for approximately 10 min in 400 μl methanol, and filtered through 0.45 μm Minisart RC4 filters (Sartorius, Germany) into clean vials

before analysis. The extracts were analysed by reverse phase high-performance liquid chromatography (HPLC) using 10-μl injections on a HP1090M series II liquid chromatograph (Hewlett Packard, Germany) with a built-in diode array detector measuring full UV-VIS spectra (200–600 nm), twice per second, with a bandwidth of 4 nm. The column was a 100 × 4 mm HP Hypersil BDS-$C_{18}$ (3-μm particles) (Hewlett-Packard, USA) including a 4 × 4 mm guard column. The gradient ran from 15.0% acetonitrile in water to 100% acetonitrile and contained 0.005% trifluoracetic acid. It was held at a flow rate of 1 ml/min. All chemicals used were analytical grade from Merck and all water used was double distilled.

Metabolites were quantified by peak area (milli-absorption units (mAu) × s) at 210 nm. Retention indices (RIs) of fungal metabolites were calculated according to Frisvad and Thrane (1987), and identification was based on comparison of UV spectra and RI with in-house library.

### 2.7. Statistical analysis

#### 2.7.1. Data analysis of rye bread experiment

Data were analysed by partial least squares (PLS) regression using Unscrambler 7.6 SR-1 (Camo ASA, Norway). Wold et al. (1984) have described the statistical principle of the method. X matrix was the design variable, and the response variable, *Y*, indicates colony diameters for all measured days for all fungi. All variables were standardized by auto-scaling prior to statistical analysis (subtraction of the mean and division of the variance).

Metabolite data was expressed as the logarithm of peak area. Student's *t* tests were used for testing significant effects on metabolite production.

#### 2.7.2. Data analysis of screening experiment

Survival analysis methodology was applied in S-Plus 6.0.2 for Windows, Release 1 (Insightful, USA). Survival analysis is concerned with distribution of 'lifetimes' for a group or several groups of individuals for which there is a defined point event in time (as death, failure or in this case 'visible growth') that can occur only once for each individual. The major distinguishing feature of survival analysis is censoring. In this case, it will take place if mould growth was not observed within the timeframe of the trial period (day 30). The parametric hazards model with Weibull distribution was employed:

$$h(t) = \lambda^{\alpha}\alpha t^{\alpha-1} = \alpha t^{\alpha-1}\exp(\alpha\beta^{T}x)$$

where $h(t)$: hazard (probability of no growth at time $t$); $\lambda$: reference value of hazard; $\alpha$: 1/scale; $t$: time (days); $\beta$: transposed vector of parameters ($a_w$, pH, preservative type, preservative concentration); $x$: covariate vector. The event modelled was 'spoilage-free time', defined as the last day, wherein no visible growth was observed. Preservatives are known to act inhibitory, extending lag phase, rather than killing spoilage organisms (Lambert and Stratford, 1999). Thus, the 'hazard'/'spoilage-free time' in this model expressed how long the inhibitory effect would last at the given parameters. The parameters of the model was as listed above the factors of the experiment. For a thorough description of survival analysis theory and model building, see Cox and Oakes (1984).

## 3. Results

### 3.1. Rye bread experiment

The three *P. roqueforti* isolates and *Eu. rubrum* were the only fungi that grew on all media in the rye bread experiment. The remainder of the tested fungi were not able to grow at the highest concentration of propionate (0.3%), or were strongly inhibited at high $a_w$ (0.97) and high pH (4.8) at this concentration. Fig. 1 shows growth (colony diameter) on the media at day 15.

PLS regression analysis showed that propionate concentration was the most important factor for growth for all tested fungi, except for the *P. roqueforti* isolates, which were more or equally sensitive to $a_w$. Overall, higher $a_w$ enhanced growth of the fungi, with the exception of the *Eurotium* species, and pH had only minor effect within the range tested. Propionate levels below 0.3% had poor growth inhibiting effects (Fig. 1). The *Penicillium* ssp. even showed stimulated growth at some intermediate propionate concentration treatments. All three *P. roqueforti* isolates were stimulated at high $a_w$ (0.97) and high propionate concentrations (Fig. 1), and this positive interaction effect of $a_w$ and propionate for *P. roqueforti* isolates was significant (by jack-knifing in the PLS regression

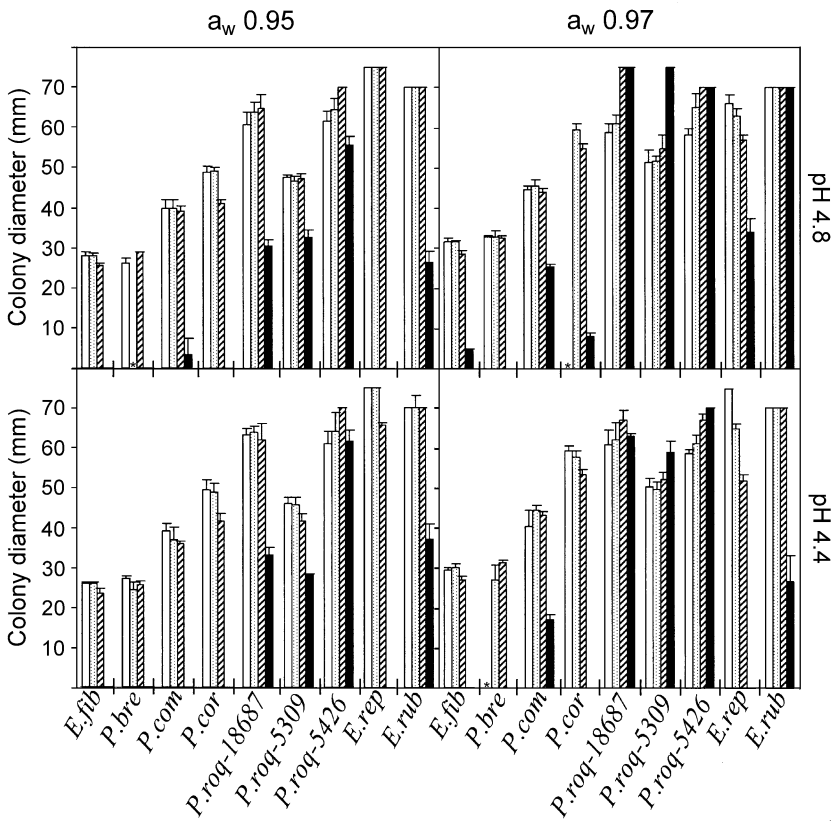



Fig. 1. Colony diameter (mm) day 15 at water activity ($a_w$) 0.95 and 0.97, pH levels 4.4 and 4.8 and propionate concentrations (□) 0%, (▨) 0.003%, (▨) 0.03% and (■) 0.3%. *E.fib*: *En. fibuliger*; *P.bre*: *P. brevicompactum*; *P.com*: *P. commune*; *P.cor*: *P. corylophilum*; *P.roq-18687*: *P. roqueforti IBT 18687*; *P.roq-5309*: *P. roqueforti IBT 5309*; *P.roq-5426*: *P. roqueforti IBT 5426*; *E.rep*: *Eu. repens*; *E.rub*: *Eu. rubrum*. (*) Missing data.

model). It should be noted, however, that the stimulation of growth occurred relatively late in the growth phase, as the lag phase was prolonged approximately one week by high propionate (0.3%) as illustrated in Fig. 2. The other moulds, represented by *P. corylophilum* in Fig. 2 only showed small (non-significant) growth stimulation at very low concentrations of propionate ($\leq 0.003\%$).

### 3.1.1. Effect of calcium propionate, pH and $a_w$ on metabolite production

Roquefortine C and PR-toxin was not produced in detectable levels by the *P. roqueforti* isolates on the media, but mycophenolic acid was produced consistently by two isolates (IBT 5309 and IBT 18687) and more scarcely by the third (IBT 5426). *P. brevicompactum* also produced mycophenolic acid, and *P. commune* produced rugulovasine. No other toxic metabolites were identified among the secondary metabolites produced by the penicillia. Echinulin and flavoglaucin and other flavoglaucin family compounds were among the metabolites identified in the *Eurotium* extracts.

*P. roqueforti* generally produced around 10% more mycophenolic acid at $a_w$ 0.97 as compared to $a_w$ 0.95 ($P = 0.5$ for isolate 5309 and $P < 0.001$ for isolate 18687 by $t$ test). The pH did not significantly affect production. At $a_w$ 0.95, the highest propionate concentration (0.3%) affected mycophenolic acid production by either reducing it (Fig. 3a, isolate 18687) or stimulating it (Fig. 3b, isolate 5309). Growth was also reduced by this concentration of propionate in both cases. This phenomenon was not seen at $a_w$ 0.97 (data not shown).

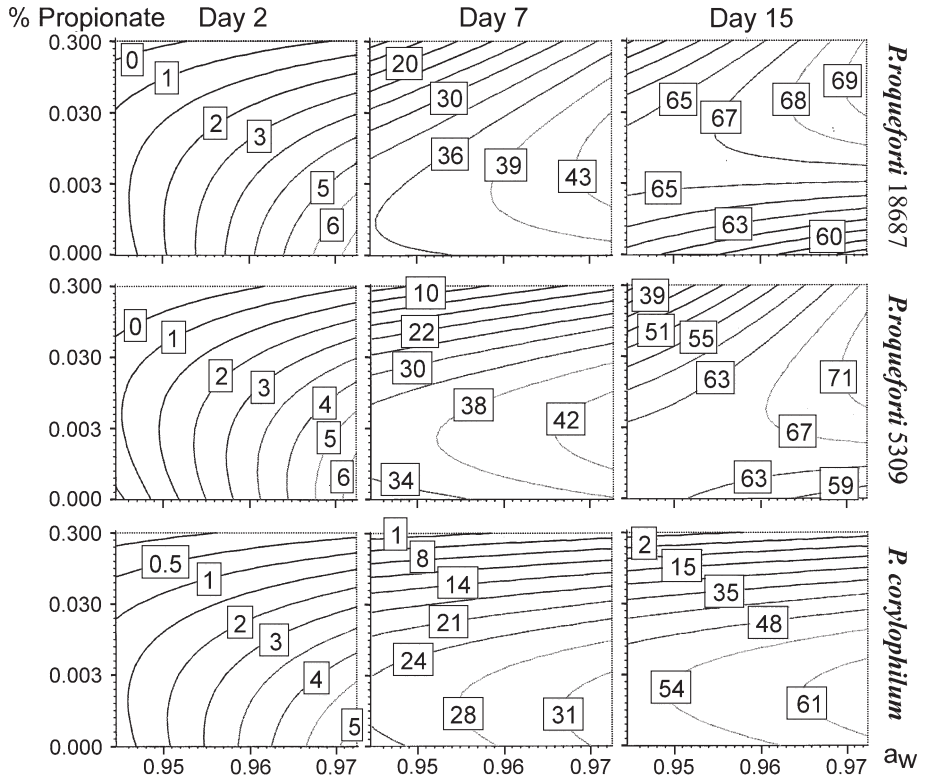



Fig. 2. Colony diameter (mm) modelled with PLS regression days 2, 7 and 15 at propionate concentration 0–0.3% and water activity ($a_w$) 0.95–0.97 at pH 4.6. PLS model for *P. roqueforti* isolates explained 89% of the variation with significant terms; propionate, propionate$^2$, propionate × $a_w$ and $a_w$. PLS model for *P. corylophilum*—a model for all penicillia except *P. roqueforti*—explained 95% of the variation with $a_w$, propionate and propionate$^2$ as significant terms.

Mycophenolic acid production by *P. brevicompactum* was also higher (on average 8%) at $a_w$ 0.97 than at 0.95. Higher pH seemed to induce higher production (on average 4% at pH 4.8 compared to 4.4) except for the media with no propionate added. The same tendency, enhanced production at higher pH, was seen for a terpene metabolite (RI = 1024) produced by *P. corylophilum* in all media containing propionate.

Rugulovasine production by *P. commune* was not significantly influenced by propionate concentration, $a_w$ or pH, but media containing 0.3% propionate was not included in the analysis due to absence of growth at low $a_w$ and poor growth at high $a_w$.

Production of echinulin and flavoglaucin by *Eu. repens* and *Eu. rubrum* was not dependent on water activity and pH of the media. *Eu. repens* did not grow on media containing 0.3% propionate, with the exception of media with high pH (4.8) and $a_w$ 0.97. Growth decreased with increasing propionate concentrations whereas the opposite was the case of metabolite production (data not shown). Growth of *Eu. rubrum* was inhibited by 0.3% propionate, although the effect was lost at high pH (4.8) and high $a_w$ (0.97) (Fig. 4). Metabolite production was significantly higher on media containing 0.3% propionate compared to media with lower concentrations as shown for echinulin and flavoglaucin (Fig. 4).

### 3.2. Modelling spoilage-free time on wheat flour agar (screening experiment)

Water activity was the most important factor controlling growth in the screening experiment. Media with $a_w$ 0.80 did not support mould growth in the 30-day observation period with the exception of the *Eurotium* isolates. Censored data (i.e., media which

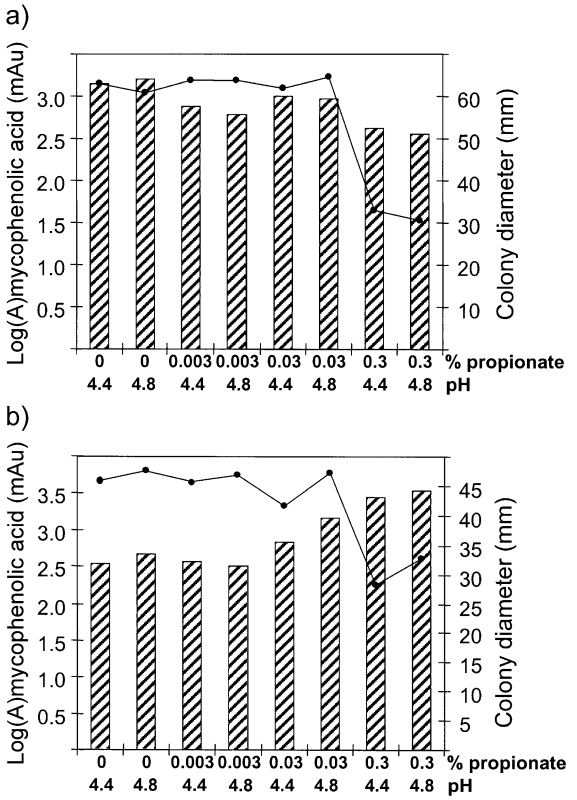



Fig. 3. Production of mycophenolic acid and colony diameter (mm) day 15 of *P. roqueforti*. (a) Isolate IBT 18687 and (b) isolate IBT 5309. (▨) Log(A) mycophenolic acid; (—●—) colony diameter, mm.

showed no mould growth for 30 days) were also frequent at the higher $a_w$ levels at low pH (4.7) when preservatives were added in high concentration (0.3%). The design of the screening experiment and the corresponding responses of the fungi are shown in Table 1. One of the *Eurotium* species, *Eu. rubrum*, was omitted from the analysis because of unacceptable high variation. The shortest time to spoilage was observed at the highest $a_w$ (0.95). Here, growth was detected within 1 week, except for media with high preservative concentration at low pH. The response used for survival analysis was the exact day of first visible growth and not the levelled responses shown in Table 1. ANOVA testing of the terms used in the parametric hazard function is shown in Table 2. It showed no statistically significant difference between the tested fungi. All other terms were significant. The hazard function obtained distinguished benzoate from sorbate ($P<0.01$), while propionate differed even more from both of them ($P<0.001$). With respect to the concentration of preservatives, 0.3% differed significantly from 0.003% ($P<0.001$) and 0.03% ($P<0.01$), whereas the difference between the effect of 0.003% and 0.03% was not significant.

Numerically, residuals of the model were on average 1.9 days measured as observed minus predicted value; 38% of the samples had residual values $<1$ day and 72% had values $<3$ days.

Preservative concentrations of 0.003% showed little efficacy, and the effect of pH was also less notable

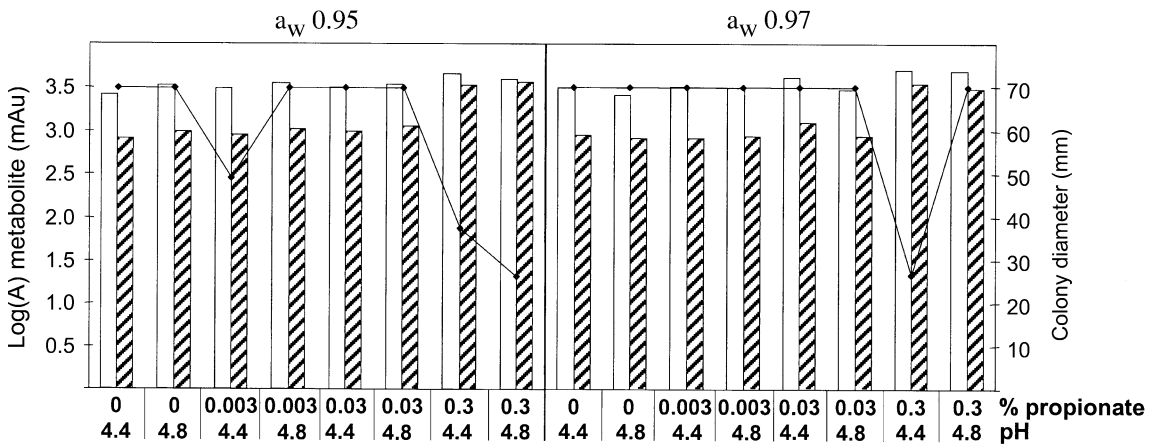



Fig. 4. Production of echinulin, flavoglaucin, and colony diameter (mm) day 15 of *Eu. rubrum*; (▭) echinulin; (▨) flavoglaucin; (—◆—) colony diameter, mm.

Table 2
ANOVA analysis of the terms used for the hazard function modelling

| Term | *df* | $P(\chi)$ |
|---|---|---|
| $a_w$ | 3 | <0.001 |
| $a_w^2$ | 4 | <0.001 |
| pH | 5 | <0.001 |
| $pH^2$ | 6 | <0.001 |
| Preservative concentration | 8 | <0.001 |
| Preservative type | 10 | 0.02 |
| Fungi type | 18 | 0.19 |
| pH × preservative concentration | 20 | <0.001 |
| $a_w$ × preservative concentration | 22 | 0.013 |
| pH × $a_w$ | 23 | 0.049 |
| Fungi × $a_w$ | 31 | <0.001 |
| Fungi × pH | 39 | 0.003 |
| pH × preservative type | 41 | <0.001 |
| Preservative type × concentration | 45 | <0.001 |
| Fungi × preservative concentration | 61 | <0.001 |
| $a_w$ × pH × preservative concentration | 61 | 0.013 |

at this concentration. A decrease in the inhibitory effect was evident with an increase in pH in media containing 0.03% and 0.3% preservative, regardless of the $a_w$. Propionate was not as effective as sorbate and benzoate (Fig. 5). The inhibitory effect of low $a_w$ was also clear, e.g., spoilage-free time of 0.3% propionate at pH 6 was on average 29.5 ± 16.1 days at $a_w$ 0.88 but reduced to 3.5 ± 2.6 days at $a_w$ 0.95 . Neither sorbate nor benzoate at 0.3% allowed growth at pH 4.7 within a realistic time frame. At pH 6, the spoilage-free times were 38–259 days for 0.3% sorbate at $a_w$ 0.95. However, *P. roqueforti* isolates and *P. commune* showed increased tolerance compared to the other moulds with spoilage-free times of less than 60 days. These isolates were also the most tolerant towards propionate at 0.3% and pH 4.7 $a_w$ 0.95 (spoilage-free times less than 25 days). The inhibitory effect of sorbate and benzoate was drastically reduced at pH 7.4, e.g., average spoilage-free time was 17.9 ± 11.5 days at $a_w$ 0.88 and 0.3% sorbate and benzoate.

At the lowest $a_w$ level (0.80), the only spoilage organism capable of visible growth before day 30 was *Eu. repens*. Preservatives were inhibitory at levels higher than 0.003% at pH 6, but the effect was strongly reduced at pH 7.4.The tendency of propionate being

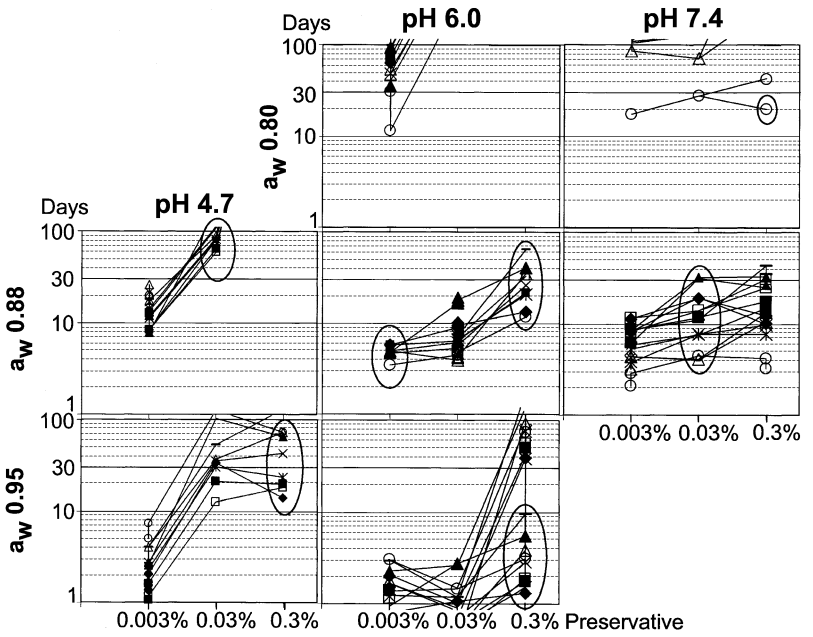



Fig. 5. Predicted 'spoilage-free time' (days) according to the hazard model at preservative concentration 0.003–0.3%, water activity ($a_w$) 0.80–0.95 and pH levels 4.7–7.4. Predicted observations marked with bold circle (⓪) are propionate treatments, 'un-circled' are sorbate or benzoate. (—○—) *Eu. repens*; (—▲—) *H. burtonii*; (—△—) *En. fibuliger*; (—✳—) *P. commune*; (—▬—) *P. brevicompactum*; (—✕—) *P. corylophilum*; (—□—) *P. roqueforti* IBT 18687; (—■—) *P. roqueforti*, IBT 5309; (—◆—) *P. roqueforti* IBT 5426.

less effective than the two other preservatives at high levels was also seen at $a_w$ 0.80, and in particular for other moulds than *Eu. repens*, although their spoilage-free times were highly theoretical, i.e., more than 210 days at this $a_w$.

## 4. Discussion

The simulated rye bread conditions showed that high propionate concentration (0.3%) generally had a strong inhibitory effect on all fungi tested, but after an increased lag phase the *P. roqueforti* isolates emerged with stimulated growth (Fig. 2), which furthermore was enhanced at higher $a_w$. The inhibitory effect of high propionate level was decreased at high $a_w$ and high pH for the majority of the remaining spoilage fungi. Due to the spoilage organisms' initial physiological and metabolic requisites from living in an acid (rye bread) environment, their abilities to adapt and even circumvent weak acid preservatives as propionate could have been expected. Thus, some authors have also linked *P. roqueforti* in particular with rye bread added propionic acid and/or sorbic acid (Hartog and Kuik, 1984).

The production of toxic secondary metabolites by *P. roqueforti* growing on rye bread agar was limited to mycophenolic acid. Mycotoxins (citrinin, aflatoxins, ochratoxin, penicillic acid) have been isolated from inoculated bread (Reiss, 1981, 1988), but surveys of mycotoxin content in naturally moulded bread have not been alarming (Osborne, 1980; Legan, 1993). Health hazards of mycotoxins in bread can therefore not be regarded as threatening in developed countries—unless the mycotoxins are already present in the flour used for baking. In developing countries, however, where people do eat mouldy bread, it represents a true risk (Brun et al., 1989).

The three *P. roqueforti* isolates showed a somewhat different behaviour in regard to growth and secondary metabolite production. Mycophenolic acid production by isolate IBT 5309 was enhanced at higher propionate concentration in media at $a_w$ 0.95, whereas isolate IBT 18687 had inhibited production (Fig. 3). The production of mycophenolic acid by IBT 5426 was not as consistent on all media as the two other isolates, perhaps due to degradation or transformation to other compounds by this the fastest growing isolate. Conflicting results were also found in the literature where some authors find that preservatives at sub-inhibitory levels stimulate mycotoxin production (Yousef and Marth, 1981; Gareis et al., 1984; Bullerman, 1985), whereas the opposite—inhibition—have been reported by others (Ghosh and Hagg-blom, 1985; Liewen and Marth, 1985b; Skrinjar et al., 1995; Combina et al., 1999). Thus, the mechanisms of regulation seem to be complex and not easily generalised, and most probably species, media, and concentration dependent. The *Eurotium* species showed a tendency of stimulated production of echinulin and flavoglaucin family compounds at inhibited growth conditions by high propionate concentration (Fig. 4). Further studies are necessary before firm conclusions about the effect of preservatives on metabolite production can be drawn.

The advantage of using survival analysis techniques is that censored data can be modelled, which was most appropriate for this study. Even though the screening experiment extensively expanded $a_w$ and pH area of bakery products and with only relatively few data points, an acceptable model with low residual values was obtained. The spoilage-free time modelled for propionate in the 'rye bread region' (high $a_w$–low pH) was in accordance with the results obtained in the first experiment—with a notable exception of *P. roqueforti* isolates. These isolates occurred approximately one week earlier on the rye bread media than predicted by the hazard function on wheat flour agar. This inaccuracy is most probably due to differences of media—with *P. roqueforti* having strong affinity with rye bread—as well as the relatively poor fungi differentiation ability shown by the hazard model.

The screening experiment confirmed that *Eurotium* species represent the potential spoilage organisms at low water activity levels ($a_w$ 0.80). This is in accordance with investigation of such products, e.g., cakes (Seiler, 1988). Growth of *Eurotium* was inhibited >300 days at pH 6 and $\geq$0.03% preservative, but the inhibitory effect was lost when pH was raised to 7.4 (Fig. 5). Thus, addition of preservatives to dry cake types can be inefficient, as values reaching pH 8.8 have been reported from, e.g., Spanish sponge cakes (Guynot et al., 2002).

The decrease in spoilage-free times observed and modelled at higher $a_w$ values was expected, as most moulds have optimum for growth near $a_w$ 1 (except strains of *A. glaucus* group (e.g., *Eurotium*), which have optimum near $a_w$ 0.9) (Corry, 1987). Enhanced

efficacy of the preservatives at lower pH was also expected, as the inhibitory effect of these weak acids resides mainly in the undissociated form entering the cell of the microorganism (Gould, 2000), albeit the dissociated form also shows inhibitory—but less—effect (Eklund, 1983, 1985). At pH 6 will only 7% of the propionic acid be undissociated, compared to 71% at pH 4.5. Growth of all fungi appeared $<30$ days at 0.3% propionate, pH 6 and $a_w$ 0.95 (Table 1—media N8), but not at pH 4.7 (Table 1—media N3). A striking feature was the *P. roqueforti* isolates' ability to grow at high preservative levels and low pH when $a_w$ was high (0.95) (Table 1—media N3, N19, N22). This trait was also modelled for *P. commune* with propionate to some extend (Fig. 5—$a_w$ 0.95, pH 4.7, 0.3%), and in accordance with the observations from the rye bread media experiment. Sorbate resistant isolates of *P. roqueforti* and other *Penicillium* species have been isolated from sorbate-treated cheeses where they were able to metabolise sorbate and grow in the presence of 9000 ppm in YM broth (Liewen and Marth, 1985a). Thus, the variety between isolates of the same species and the ability of environmental adaptation should not be underestimated.

Generally, propionate was the weakest acting preservative (Fig. 5) in accordance with results from other studies (Brachfeld, 1969; Razavi-Rohani and Griffiths, 1999). In a study of *P. brevicompactum* and sorbic acid, propionic acid and sodium benzoate at $a_w$ 0.90, no difference between propionic and sorbic acid was found (both applied at 0.2%) and benzoate was found to be less effective (at 0.05%) (Membre et al., 2001). In the present study, propionate was generally less effective than sorbate and benzoate, and the difference between the latter two was vague. Thus, differences in media composition and the concentrations applied are of major importance when testing the efficacy of preservatives.

The lack of effect by preservatives at 0.003% dosages has also been found by others in studies with wheat flour agar (Guynot et al., 2002).

To conclude, it was found that water activity levels and pH values are of paramount importance for the efficacy of preservatives in bakery products. Preservation of rye bread with weak acid preservatives as the traditionally used calcium propionate could not be recommendable in the long run, as an extended lag phase was followed by stimulated growth by the major spoilage organism *P. roqueforti*. This could lead to development of resistant isolates in the production environment. Other measures as good hygiene in the bakeries and if necessary complementary post packaging heat treatments or modified atmosphere packaging are better alternatives.

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