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The application of growth-no growth models to directly assess the stability of wholemeal multigrain bread towards *Penicillium paneum* LMQA-002 and *Paecilomyces variotii* LMQA-001

Juliana Lane Paixão dos Santos[a,b], Beatriz S. Silva[a], Marianna M. Furtado[a], Letícia L.P. Morassi[a], An Vermeulen[b], Anderson S. Sant'Ana[a,*]

[a] Department of Food Science, Faculty of Food Engineering, University of Campinas. Campinas, SP, Brazil
[b] Laboratory of Food Microbiology and Food Preservation, Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University, Coupure links 653, B-9000, Ghent, Belgium

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ABSTRACT

This study aimed to develop predictive models to assess the growth response of *Pencillium paneum* LMQA-002 and *Paecilomyces variotii* LMQA-001 in wholemeal multigrain bread regarding moisture, pH, calcium propionate, temperature and time of storage. A complete factorial design was performed using slices of bread with different formulation: moisture (36; 38; 40; 42%), pH (5.1; 5.3; 5.5) and calcium propionate (0.6; 0.8 and 1%). The slices were inoculated with 10^2-10^3 spores following storage at 20 °C, 25 °C and 30 °C for up to 21 days. The degree of agreement between predictions and observations was > 90% for both species. According to the results, the growth of *P. paneum* LMQA-002 and *P. variotii* LMQA-001 was inhibited for up to 21 days in bread formulations presenting pH (5.1), 36% moisture and 0.6% calcium propionate. The lower temperatures (20 °C and 25 °C) contributed to the growth of *P. paneum* LMQA-002 and storage at 30 °C favored the growth of *P. variotii* LMQA-001. Validation results showed correct predictions in 80% of the cases. A significant achievement of this study comprises the extension of wholemeal multigrain bread shelf-life from 12 to 21 days (> 70% increase).

1. Introduction

Bread is prepared by mixing flour dough, water, yeast and other ingredients, following fermentation and baking (Young & Cauvain, 2007). Due to their intrinsic factors (high carbohydrate content and slightly low pH), these products are highly susceptible to spoilage by filamentous fungi, leading to economic losses (Legan, 1993; Smith, Daifas, El-Khoury, Koukoutsis, & El-Khoury, 2004). Although different fungal species are associated to bread spoilage, some of them are also resistant to hurdles used by the food industry, such as propionates and sorbates. Therefore, these fungi represent a threat for the microbial stability of bakery products such as breads.

Current consumer demands for healthier (Ronteltap, Sijtsema, Dagevos, & de Winter 2012), high-fiber products (Hellyer, Fraser, & Haddock-Fraser, 2014) and clean label trends (Asioli et al., 2017) have been driving changes in the bakery segment. For instance, the use of cereal grains and integral ingredients has grown despite the challenges faced by the bakery industries to control fungal growth during shelf-life, mainly by fungi belonging to the genera *Penicillium* sp. and *Aspergillus* sp. such as *P. paneum*, *P. roqueforti*, *P. polonicum*, *P. commune*, *P variotii* and *A. sydowii* (Santos et al., 2016; Suhr & Nielsen, 2004).

The extension of shelf-life of breads and bakery products has been targeted through the application of different approaches such as, addition of alternative additives/ingredients/enzymes, encapsulated essential oils, use of cell free supernatants of lactic acid bacteria, active packaging and modified atmosphere (Gonçalves et al., 2017; Janjarasskul, Tananuwong, Kongpensook, Tantratian, & Kokpol, 2016; Luz et al., 2017; Russo et al., 2017; Scarnato et al., 2017; Secchi et al., 2017). However, a few studies have used predictive models to assess the behavior of fungi isolated from spoiled bakery products (Stéphane Dagnas, Onno, & Membré, 2014; Stéphane Dagnas, Gauvry, Onno, & Membré, 2015; Stéphane Dagnas, Gougouli, Onno, Koutsoumanis, & Membré, 2017; Huchet et al., 2013). Nevertheless, most of them have studied fungal behavior in synthetic media and not specifically in a bakery matrix. The development of a predictive model in a target food may allow for uncertainty and variability associated with the behavior of fungi in that food to be taken into account in model's prediction.

Boundary models are a case of secondary predictive models through

* Corresponding author. Rua Monteiro Lobato, 80, CEP: 13083-862, Campinas, SP, Brazil.
E-mail address: and@unicamp.br (A.S. Sant'Ana).

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which the interfaces of growth-no growth of a microorganism are determined (Pérez-Rodríguez & Valero, 2013; Ratkowsky & Ross, 1995). The use of boundary models in the development of food formulations has as an underlying concept the study of a combination of different factors to inhibit microbial growth (Tapia de Daza, Alzamora, Chanes, & Gould, 1996), as preconized by the hurdle technology (L. Leistner, 1992; Leistner, 2000). In this study, probabilistic growth-no growth models for two fungal species (*Penicillium paneum* and *Paecilomyces variotii*) were developed and validated using data from growth response of both fungi on wholemeal multigrain bread as affected by pH, preservative concentration, moisture, temperature and storage time.

2. Material and methods

2.1. Strains and spore suspension

Strains of *Penicillium paneum* (LMQA-002) and *Paecilomyces variotii* (LMQA-001) isolated from wholemeal multigrain bread (Santos et al., 2016) and previously selected based on their faster growth rates compared to other fungal strains were used (Santos, Chaves, & Sant'Ana, 2017). *P. paneum* LMQA-002 and *P. variotii* LMQA-001 suspensions were prepared as previously described (Silva, Sant'Ana, & Massaguer, 2010; Delgado, Sant'Ana, Granato, & Massaguer, 2012).

2.2. Bread production

Multigrain wholemeal bread formulations were produced in a pilot plant. The basic formulation was composed of 40% whole wheat flour, 7% grain, 2% other flours and cereals, 36% water, 6% sugar, 6% yeast, 1% salt and 1% vegetable oil. For the production of bread formulations, the sponge method was used (Kulp, 2003), in which the sponge cake was allowed to rest at 30 °C for 2 h, following addition of the other ingredients (including the addition of calcium propionate, i.e., % of the flour, according to the desired formulation), beating for 20 min, partitioning, moulding, mass fermentation at 43 °C for 60 min. Baking was done at 220–240 °C for 15–25 min, according to the desired moisture, followed by cooling down (60 min), slicing and packaging. The desired values of moisture were achieved by adjusting the absorption (decrease and increase) of water during preparation of the dough. The pH values were obtained through formulation adjustments, using acidulants and acidity regulators as well as through process parameters related to the sponge/fermentation formation time.

2.3. Experimental planning

A complete factorial design was used to assess the effect of the following factors on the growth-no growth of *P. paneum* LMQA-002 and *P. variotii* LMQA-001 in wholemeal multigrain bread: pH (5.1, 5.3 and 5.5), moisture (36%, 38%, 40% and 42%), calcium propionate concentration (0.6, 0.8 and 1% – flour base), storage temperature (20 °C, 25 °C and 30 °C) and storage time (4, 8, 12 and 21 days). The parameters were confirmed one day after the bread production. A total of 36 different formulations, three temperatures, six replicates per formulation for each of the two fungi strains were analyzed for 21 days (twice the shelf life of wholemeal multigrain bread before the study was carried out).

2.4. Bread inoculation and storage

Slices of approximately 6 g of bread were aseptically transferred to sterile 60 × 15 cm Petri dishes (named plates A). Sets of three plates A were inserted into 140 × 15 cm Petri dishes (named plates B). The control of moisture was performed through the determination of initial and final values of a_w, by the analysis of 2–3 repetitions per formulation using an Aqualab CX-2 moisture meter (Decagon Devices, Pullman, USA). In order to maintain the specific a_w value of each formulation during the storage period, sterile hydrophilic cotton swabs soaked in water/glycerol solutions with moisture identical to the values found in the bread were added within plates B. Each bread slice was then centrally inoculated with 5 μl (10^2-10^3 spores) of standardized suspensions (1–7 × 10^5 spores/mL). The plates were hermetically sealed with Parafilm®, following storage at 20 °C, 25 °C and 30 °C for up to 21 days. Plates containing non-inoculated bread slices were used as controls.

2.5. Growth measurement and data analysis

The growth response modeled in this study was the time until the appearance of mycelium (colony diameter = 3 mm) in the set of bread slices of each studied condition (see 2.3). Values equal to 1 were attributed to growth and 0 to no-growth. Thus, in a set of six replicates, if three presented growth and three presented no-growth, the probability of fungal growth for this condition was 50%, and the data were expressed as follows: 3 replicates = 0, and 3 replicates = 1. These results were then used as input to the fit a logistic regression model to the study variables. The equation of the secondary logistic regression model including 13 terms is shown in Equation (1).

$$Logit\ (P) = \ln\left[\frac{P}{1-P}\right] = b_0 + b_1 T + b_2 Moist + b_3 pH + b_4\ prop + b_5 Moist^2 + b_6 TMoist + b_7 TpH + b_8 T\ prop + b_9 Moist\ pH + b_{10} Moist\ prop + b_{11} pHprop + b_{12} time \quad (1)$$

where P (t) is the probability (0–1) of growth-no growth of the fungus in relation to time, T is the storage temperature, *Moist* is the bread moisture, pH, the bread pH, *prop* is the concentration value (%) of calcium propionate, *time*, is storage time, and b_i, are the coefficients that will be estimated for each term.

The binary logistic regression model was fitted to the data using SPSS version 24 (SPSS, US). The selection of variables was performed in two blocks: the first block, enter, was used to include all the main effects (temperature, moisture, pH, propionate, and time) to the model. The second block selected, forward LR, was used to select the quadratic and interaction terms. These were chosen according to the degree of significance ρ = 0.05. The quadratic term was only obtained for the variable moisture due to the limitation in the number of levels studied within each variable. Once only three levels were investigated for the variables temperature, pH, and calcium propionate, it was not possible to obtain quadratic terms for these factors. The adjustment and performance of the models were then evaluated according to the degree of agreement (%) between observations and predictions, Nagelkerke R^2, receiver operating curve (ROC-curve) c-value statistics. The obtained models were then transformed into growth-no growth interface charts containing their respective contour curves for probabilities P = 0.1, 0.5 and 0.9 using Microsoft Excel 2016 Solver.

2.6. Validation of the predictive growth-no growth models developed in wholemeal multigrain bread

The models developed to predict growth-no growth of *P. paneum* LMQA-002 and *P. variotii* LMQA-001 were validated through a new set of experiments following the same protocols described from 2.1 to 2.4. The predictive power of the models was evaluated by comparing the observed growth-no growth probability responses with the responses predicted by the models.

3. Results

The growth-no growth responses of *P. paneum* LMQA-002 and *P. variotii* LMQA-001 could be characterized by a marked definition of the growth regions between 8 and 12 days of storage (Table 1).