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Using the gamma concept in modelling fungal growth: A case study on brioche-type products

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ABSTRACT

Fungi are common spoilers of intermediate moisture foods such as bakery products. Brioche are bakery products prone to fungal spoilage due to their pH (5.8–6.2) and water activity (a_w) (0.82–0.84). The aims of the present study were: (i) the identification of fungal species occurring in brioche products, (ii) the *in vitro* assessment of their growth potential, and (iii) the development of a validated growth model following the gamma concept. A total of 102 fungal strains were isolated, with *Penicillium* sp., *Cladosporium* sp., and *Aspergillus* sp. being the main genera, representing 90% of the isolates. Given the isolation frequency, any potential fungal prevalence throughout the bakery processs and/or the results of *in vitro* assessment of fungal growth potential under conditions mimicking brioche (pH, a_w, temperature), A*spergillus flavus, Aspergillus fumigatus*, and *Penicillium* sp. were selected for the development of the gamma model. According to *in vitro* validation, the model successfully predicted fungal growth, while on *in situ* experiments, the intrinsic parameters (a_w and/or level of used preservative) of brioche in combination with packaging conditions (modified atmosphere) did not allow fungal growth.

1. Introduction

Fungal spoilage is a major concern for the bakery industry. The primary source for fungal contamination is related to the high occurrence of molds in stored agricultural ingredients, which are used as raw materials (Biro et al., 2009; Chehri et al., 2010; Eglezos, 2010). Moreover, fungi have key characteristics such as adaptation and persistence in different environments, mechanisms for wide dispersion of spores, which render them capable of causing post-baking contamination. Thereafter, visible mycelia would occur on the products' surface consequently leading to rejection either during inspection by quality control or after distribution, by the consumer. Both cases are considered undesirable by the bakery industry as they cause significant economic losses due to product rejection or recalls (Melikoglu and Webb, 2013). These losses vary 1–5% of the products depending on season, type of product, method of processing, and/or potential reputation issues (Saranraj and Sivasakthivelan, 2015).

During the last years, sweet bakery products such as croissant or brioche-type products filled with various kind of creams or marmalades have become popular, especially to children. Their moderate water activity (a_w 0.82–0.84) classifies them among intermediate moisture foods (IMF) (a_w 0.65–0.90) (FAO, 2003), rendering them highly susceptible to fungal growth (Pitt and Hocking, 2009) (Table 1). Other factors like their pH (5.8–6.2) along with the fact that they are being distributed as products placed on the shelf, enhance the probability for fungal growth (Table 1). However, the peculiarity of these products is mainly focused on their complex macrostructure due to the presence of different ingredients i.e., flour, water, sweet or savory cream fillings, chocolate chips, dried fruits, marmalades (Osimani et al., 2017). In fact, the post-baking addition of some ingredients like cream fillings accentuates the need for research on the hygiene level and fungal proliferation during subsequent storage. The probability of mold spoilage may become greater due to food regulations of importing countries such as Middle East, where zero tolerance regarding alcohol is required, as well as the contemporary trend for clean label products, resulting in production of preservatives-free fillings or without (post-processing) surface spraying with alcohol for protection from fungi. Previous studies have reported that spoilage of sweet bakery products is mainly caused by *Penicillium* sp. (80–90% prevalence), *Aspergillus* sp. (especially in warm climates), *Eurotium*, and *Cladosporium* sp. (Abellana et al., 2001; De Clercq et al., 2014; Le Lay et al., 2016; Marín et al., 2002; Vytrasova et al., 2002). However, there are limited studies that

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Table 1
The technological characteristics of the three studied brioche products and their respective cream-fillings, as provided by the bakery industry.

	TYPE	a_w	pH	PRESERVATIVE	PACKAGING
CREAM-FILLINGS	Praline	0.78–0.81	6.1–6.3	Potassium sorbate	
	Biscuit	0.82–0.83	5.9–6.1		–
	Strawberry	0.78–0.79	3.2–3.4		
BRIOCHE	Praline	0.82–0.84	6.1–6.3	Calcium propionate (400 or 800 ppm)	10% O_2: 10% CO_2: 80% N_2
	Biscuit	0.82–0.84	5.9–6.1		
	Strawberry	0.81–0.83	4.7–4.8		

systematically assess the role of critical intrinsic or extrinsic food-related parameters on the shelf-life of cream-filled brioche or croissant-type products (Hozová et al., 2002).

Considering the above and given that in-factory contamination levels may remain low when good sanitary practices are applied, the probability of mold growth may be reduced by applying technological hurdles (i.e., pH, a_w, preservatives, temperature, moisture, modified atmosphere packaging-MAP) during the process, storage, and distribution supply chain steps (Akgün et al., 1997; Dagnas et al., 2014; Smith and Simpson, 1995; Suhr and Nielsen, 2004). Among the technological barriers, a_w, pH, and temperature have been reported as the most effective on inhibiting fungal growth on bakery products (Abellana et al., 1999, 2001; Guynot et al., 2003; Huang et al., 2010). In order to assess quantitatively the effect of the aforementioned factors on mold growth (growth rate and/or lag time), the use of predictive models is valuable, since they may provide the bakery industry a potential decision tool, capable of determining products' shelf-life. Among the existing secondary models, Gamma type models have been often used in order to assess the extent of fungal growth on several products (Dagnas and Membré, 2013). However, there are limited reports with regard to bakery products and especially multi-ingredient foodstuffs such as cream filled brioche-type products (Dagnas et al., 2014; Huchet et al., 2013). In addition, only a few of these models have been validated with observation data on real food matrices (Huchet et al., 2013).

Given the need to assess and quantify the risk of potential fungal growth on brioche-type products, a case study was performed to holistically approach the following objectives: (i) monitoring, isolation, and identification (*via* phenotypic and molecular characterization) of the risk (potential fungal growth) during an 18-month screening of raw materials and final products, (ii) *in vitro* growth potential of a representative number of isolated fungi as a function of a_w and storage temperature, (iii) quantifying the risk by modelling, following the gamma concept, the effect of abiotic factors (temperature, pH, and a_w) on growth parameters (growth rate and lag time) of three species belonging in *Aspergillus* and *Penicilium* genera, and (iv) validation of the developed model through *in vitro* (on laboratory media with adjusted a_w and pH to mimic the food product) and *in situ* (on commercially packaged brioche) experiments in order to provide the bakery industry with a useful validated tool.

2. Materials and methods

2.1. Isolation and identification of fungal strains

During an 18-month survey (from February 2015 until August 2016), 650 samples of raw materials and final products were provided by a commercial Greek bakery on a regular basis and were screened for putative fungi. The tested raw materials were ingredients such as flour, all three types of cream fillings, and enhancers obtained in bulk packages. With regard to the final products (packages of 75 g), two different approaches were followed: freshly produced brioche (filled with praline, biscuit cream or strawberry marmalade) were either tested for fungal presence upon their arrival at the laboratory or during storage at 20, 30, and 37 °C for 60-days (recommended shelf-life by the bakery industry at ambient temperature), resulting in extra 350 samples. The technological characteristics of the three studied brioche products and their respective cream fillings, as provided by the bakery industry, are shown on Table 1. With regard to the isolation media, Malt Extract Agar (MEA; LabM, Lancashire, UK) and Rose Bengal Chloramphenicol agar (RBC; LabM, Lancashire, UK) were used. It is well-known that Dichloran (18%) Glycerol Agar (DG-18) is recommended for the selection of fungi in food products with $a_w < 0.95$ (Hocking and Pitt, 1980; Samson et al., 1992). However, it was not selected as an isolation medium, based on reports which indicate that media with high a_w (i.e., PDA and RBC) and moderate a_w (DG-18) are generally equivalent for recovering total molds in similar to brioche IMF products (i.e., bread) (Taniwaki et al., 2001). Specifically, prior to sampling, raw materials or brioche were macroscopically examined for fungal growth. In case of visible fungal growth, hyphae or spores were taken with an inoculation loop and cultivated on MEA (known as general-purpose medium for detection and enumeration of fungi) at 25 °C for 7 days. On samples that appeared to be mould-free, microbiological analysis was performed as follows. Specifically, 10 g of each sample (collected from at least 3–4 different sites of the product) and 90 mL of sterile ¼ strength Ringer's solution (LabM, Lancashire, UK) were homogenized in a stomacher for 60 s (Interscience, France). Following homogenization, decimal dilutions in Ringers' solution were prepared and 0.1 mL of the appropriate dilution was spread on RBC followed by incubation at 25 °C for 5 days. Macroscopically different fungal colonies were then purified by transfer and streaking onto MEA using an inoculation loop, following incubation at 25 °C for 7 days. Afterwards, fungal spores were obtained by adding 10 mL sterile distilled water containing 0.1% Tween 80 on MEA and gently scratching the colony surface with a sterile spatula. Spores suspensions were filtered through sterile gauze to remove any remaining hyphae and were kept in 60% v/v glycerol at - 80 °C, giving rise to 102 isolates.

The isolates were first classified to genus level based on their phenotypical characteristics, namely macroscopic (colony diameter and color) as well as microscopic characteristics (Pitt and Hocking, 2009). Afterwards, molecular characterization was performed by sequencing the Internal Transcribed Spacer (ITS) region according to Schoch et al. (2012) and White et al. (1990). More specifically 10 μL of spore suspension was inoculated in petri dishes containing Malt Extract Broth, MEB (LabM, Lancashire, UK) and incubated at 25 °C for 48–72 h. Mycelia were then transferred to sterile filter paper in order to remove unwanted broth and finally placed in sterile 1.5 mL tubes. For DNA extraction, 500 μL of Lysis Buffer (400 mM Tris-HCl pH 8.0, 60 mM EDTA pH 8.0, 150 mM NaCl, 1% SDS) was added following incubation at room temperature for 10 min. Then, 150 μL potassium acetate (pH 4.8) were added, samples were briefly vortexed and then centrifuged at 10000 × g for 5 min. The supernatant was transferred to a new eppendorf tube and then centrifuged again at 10000 × g for 5 min. The supernatant was transferred to a new eppendorf tube and 1 V (volume) isopropyl alcohol was added. Samples were then mixed by inversion, incubated at room temperature for 5 min and centrifuged at 10000 × g for 5 min. The supernatant was then discarded, the pellet was washed with 300 μL ethanol (70% v/v) and centrifuged at 10000 × g for 5 min. Supernatant was discarded and the pellet was left to dry. The DNA pellet was finally dissolved in 50 μL water. 1–2 μL aliquots of the DNA suspension were then used as template in downstream polymerase chain reaction (PCR) in order to enhance ITS region using the primers ITS1 –ITS4 (White et al., 1990). PCR products were then analyzed in 1% w/v agarose gel, purified, and subsequently sequenced. Sanger sequencing was performed on the ITS amplicons using ITS1 as sequencing primer, while the identification of the fungal was performed using NCBI-BLAST by aligning the unknown sequences to the known sequence database.

2.2. In vitro determination of fungal growth potential in function with a_w and storage temperature

In order to determine the *in vitro* growth potential of the fungal isolates, 40 representative strains based on basic ingredient (praline, biscuit, and strawberry), sample type (final products: freshly produced or after storage; raw materials), and isolation frequency were selected (Fig. 2). MEA was used as basal medium. The pH was adjusted to pH 6.0 using NaOH (1 N), prior sterilization, while a_w of the medium was adjusted to 0.99 (optimum value for fungal growth) and 0.82 (corresponding to the lower level of a_w that can be encountered on the commercial product) by adding 0 and *ca.* 38% v/v glycerol, respectively. Reported pH and a_w values were verified after autoclaving using a digital pHmeter (pH 526, Metrohm Ltd, Switzerland) and a a_w-meter (Hydrolab rotronic, Basserdorf, Switzerland), respectively. The inoculated plates were sealed with Parafilm® and stored aerobically at 25 °C (*max.* recommended storage temperature by the bakery industry) and 37 °C (to simulate potential temperature abuse especially during summer). Fungal radial growth was recorded on daily basis. Growth curves were generated by plotting radial growth data against storage time, after which the radial growth rates were estimated by fitting the experimental data to the linear regression model.

2.3. Fungal strains and inoculum preparation

Three isolates, *Aspergillus flavus*, *Aspergillus fumigatus*, and *Penicillium* sp. were selected for modelling. The selection criteria were: i) the isolation frequency, ii) any potential fungal prevalence throughout the bakery process, and/or iii) the overall *in vitro* growth potential. Each isolate was inoculated on Potato Dextrose Agar – (PDA; LabM, Lancashire, UK) and subsequently incubated for 7 days at 25 °C. Fungal spores were obtained by adding 10 mL sterile distilled water containing 0.1% v/v Tween 80 and gently scratching the colony surface with a sterile spatula. Afterwards, spores suspension was filtered through sterile gauze to remove any remaining hyphae. The final spore concentration was determined at *ca.* 10^6–10^7 spores/mL by performing decimal serial dilutions followed by plating on MEA.

2.4. Inoculum preparation and determination of cardinal values

The cardinal growth parameters for the three aforementioned fungal isolates were determined (Rosso and Robinson, 2001) by modelling the kinetic growth parameters (growth rate, μ_{max}; lag phase, λ) as a function of T and a_w, following a gamma model approach. In order to determine the cardinal values (T_{min}, T_{max}, T_{opt}, a_{wmin}, a_{wmax}, a_{wopt}), PDA plates were spot inoculated with 10 μL of spore suspension of each fungus (initial inoculum: 10^3 spores) under aseptic conditions, sealed with Parafilm® to minimize potential media dehydration and incubated at the appropriate temperature. The different a_w levels were obtained by partially replacing the water in the media with glycerol (v/v), which is commonly used in bakery industry as a humectant. In order to assess the effect of temperature on fungal growth, PDA plates (pH 5.7 ± 0.2) with optimal a_w were incubated at 15, 20, 22, 25, 28, 30, 35, and 37 °C for *A. flavus* and *A. fumigatus*, while at 10, 15, 20, 22, 25, 28, 30, and 35 °C for *Penicillium* sp. The effect of a_w on the fungal growth was assessed at different levels of a_w (0.981, 0.958, 0.927, 0.885, 0.854, 0.839, 0.827, 0.809, 0.800, 0.778 adjusted by adding 0, 10, 20, 25, 30, 35, 40, 42, 45, 47, and 50% v/v glycerol, respectively) at the optimum temperature for growth of each fungus (*Aspergillus flavus*, 30 °C; *Aspergillus fumigatus*, 37 °C; *Penicillium* sp., 25 °C). Reported a_w values were verified after autoclaving using a a_w-meter. Fungal growth was monitored *via* measurement of colony diameter on a daily basis. A total of fifteen ($n = 15$) replicates were performed for every experimental condition, corresponding to three experimental reproductions (biological replicates) with five technical replicates within each.

2.5. Mathematical modelling

Growth curves were generated by plotting colony diameter against storage time. Growth kinetic parameters (growth rate, μ_{max}, cm/day; lag phase, λ, day) were determined by fitting Baranyi and Roberts (1994) equation to the experimental data using the excel-based tool DMFit version 2.1 (www.ifr.ac.uk/safety/DMfit). The cardinal growth parameters (T_{min}, T_{max}, T_{opt}, a_{wmin}, a_{wmax}, a_{wopt}), regarding temperature and a_w, for the three fungal strains were determined by fitting the secondary model of Rosso and Robinson (2001) to the growth kinetic parameters by using the Table Curve 2D software (Systat Software Inc., San Jose, CA, USA). For modelling purposes and in order to stabilize the variance between μ_{max} and λ, the inverse lag phase (1/λ) was used (Zwietering et al., 1994; Huchet et al., 2013). Regarding a_w, the higher value a_{wmax} was considered to be 1.0, which corresponds to pure water.

In order to model the effect of additional extrinsic (T, CO_2) and intrinsic parameters (a_w and calcium propionate) on the μ_{max} and λ of the isolates, the gamma concept approach was used (Le Marc et al., 2002; Mejlholm et al., 2010). Two models were evaluated describing the effect of the aforementioned factors on the growth rate (μ_{max}) and the inverse lag phase (1/λ), respectively. The equation regarding the gamma model for growth rate (μ_{max}) was formed as follows:

$$CM_n(x, P_{\min}, p_{opt}, p_{\max}) = \begin{cases} x \le p_{\min}, & 0.0 \\ p_{\min} < x < p_{\max}, & \dfrac{(x - p_{\min})^n (x - p_{\max})}{\left(p_{opt} - p_{\min}\right)^{n-1} \left\{ \left(p_{opt} - p_{\min}\right)(x - p_{\min}) - \left(p_{opt} - p_{\min}\right)\left[(n-1)p_{opt} + p_{\min} - nx\right] \right\}} \\ x \ge p_{\max}, & \end{cases} \quad (1)$$

where x is the environmental variable, namely temperature (T) and a_w and; p_{min}, p_{opt} and p_{max}, are respectively the theoritical minimum, optimum and maximum value for each variable; n is a shape parameter; CM the growth parameters (μ_{max} and 1/λ).

According to the records of the bakery industry the inhibitory factors included in the equation above, temperature and a_w are the most variable, due to the fluctuating storage conditions and the variation in water holding capacity of the dough supplemented with the cream fillings. As for the others, the manufacturer uses a constant flushed packaging atmosphere (10% O_2: 10% CO_2: 80% N_2) and either 400 or 800 ppm calcium propionate (Table 1), with 400 ppm being the preferable level in the context of minimizing the use of chemical preservatives. Therefore, we limited the experiments regarding cardinal values estimation to those for T and a_w and did not collect any experimental data for modelling the impact of CO_2 and calcium propionate on fungal growth. Instead, the CO_{2max} and the minimum inhibitory concentrations (MIC) of propionate were obtained from literature and set at 100% and 0.25%, respectively (Matsuda et al., 1994).

An alternative to using specific gamma terms for propionate, is to express its inhibitory effect through a reference value for μ_{max} and 1/λ. In this context, and aiming to increase the robustness of models close to the growth boundaries, two a_w reference conditions were used to calculate μ_{ref} and $1/\lambda_{ref}$, in order to encompass the combined impact on fungal growth of those factors (e.g., preservative and packaging atmosphere) that are present in commercial brioche package, but are not accounted for by the gamma terms for a_w and T. This step is necessary to calibrate the model in relation to the total brioche environment. The two a_w reference conditions were chosen to be growth-supporting, while representing a low level (0.84), close to the target a_w, achieved in adequately baked brioche (0.82) and an uncommon high a_w (0.90) that may occur either in a less-than-desired baked brioche that maintains high levels of moisture, or may be associated with technical specifications of raw materials, such as cream filling of high a_w. Both scenarios were expected to favour fungal growth. The reference kinetic parameters (μ_{ref} and $1/\lambda_{ref}$) were determined in PDA with a_w (0.84 and 0.90) and 400 ppm of calcium propionate under aerobic conditions at 25 °C.

2.6. Evaluation of model performance

In order to validate the developed mathematical model regarding fungal growth, laboratory media imitating the characteristics of the brioche with regard to pH, a_w, and preservative presence (*in vitro* validation) as well as final products (*in situ* validation), were inoculated with *A. flavus*, *A. fumigatus*, and *Penicillium* sp. Moreover, the model was used in two versions regarding the gamma factors included in the model for its validation on laboratory media (*in vitro*) and final products (*in situ*), respectively.

The *in vitro* validation aimed to test the applicability of the model as a generic tool for various bakery products and thus, it was performed on PDA plates of pH 5.8 (adjusted with NaOH 1 N), a_w 0.84 and a_w 0.90 (by adding 40% and 25% v/v glycerol, respectively) and 400 ppm calcium propionate. Reported pH and a_w values were verified after autoclaving using a digital pH meter and a a_w meter, respectively. PDA plates were spot inoculated (10 μL; initial inoculum of *ca.* 10^3 spores), packaged under aerobic conditions, and incubated for at least 35 days at temperatures between 20 and 30 °C (8 h at 20 °C, 8 h at 25 °C, 8 h at 30 °C). Radial growth of each fungi was recorded on a daily basis. Considering, though, that the two aforementioned reference values of μ_{ref} and $1/\lambda_{ref}$ include the effect of 400 ppm propionate and the fact that the validation was performed under aerobic conditions, for model simulations, the gamma model with the T and a_w terms only (without gamma functions for propionate and CO_2), was used as secondary model for obtaining predictions for μ_{max} and $1/\lambda$ (Eq. (2)). The agreement between growth simulation by the model and the observed data on radial growth were further evaluated using the accuracy and bias factors as proposed by Ross (1996).

Regarding the *in situ* (brioche-specific) evaluation of model performance, fresh produced brioche containing 800 ppm of calcium propionate were obtained directly from the bakery industry. Following unpacking under aseptic conditions, brioche were inoculated (10 μL; initial inoculum of *ca.* 10^3 spores: being representative in terms of natural contamination level) with each fungal strain separately, on eight (8) different spots, namely at the top, bottom, and sides of brioche. Each fungal strain was inoculated on three brioche (n = 3). Inoculated brioche were then packaged under MAP conditions of 10% O_2: 10% CO_2: 80% N_2 (simulating the original packaging conditions) and stored under dynamic temperature program (8 h at 20 °C, 8 h at 25 °C, 8 h at 30 °C). Dynamic temperature profile was used to simulate the fluctuating conditions that may occur during product distribution in the retail market and kiosks, including the summer when elevated temperatures are observed. The inoculated brioche products were visually inspected on daily basis for fungal growth for at least 3 months. For growth simulations, the full gamma model (Eq. (3)), including calcium propionate and CO_2 terms with the two aforementioned reference values of μ_{ref} and λ_{ref} was used as secondary model for obtaining predictions for μ_{max} and $1/\lambda$.

$$\mu_{max} = \mu_{ref}\cdot CM_2(T)\cdot CM_2(a_w)\cdot \xi(T,a_w)$$
$$1/\lambda = 1/\lambda_{ref}\cdot CM_2(T)\cdot CM_2(a_w)\cdot \xi(T,a_w) \quad (2)$$

$$\mu_{max} = \mu_{ref}\cdot CM_2(T)\cdot CM_2(a_w)\cdot\left(1-\frac{[PAU]}{MIC_u propionate}\right)\cdot\frac{(CO_{2\,max}-CO_2)}{CO_{2\,max}}\cdot\xi(T,a_w, propionate, CO_2)$$
$$1/\lambda = 1/\lambda_{ref}\cdot CM_2(T)\cdot CM_2(a_w)\cdot\left(1-\frac{[PAU]}{MIC_u propionate}\right)\cdot\frac{(CO_{2\,max}-CO_2)}{CO_{2\,max}}\cdot\xi(T,a_w, propionate, CO_2) \quad (3)$$

where ξ is the term describing the quantitative effects of the interactions of the tested variables on the μ_{max} values (Le Marc et al., 2002).

Changes in radial growth of the inoculated fungi on PDA were predicted by the following linear model:

$$D_t = D_o,\ t \le \lambda \quad (4)$$

$$D_t = D_o + \mu_{max}\cdot(t-\lambda),\ t > \lambda \quad (5)$$

where D_o and D_t are the diameter of fungi (cm) at the beginning of the experiment (time 0) and any time *t* during storage, respectively and λ is the lag time. D_o is the diameter of the inoculated spore suspension drop (0.5 cm).

The prediction of growth was based on the assumption that after a temperature shift, the growth rate is adopted instantaneously to the new temperature environment. Therefore, after the completion of the initial "adaptation work", no further induced lag due to temperature shifts was considered. To estimate lag time under non-isothermal conditions, an average "adaptation work" for each fungus studied (Table 4) was estimated as the product of μ_{max} times λ, based on the fact that this product was relatively constant *per* fungus (Dagnas et al., 2014; Gougouli and Koutsoumanis, 2010) for different isothermal condition (Fig. 6). Lag time was cumulatively calculated *via* the integral rule (Gougouli and Koutsoumanis, 2010; Zwietering et al., 1994), adding the percentage of the "adaptation work" that was produced during each temperature step (according to the reading frequency of data loggers), defined as the ratio of the duration of a given temperature step over the predicted (momentary) lag time for the same temperature. Lag time was considered to have totally lapsed, the first time that the cumulative ratio of adaptation work exceeded the value of 1.

Table 2
Cardinal values of *Aspergillus flavus, Aspergillus fumigatus,* and *Penicillium sp.,* regarding temperature and a_w as stated in literature (Pitt and Hocking, 2009) as well as experimentally determined on PDA medium. Cardinal values were estimated by fitting the cardinal growth model (Rosso and Robinson, 2001) to the experimental growth parameters (μ_{max}) using the Table2Dcurve software. μ_{opt} corresponds to the average μ_{opt} determined for T and a_w.

Cardinal values based on growth rate (μ_{max})						
Cardinal parameter	*Aspergillus flavus*		*Aspergillus fumigatus*		*Penicillium* sp.	
	Experimental data	Literature data	Experimental data	Literature data	Experimental data	Literature data
T_{max} (°C)	40.0 ± 0.6	48	45.0 ± 14.9	55	37.3 ± 0.5	37
T_{min} (°C)	12.5 ± 0.7	12	10.8 ± 3.4	12	12.0 ± 2.4	4
T_{opt} (°C)	30.5 ± 0.6	30–37	37.3 ± 4.7	40–42	25.8 ± 0.84	23
$a_{w\ min}$	0.794 ± 0.01	0.78	0.836 ± 0.006	0.82	0.750 ± 0.015	0.78–0.81
$a_{w\ opt}$	0.958 ± 0.003	–	0.975 ± 0.002	–	0.954 ± 0.004	–
μ_{opt}	1.60 ± 0.14		2.47 ± 0.10		0.71 ± 0.06	–

Table 3
Cardinal values of *Aspergillus flavus, Aspergillus fumigatus,* and *Penicillium sp.,* regarding temperature and a_w as stated in literature (Pitt and Hocking, 2009) as well as experimentally determined on PDA medium. Cardinal values were estimated by fitting the cardinal growth model (Rosso and Robinson, 2001) to the experimental growth parameters (1/λ) using the Table2Dcurve software. $1/\lambda_{opt}$ corresponds to the average $1/\lambda_{opt}$ determined for T and a_w.

Cardinal values based on reverse lag (1/λ)

Cardinal parameter	*Aspergillus flavus*		*Aspergillus fumigatus*		*Penicillium* sp.	
	Experimental data	Literature data	Experimental data	Literature data	Experimental data	Literature data
T_{max} (°C)	40.7 ± 2.2	48	42.0 ± 13.7	55	42.2 ± 1.8	37
T_{min} (°C)	10.7 ± 3.3	12	10.0 ± 6.0	12	8.0 ± 2	4
T_{opt} (°C)	30.0 ± 1.0	30–37	37.3 ± 8.0	40–42	25.0 ± 2.3	23
$a_{w\ min}$	0.770 ± 0.026	0.78	0.767 ± 0.030	0.82	0.767 ± 0.017	0.78–0.81
$a_{w\ opt}$	0.990 ± 0.003	–	0.989 ± 0.020	–	0.959 ± 0.005	–
$1/\lambda_{opt}$	1.99 ± 0.40		2.58 ± 0.43		1.44 ± 0.08	–

Table 4
Parameters used for the development of gamma model describing growth of *Aspergillus flavus, Aspergillus fumigatus,* and *Penicillium* sp.

Fungus	Average adaptation work	μ_{ref}		$1/\lambda_{ref}$	
		$a_{w\ ref}$ 0.90	$a_{w\ ref}$ 0.84	$a_{w\ ref}$ 0.90	$a_{w\ ref}$ 0.84
Aspergillus flavus	0.77	0.49	0.025	0.6	0.07
Aspergillus fumigatus	1.17	0.24	0.0001	0.33	0.03
Penicillium sp.	0.58	0.41	0.07	0.75	0.31

3. Results and discussion

3.1. Fungal isolation and identification

A total of 102 fungal strains were isolated from fresh-produced brioche, brioche during storage at 20, 30, and 37 °C and raw materials. Then, they were grouped based on phenotypical characteristics and further identified using molecular based methods (Schoch et al., 2012; White et al., 1990). *Penicillium* sp., *Cladosporium* sp., and *Aspergillus* sp. were the main genera isolated representing 90% of the isolates, regardless of the isolation origin or treatment (Fig. 1). The remaining 10% comprised of other fungi such as *Dothideomycetes* sp., *Talaromyces rugulosus, Epicoccum nigrum, Rhizopus stolonifera* (Fig. 2).

3.2. In vitro determination of fungal growth potential in function with a_w and storage temperature

The main objective of the present study was to select representative isolates *per* genus of *Penicillium*, *Aspergillus*, and *Cladosporium* for modelling purposes, as they represented *ca.* 90% of total isolates. Within this context, *in vitro* determination of the growth capacity of selected isolates (n = 40) was conducted on agar media of a_w 0.99 and a_w 0.82 at 25°Cand 37 °C (Fig. 3), representing optimal a_w and brioche-related a_w conditions, respectively. The selection criteria for fungal isolates were: i) the isolation frequency, ii) any potential fungal prevalence throughout the bakery process, and/or iii) the overall *in vitro* growth potential. *Cladosporium* isolates were excluded, due to their inability to grow at 37 °C, at both studied a_w (0.99 and 0.82) (data not shown). Thus, the selection of isolates, namely *A. flavus*, *A. fumigatus*, and *Penicillium* sp., was carried out from the 2 remaining genera. For the selection of fungal isolates, emphasis was given to the first two criteria, aiming to represent realistic contamination scenarios, while the third one was used supplementary, since the growth potential was assessed on culture media. As such, *Penicillium* sp. was selected because it satisfied both, first two criteria (Fig. 2). However, due to the wide spread and prevalence of *Penicillium* in the industrial environment of bakery products, the *Penicillium* strain that showed an average-to-fast growth response in all screening conditions was the one selected for further modelling purposes (Fig. 3c). In case of *Aspergillus* sp., *Aspergillus flavus* and *Aspergillus fumigatus* were selected for downstream modelling approach as they were the most frequently isolated species for *Aspergillus* genus and showed high growth potential in both 25 °C and 37 °C (Figs. 2 and 3). Even though *Aspergillus* genera represented the minority of isolates from brioche-like products, in the literature, they are recognized as highly important genera associated with both spoilage and safety issues (Abellana et al., 2001; De Clercq et al., 2014; Marín et al., 2002; Vytrasova et al., 2002).

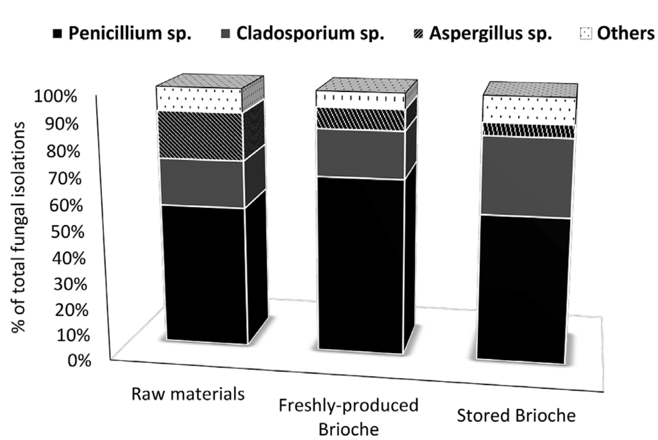

Fig. 1. Distribution of % main fungal genera (n = 102) identified in function to isolation origin or treatment (raw materials or brioche; freshly produced or stored at 20 °C, 30 °C, and 37 °C).

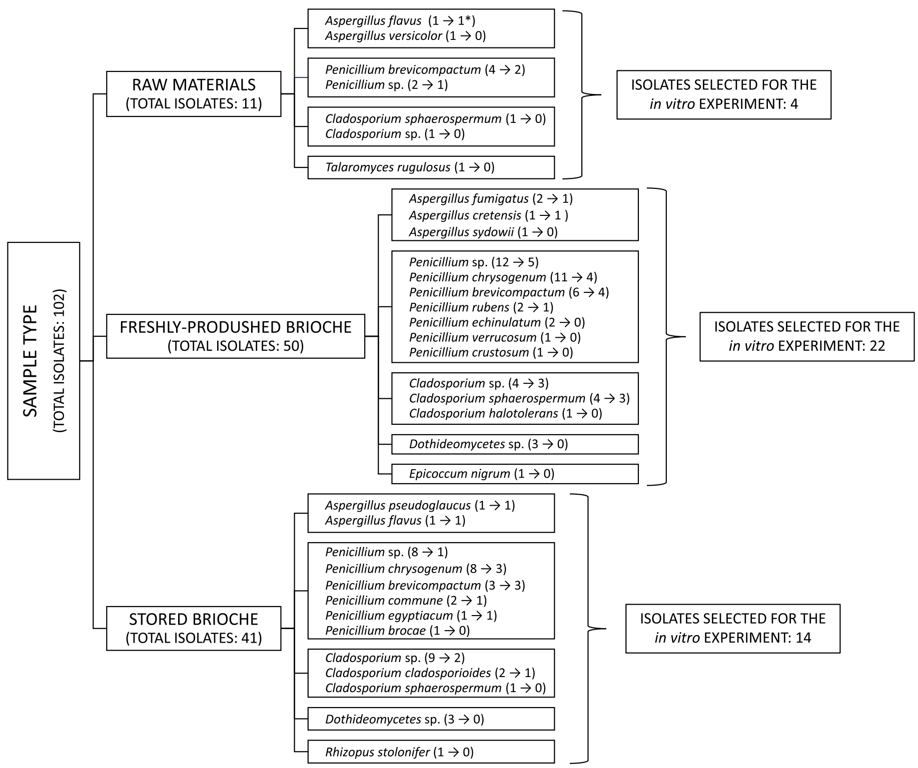

Fig. 2. Grouping of the 102 fungal isolates (in terms of genus or species according to phenotypical and molecular characterization) according to sample type (raw materials or brioche; freshly produced or stored at 20 °C, 30 °C, and 37 °C). * Total number of isolates → Number of isolates selected for the *in vitro* experiment.

3.3. Development of fungal growth model

Cardinal values of temperature and a_w for the lag time (λ) and the growth rate of the three selected strains were determined with two cardinal models (Tables 2 and 3; Figs. 4 and 5). Fitted models performed well in all data sets and the CM values were close to the limiting for growth values of temperature and a_w that were observed experimentally. The models used have been successfully applied for fungi associated with cheese, bakery products or wood (Dagnas et al., 2014; Maurice et al., 2011; Morin-Sardin et al., 2016). In addition, the estimated CM values of the models for 1/λ were close to the respective values for μ_{max}, with the exception of a_{wmin} for *A. flavus*, indicating similar temperature and a_w dependence for the growth rate and the lag time for mycelial growth. The estimates for the two *Aspergillus* strains were closer to the reported values in the literature, whereas deviation from the published values was observed for the cardinal parameters of

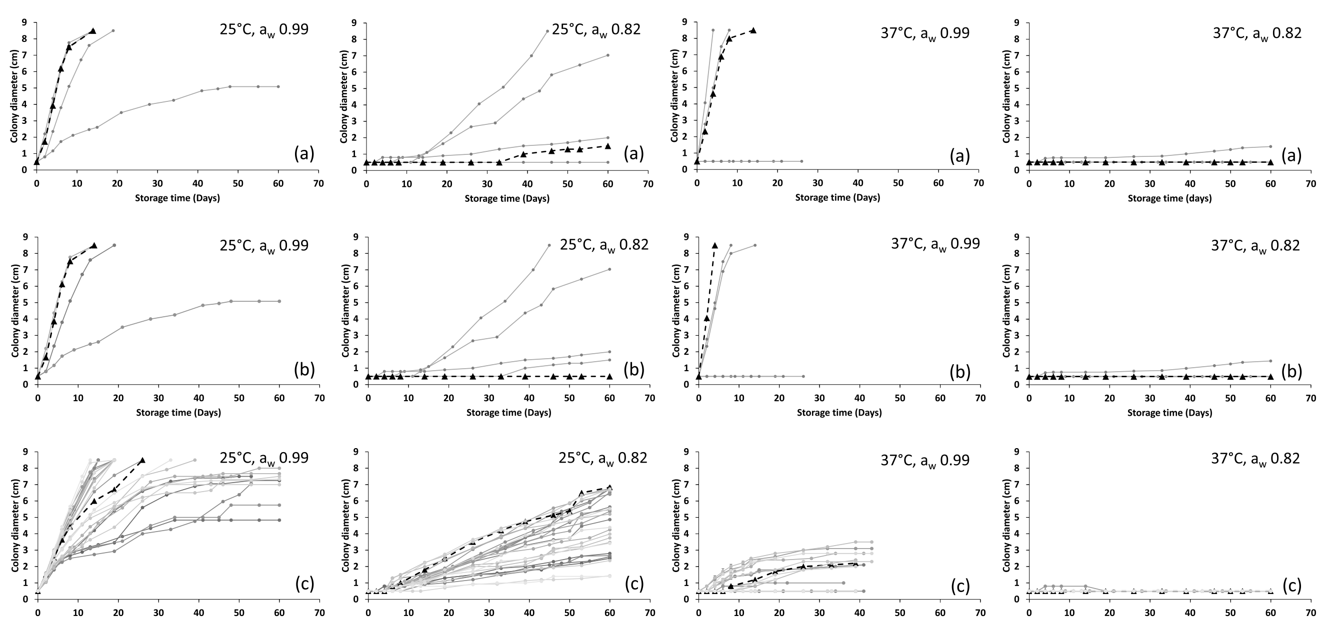

Fig. 3. *In vitro* growth potential of fungal isolates of *Aspergillus* and *Penicillium* sp. under optimal and brioche mimicking a_w (0.99 and 0.82, respectively) stored at 25 °C and 37 °C. Black dotted lines correspond to the selected isolates (a: *A. flavus*, b: *A. fumigatus,* and c: *Penicillium* sp.) for further development of growth model.

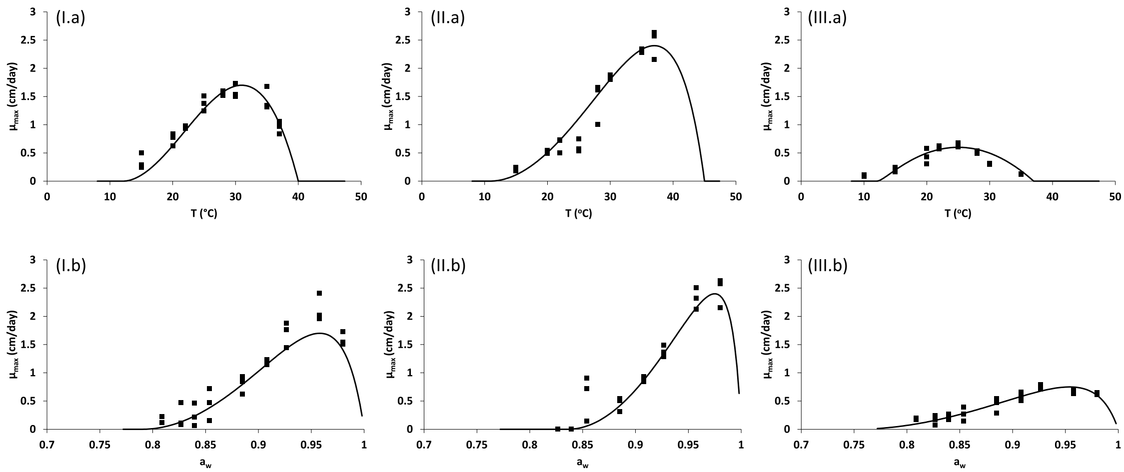

Fig. 4. Effect of (a) temperature and (b) a_w on the maximum radial growth rate (μ_{max}; cm/day) of (I) *Aspergillus flavus,* (II) *Aspergillus fumigatus,* and (III) *Penicillium* sp. Cardinal growth model (Rosso and Robinson, 2001) was fitted to the experimental data using the Table2Dcurve software.

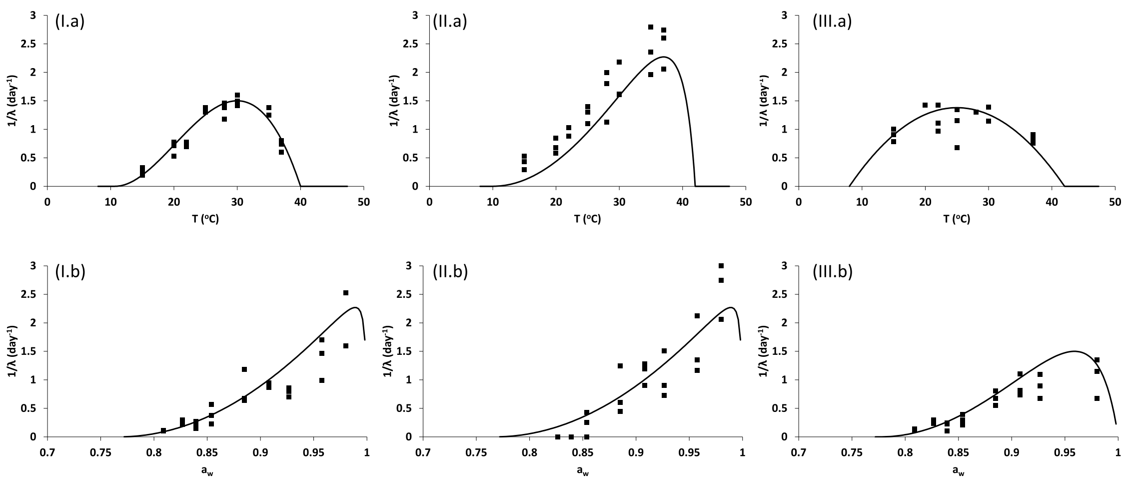

Fig. 5. Effect of (a) temperature and (b) a_w on the reverse lag phase ($1/\lambda$; day^{-1}) of (I) *Aspergillus flavus,* (II) *Aspergillus fumigatus,* and (III) *Penicillium* sp. Cardinal growth model (Rosso and Robinson, 2001) was fitted to the experimental data using the Table2Dcurve software.

the *Penicillium* strain. The determination of cardinal values is of vital importance for the performance of gamma models, since small changes in these values may lead to remarkable deviations of model predictions from observed independent data (Morin-Sardin et al., 2016). Factors known to influence the estimation of cardinal values are the model structure (i.e., the respective gamma terms of each growth-controlling factor), the growth medium, the fungal strain and the humectant used for adjustment of the a_w of the substrate (Dagnas et al., 2014; Maurice et al., 2011; Morin-Sardin et al., 2016). As such, selection of strains and substrate are critical for generating relevant modelling data. Further to the growth limiting values of temperature and a_w, there are other substrate-specific factors that impact the optimum (or reference) values of the kinetic parameters of fungal growth, without really affecting minimum, optimum or maximum values for growth. To compensate for such variations, the gamma models need to be calibrated in relation to reference conditions that introduce into the model the effect of factors that are not considered by the fitted gamma terms of the original model. This also enables increase in model performance and its accountability for growth on real foods without expenditure of resources to collect data for adding new gamma factors. This practice was applied in the present study to calibrate the model at conditions that closely resemble the commercial cream-filled brioche and encompass the effect of

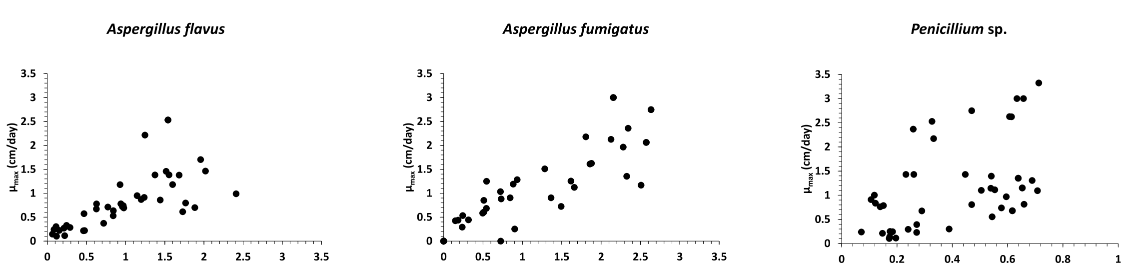

Fig. 6. Correlation curves of μ_{max} and $1/\lambda$ of *Aspergillus flavus, Aspergillus fumigatus,* and *Penicillium* sp.

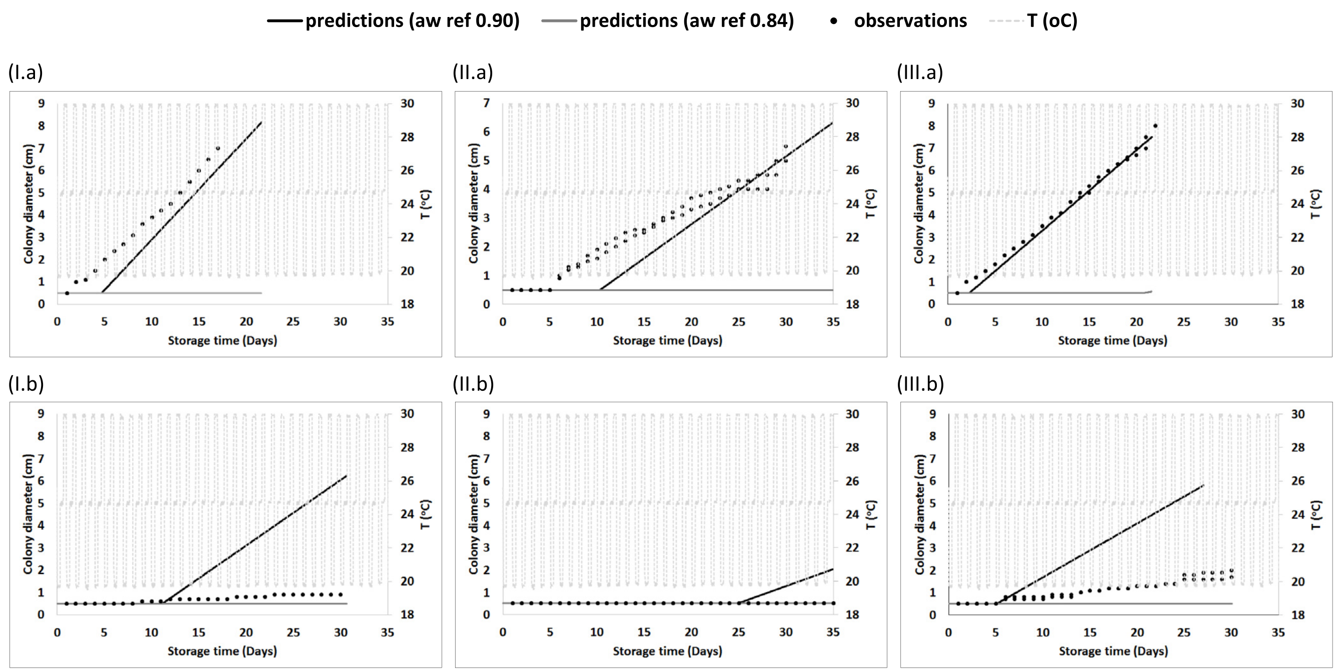

Fig. 7. *In vitro* observations and predictions of time to visible growth of (I) *Aspergillus flavus*, (II) *Aspergillus fumigatus*, and (III) *Penicillium* sp. on PDA of a_w 0.90 (a) and 0.84 (b) stored under dynamic temperature conditions.

Table 5
Accuracy and bias factors regarding *in vitro* model validation of *Aspergillus flavus, Aspergillus fumigatus,* and *Penicillium* sp. as determined according to Ross (1996).

Fungus	a_w 0.90				a_w 0.84			
	$a_{w\ ref}$ 0.90		$a_{w\ ref}$ 0.84		$a_{w\ ref}$ 0.90		$a_{w\ ref}$ 0.84	
	Af	*Bf*	*Af*	*Bf*	*Af*	*Bf*	*Af*	*Bf*
Aspergillus flavus	1.42	0.71	5.77	0.17	3.60	3.60	1.47	0.68
Aspergillus fumigatus	1.35	0.75	4.93	0.20	1.34	1.348	1.00	1.00
Penicillium sp.	1.068	0.96	6.51	0.15	2.55	2.55	2.11	0.47

preservative and the combined outcome of complex brioche ecosystem on fungal growth, without any further experimentation (Table 4). The benefits of this approach are reflected on the model performance against independent data, as detailed in the next paragraph.

3.4. Evaluation of model performance under non-isothermal conditions

Considering that sealed-packaged brioche (under MAP conditions) represent standardized and relatively stable ecosystems, the major factor that is likely to vary on the shelf is temperature. As such, evaluation of model performance took place under non-isothermal conditions at two different a_w scenarios, representing the baseline scenario of the well-baked brioche (with a_w 0.84) and that of a softer product, that is less cooked and has a_w around 0.90. The first part of non-isothermal trials were performed on PDA plates (*in vitro* validation). This is also an element of originality of the present approach, since it extents and integrates the application of gamma models for fungi in the context of dynamic storage conditions.

According to Fig. 7, calibration of model to the two reference a_w values and the presence of calcium propionate seemed to enhance the agreement of growth simulation with observed data, both regarding the time of initiation of mycelium expansion and the increase in colony diameter (Table 6). Notably, on PDA plates with a_w 0.84, the model using the reference values of μ_{max} and λ estimated at a_w 0.90 seemed to

Table 6
In vitro observations and predictions of λ (days) and time to visible growth (when colony diameter is equivalent to 1 cm; days) of *Aspergillus flavus, Aspergillus fumigatus,* and *Penicillium* sp. on PDA plates stored under aerobic conditions.NG: No growth.

Fungus	$a_{w\ ref}$	Validation conditions			Predictions		Observations	
		a_w	Propionate (ppm)	T (°C)	λ (days)	$t_{d=1\ cm}$ (days)	λ (days)	$t_{d=1\ cm}$ (days)
Aspergilllus flavus	0.90	0.90	400	Dynamic	4.82	6.0	1	2
	0.84	0.90	400	Dynamic	NG	NG		
	0.90	0.84	400	Dynamic	11.8	12.8	8	31
	0.84	0.84	400	Dynamic	NG	NG		
Aspergilllus fumigatus	0.90	0.90	400	Dynamic	10.5	13.0	5	6
	0.84	0.90	400	Dynamic	NG	NG		
	0.90	0.84	400	Dynamic	26.0	28.0	NG	NG
	0.84	0.84	400	Dynamic	NG	NG		
Penicillium sp.	0.90	0.90	400	Dynamic	2.44	3.01	1	2
	0.84	0.90	400	Dynamic	NG	NG		
	0.90	0.84	400	Dynamic	5	7.3	5	14
	0.84	0.84	400	Dynamic	30	35		

Table 7
In situ observations and predictions of λ (days) and time to visible growth (when colony diameter is equivalent to 1 cm; days) of *Aspergillus flavus, Aspergillus fumigatus,* and *Penicillium* sp. on brioche stored under MAP (10% O_2: 10% CO_2: 80% N_2).

Fungus	$a_{w\ ref}$	a_w	Validation conditions		Predictions		Observations	
			Propionate (ppm)	T (°C)	λ (days)	$t_{d=1\ cm}$ (days)	λ (days)	$t_{d=1\ cm}$ (days)
Aspergillus flavus	0.84	0.84	800	Dynamic	NG	NG	NG	NG
Aspergillus fumigatus	0.84	0.84	800	Dynamic	NG	NG	NG	NG
Penicillium sp.	0.84	0.84	800	Dynamic	32	37	NG	NG

NG: No growth.

overestimate fungal growth, as manifested also by the estimated accuracy (A*f*) and bias (B*f*) factors, suggesting a potential synergistic effect of the preservative and low a_w that cannot be accounted for by the gamma term for a_w alone and requires additional factors explaining fungal growth in this area (Fig. 7 and Table 5). This was further supported by the agreement of the predictions of the model calibrated against the same a_w of 0.84 (Fig. 7 and Table 6).

The major advantages of gamma models include: (i) the parsimony and interpretability of the model parameters, and (ii) the possibility to complete the model by adding the effect of an extra preservative factor interfering with the existing model structure. Therefore, in the non-isothermal trials with commercial brioche, the full gamma model with calcium propionate and CO_2 terms was used (Eq. (3)), in order to predict the time to have visible fungal growth. Following preliminary trials and based on the available literature reports for MIC of propionate and CO_2 around 0.25% and 10–20%, respectively, (Matsuda et al., 1994; Guynot et al., 2003), a recommendation was made to the industry for adding a maximum level of 800 ppm calcium propionate and the MAP composition of Table 1. These conditions resulted in no visible fungal growth, which was also correctly predicted by the model (Table 7).

4. Conclusions

Overall, the study constitutes a case study demonstrating the cost-effective and simplified application of gamma concept on predicting fungal growth in bakery products, especially under non-isothermal conditions. The ultimate goal is to upgrade such models into decision making support tools for use during product formulation, shelf-life determination, stability and logistics management based on the minimum necessary inputs of product parameters (e.g., a_w and preservative level). Combining proper strain selection, substrate for growth kinetics estimation and product-specific calibration of model may assure the desired model performance. Further experimentation with testing the model performance at the extremes of growth both for temperature and different levels of a_w and preservatives may improve and increase the robustness of the proposed models.

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