Evaluation of different hurdles on *Penicillium crustosum* growth in sponge cakes by means of a specific real time PCR

Mariana Gonda¹ · Caterina Rufo² · Gianna Cecchetto^{1,3} · Silvana Vero¹

Revised: 11 February 2019 / Accepted: 6 March 2019 / Published online: 21 March 2019
© Association of Food Scientists & Technologists (India) 2019

Abstract Limited shelf life of bakery products, caused by microbial deterioration, is a concern for industries due to economic losses. Fungal spoilage of sponge cakes industrially produced in Montevideo was caused mainly by *Penicillium* species, in particular by *Penicillium crustosum*. The combination of different hurdles was studied to inhibit *P. crustosum* growth in sponge cakes. A full factorial design was performed to study the effect of the concentration of potassium sorbate, pH, packaging atmosphere and storage time. The results showed that packaging atmosphere and storage time were the significant factors in the ranges tested. No growth was detected in cakes stored in modified atmosphere packaging (MAP) (N₂:CO₂ 50:50) at room temperature (25 °C) for 15 days. The effect of MAP on *P. crustosum* growth in cakes at room temperature was compared with the effect of air-packaging and storage at low temperature (4 °C) for 30 days. *P. crustosum* growth

was not detected in cakes packaged in MAP, whereas it was detected after 20 days in cakes packaged in air and stored at 4 °C. This growth was quantified by a specific real time PCR developed in this work. Specific primers were designed using the sequence of β -tubulin gene of *P. crustosum* as a target and PCR conditions were adjusted to ensure specificity. PCR efficiency was 107%, with a detection limit of 0.0014 ng of DNA. The qPCR method presented here, resulted specific and sensitive enough to detect the growth of *P. crustosum* even before biodeterioration signs were visible.

Keywords Bakery products · *Penicillium crustosum* · Hurdle technology · Real time PCR

Abbreviations

PS Potassium sorbate
MAP Modified atmosphere packaging
FFD Full factorial design

✉ Mariana Gonda
mgonda@fq.edu.uy

Caterina Rufo
crufu@fq.edu.uy

Gianna Cecchetto
giannac@fq.edu.uy

Silvana Vero
svero@fq.edu.uy

¹ Área Microbiología, Departamento de Biociencias, Facultad de Química, Universidad de la República, Gral Flores 2124, Montevideo 11800, Uruguay

² Alimentos y Nutrición, Instituto Polo Tecnológico, Facultad de Química, Universidad de la República, By Pass Ruta 8 s/n, Pando, Canelones, Uruguay

³ Microbiología, Facultad de Ciencias, Universidad de la República, Iguá 4225, Montevideo, Uruguay

Introduction

Consumption of bakery products, like cakes is increasing worldwide (Pasukamonset et al. 2018). A serious and costly problem for bakery industry is the economic loss caused by microbial spoilage which is often the main factor limiting shelf life of intermediate and high moisture bakery goods (Smith et al. 2004; Valle Garcia et al. 2019). Sponge cakes characterized by a_w values between 0.75 and 0.90 are intermediate moisture foods (Guynot et al. 2004) prone to microbial spoilage, caused mainly by moulds, which reduces their shelf life. Fungal contamination is difficult to avoid, it occurs after baking and before packaging, when fungal spores reach the product. Under adequate conditions

spores germination and concomitant mycelia growth may occur, causing visible symptoms of deterioration. The most widespread fungal species reported as spoilage agents of bakery products belong to the genera *Eurotium*, *Aspergillus* and *Penicillium* (Guynot et al. 2005). In sponge cakes, in particular, xerophilic fungi such as *Wallemia* and *Eurotium* spp. and also some species of *Aspergillus* and *Penicillium* have been frequently reported as causative agents of microbial spoilage (Smith et al. 2004). Pitt and Hocking (2009) reported several *Penicillium* species, *P. crustosum*, *P. glabrum*, *P. commune*, *P. chrysogenum*, as common causes of fungal spoilage of cakes. In addition, previous work of Gonda (2015) found that *P. crustosum*, *P. chrysogenum*, *P. brevicompactum* and *P. citrinum* represented 75% of the fungal species causing spoilage on sponge cakes industrially produced in Montevideo. In the mentioned study *P. Crustosum* represented 35% of the isolates, being the main responsible for biodeterioration. Besides the visible growth, fungi may be responsible for off-flavours and production of mycotoxins. In that sense, Tancinová et al. (2012) found that *P. crustosum* was one of the toxigenic species most frequently identified as a contaminant of bread stored in plastic bags at room temperature.

To prevent fungal spoilage of intermediate moisture bakery products and in consequence extend their shelf life, chemical preservatives are commonly used (Mohammadzadeh-Aghdash et al. 2018).

However, since today consumers are not in favour of processed food containing chemical additives an urge to reduce or replace their use has arisen within the bakery industry (Janjarasskul et al. 2016). In this context hurdle technology is often applied. Combinations of different hurdles such as temperature, water activity, pH and modified atmosphere packaging that could act synergistically to inhibit or retard microbial growth have been explored to prevent microbial spoilage in bakery products (Guynot et al. 2002, 2005; Marín et al. 2002; Qiu et al. 2019). Since most spoilage fungi are aerobic, the use of modified atmosphere packaging (MAP) with and without oxygen scavengers seems to be a promising hurdle to be more deeply evaluated, in special for intermediate moisture goods with neutral pH to be stored at room temperature (Hasan and Abdolghader 2012; Qiu et al. 2019). The main gases used in commercial MAP are nitrogen (N₂) and carbon dioxide (CO₂), which replace the air in the package reaching very low levels of oxygen (approximately 1%). The attainment of such low levels of oxygen and also the fungistatic activity of CO₂ prevent the development of fungal growth (Guynot et al. 2003).

Most of the studies on hurdle technologies for the prevention of fungal spoilage on bakeries have been carried out by determining colony diameters on culture media or

directly on the bakery product. Despite this methodology proved to be reliable, it presents the disadvantage that fungal growth could not be detected until a visible colony appeared. However, since early detection of fungal growth is relevant to food safety especially when spoilage fungi produce mycotoxins, the use of more sensitive methods of detection is needed. The measurement of biochemical markers such as ergosterol (Taniwaki et al. 2006; Mensah-Attipoe et al. 2016) could be an option. However, this is a time consuming and laborious method which lacks of specificity, since it could not distinguish between different fungal species in a mixed culture (Arquiza and Hunter 2014). On the other hand, the molecular method quantitative real time PCR appear as a fast, reliable, sensitive and specific assay that allows the quantification of low amounts of fungal DNA (Arquiza and Hunter 2014; Tannous et al. 2015).

The present work aimed to determine the effect of hurdle technology, studying the effect of MAP, pH and potassium sorbate concentration, on the growth of *P. crustosum* in sponge cakes at room temperature, evaluated by a specific qPCR method developed for this purpose. A comparison between the inhibitory effect of MAP and cold temperature on *P. crustosum* growth on sponge cakes was also carried out.

Materials and methods

Fungal isolates

P. crustosum NRRL 968, *Penicillium commune* NRRL 894 and *Penicillium chrysogenum* NRRL 1951 used in this work were provided by the Agricultural Research Service (NRRL) culture collection from the United State Department of Agriculture–Agricultural Research Service (USDA-ARS).

DNA extraction from fungal isolates

For isolation of genomic DNA, fungi were grown for 3 days at 28 °C in Yeast Extract Sucrose (Forsythe and Hayes 2000). Mycelium obtained was dried with sterile paper towels and ground with a glass rod in liquid nitrogen. About 20 mg of ground mycelium was transferred to a 1.5 mL microcentrifuge tube containing 500 µL of lysis buffer (100 mM Tris, 20 mM EDTA pH = 8, 250 mM NaCl, 2% SDS) vortexed and then incubated for 1 h at 65 °C. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to the lysis mixture and centrifuged for 10 min at 1000 rpm for 4 °C. After centrifugation, the upper layer was carefully transferred to a new 1.5 mL microcentrifuge tube. The DNA was precipitated with 300

μL of NaCl 0.5 M and an equal volume of ice-cold isopropanol. After 12 h at -20°C , the pellet obtained was rinsed with 70% ethanol, dried for 30–60 min at 35°C and suspended in 30 μL of miliQ water.

DNA was quantified with the fluorescent nucleic acid kit Quant-iT ds DNA (Invitrogen, California, USA).

Design and evaluation of specific q PCR primers for *P. crustosum*

To follow the growth of *P. crustosum* on cakes a specific qPCR method was developed. Specific primers were designed using the AlleleID 7.7[®] software (PREMIER Biosoft International, California, USA). The sequence of β -tubulin gene of *P. crustosum* strain IMI 091917 (AF003538) was used as a target. A set of specific primers was designed avoiding homology with β -tubulin sequences from other species that showed homology higher than 93% with the one used as target. Certain parameters including primers length of 20 bp, GC content of about 50%, a melting temperature about 60°C , and an amplicon length of 100 bp were set to obtain the best primer pair. Also the tendency to form primer secondary structures (hairpins, self-dimers and cross dimers) was checked. The sequences of the potential primers were compared against all other sequences available online with the Primer Basic Local Alignment Search Tool algorithm (Primer-BLAST, National Center for Biotechnology Information) (Altschul et al. 1990; Ye et al. 2012). Those primers that showed 100% homology with β -tubulin sequence of *P. crustosum* and homology levels lower than 91% with sequences of other species were selected.

A temperature gradient PCR was carried out to determine the highest annealing temperature in the range of 51°C and 60°C that allowed the amplification of β -tubulin gene using *P. crustosum* DNA as template. DNA from *P. crustosum* was extracted as mentioned in “2.2” section. PCR amplifications were performed in a gradient palm-cycler (Corbett CG1_96, Corbett Research, UK) in a total reaction volume of 25 μL consisting of 1 μL of DNA (90 ng), 2.5 μL of $10\times$ buffer, 2.5 μL of dNTPs (2 mM each), 2.5 μL of MgCl_2 (50 mM), 1 μL of each primer (25 μM), 0.1 μL of Taq DNA Polymerase (5 U/μL) (Thermo Fisher Scientific, USA) and sterile MiliQ water up to 25 μL. The PCR protocol consisted of an initial heating step of 3 min at 95°C , followed by 35 cycles of 30 s at 95°C for template denaturation, 30 s at a determined temperature in the range of 51°C to 60°C ($51, 52.9, 53.8, 54.6, 55.4, 56.3, 57.1, 58.1, 59, 60^{\circ}\text{C}$) for primer annealing and 1 min at 72°C for primer extension, with a final extension at 72°C for 10 min.

To determine specificity of the selected primer pair, PCR reactions were carried out in the conditions mentioned

above using an annealing temperature of 60°C and 90 ng of genomic DNA of *P. crustosum* NRRL 968, *P. commune* NRRL 894 and *P. chrysogenum* NRRL 1951 as templates. PCR products were visualized on 1.5% agarose gel.

Real time PCR design

Real time PCR reactions were performed in a Rotor-Gene 6000 cycler (Qiagen, Venlo, Netherlands). Reactions were performed in duplicate with total volumes of 10 μL, consisting of 5 μL of Rotor-Gene[™] SYBR[®] Green PCR Master Mix (Qiagen, Venlo, Netherlands), 0.5 μL of each primer (25 μM), 1 μL of template DNA and 3 μL sterile miliQ water. The PCR thermal cycling consisted of 5 min at 95°C and 35 cycles of 95°C for 10 s and 60°C for 20 s (during which the fluorescence was measured). Following the final amplification cycle, a dissociation curve between 50 and 99°C was constructed in order to confirm the specificity of the amplification.

A standard curve was generated by duplicate analysis of serial dilutions of DNA of *P. crustosum*. Amounts of 5.6, 0.56, 0.056, 0.0056, 0.0042, 0.0028 and 0.0014 ng of DNA were used. Dilutions of $\frac{3}{4}$, $\frac{1}{2}$ and $\frac{1}{4}$ were used with the aim to achieve the lowest detection limit. The mean cycle threshold (Ct) values were plotted against log of the corresponding DNA amount of each dilution. PCR efficiency was calculated from the slope of the standard curve with the formula $\text{Efficiency} = [10^{(-1/\text{slope})}] - 1$ (Dorak 2006). Detection limit and dynamic range were set in the linear range. Data analysis was carried out in Rotor-Gene 6000 cycler software (Qiagen, Venlo, Netherlands).

To confirm specificity of the primers designed (section “Design and evaluation of specific q PCR primers for *P. crustosum*”), a real time PCR was carried out in the conditions mentioned above on total genomic DNA of *P. crustosum* NRRL 968, *P. commune* NRRL 894 and *P. chrysogenum* NRRL 1951. DNA amounts used in each reaction were 0.42, 0.40, 0.34 and 0.55 ng respectively. Amplifications were done in duplicate.

Effect of sponge cake matrix on real-time PCR

Matrix was obtained from an extract of an uninoculated sponge cake prepared according to the protocol for DNA extraction from the Community Reference Laboratory for GM Food and Feed (CRL-GMFF 2008). The same method was used in this work for the analysis of *P. crustosum* growth on cakes. Briefly, 0.2 mL of an homogenate of cake obtained by blending the whole cake (approx. 40 g) in 400 mL of 0.1% Tween-80 for 1 min in a Stomacher 400 (Seward, UK) were extracted as indicated in the mentioned protocol.

Dilutions of DNA from *P. crustosum* NRRL 968 containing 5.6, 0.56, 0.056 and 0.0056 ng were prepared in water and in the cake extract obtained as described above. Ct values obtained in both cases were compared. Matrix effect was considered significant if the difference of Cts obtained from DNA diluted in water and diluted in matrix was more than 0.5 (Branquinho et al. 2012).

Effect of different variables on *P. crustosum* growth in packaged cakes

The effect of four variables over the growth of *P. crustosum* at 25 °C, on artificially inoculated cakes was determined using a 2⁴ full factorial design (FFD). The variables included in this study were packaging atmosphere (air or modified atmosphere) which was considered a qualitative factor, and three quantitative variables: pH of the dough (5.5, 6.0, 6.5), concentration of potassium sorbate (PS) in the cake (0, 500, 1000 ppm) and storage time (5, 10, 15 days). Immediately after the incubation time was completed, cakes were processed to obtain total DNA, according to CRL-GMFF(2008) as described in section “Effect of sponge cake matrix on real-time PCR”. All extractions were done in duplicates. The growth of *P. crustosum* in each trial was determined by quantifying *P. crustosum* DNA using the specific real time PCR method developed in this work (see sections “Real time PCR design and Effect of sponge cake matrix on real-time PCR”). The amount of *P. crustosum* DNA in each cake was used as response in the factorial design. An analysis of variance (ANOVA) was carried out in order to determine the significant factors on *P. crustosum* growth. Variables with *p* values lower than 0.05 were considered to have a significant effect. Experimental design and statistical analysis were performed using Design Expert 7.0 software (Stat Ease Inc., USA).

Preparation and inoculation of the cakes

Cakes (diameter = 7 cm, height = 4 cm, weight = 40 g) were prepared with a commercial sponge cake premix (Fleischmann, Montevideo, Uruguay) that contains wheat flour enriched with iron and folic acid, sugar, skim milk powder, maltodextrin, chemical leavening agents (INS 500ii: sodium bicarbonate, 450iii: sodium pyrophosphate, 341iii: calcium triphosphate) and stabilizers (INS 472b: glycerol mono and di-esters with fatty acids and lactic acid, 471: mono and di-glycerides of fatty acids, 415: xanthan gum). Pasteurized liquid eggs (Prodhin, Montevideo, Uruguay) and tap water was added in the quantities required by the manufacturer of the premix. Three different doughs were prepared with 0, 500 and 1000 ppm of PS. Dough initial pH was 6.5 in every case. In each treatment

pH was adjusted later with a sterile citric acid solution (0.3 g/mL) (Marín et al. 2002).

Different doughs were placed on aluminium individual plates and baked in an oven at 180 °C for 15 min. After baking, cakes were left at room temperature for 12 h and transferred to the laminar flow bench where they were exposed to UV light for 1 h to minimize microbial surface contamination.

Mould inoculum was prepared by growing *P. crustosum* in Potato Dextrose Agar (Oxoid, Hampshire, UK) (PDA) for 3 days at 28 °C to obtain a sporulating culture, from which a spore suspension of 10⁷ spores/mL was prepared in a 0.1% Tween-80 solution. Cakes were inoculated in the centre region of the upper surface with 0.1 mL of the spore suspension.

Packaging

Cakes were packaged using a SAMMIC V-410SGI professional vacuum machine (SAMMIC, Spain), by creating vacuum in the bag and then introducing the gas mixture at around 1.5 bar pressure before heat sealing. A sterilization filter of 0.2 µm (Minisart Syringe Filter, Sartorius, Germany) was adapted at gas entrance when air was used. Cakes were individually packaged in Cryovac T7335B bags (Cryovac, Sealed Air Corporation, USA), which are a co-extruded web with a nylon skin, a LLDPE sealant and an EVOH barrier. Each package was filled with air or modified atmosphere (N₂:CO₂ 50:50) in a ratio product/gas mixture 1:2 (v/v). Gas composition (N₂:CO₂ 50:50) was obtained with *Aligal 15* blend (Air Liquide, Montevideo, Uruguay). Packaged cakes were then stored at 25 °C for 5, 10 and 15 days according to the factorial design.

Control of *P. crustosum* growth on packaged cakes by packaging atmosphere and temperature

The inhibitory effect of MAP on *P. crustosum* growth at room temperature was compared with the effect of packaging under air and storage at low temperature. Cakes were prepared and inoculated as described above. In these experiments no preservative was added and pH was not adjusted. Half of the cakes were packaged under modified atmosphere (N₂:CO₂ 50:50) and stored at 25 °C, while the other half were packaged in air and stored at 4 °C. *P. crustosum* biomass was estimated by specific DNA quantification by real time PCR, as described above, at 4, 10, 16, 23 and 30 days of storage.

Results

Design and evaluation of specific primers

Primers were designed for the development of a specific real time PCR method to perform the quantification of *P. crustosum* DNA. The sequence of β -tubulin gene of *P. crustosum* was used as a target. The sequences of six potential primer pairs that comply the established parameters (see section “Design and evaluation of specific qPCR primers for *P. crustosum*”) were compared with all sequences available in the Gene Bank using Primer-BLAST (NCBI). The selected primers 2PCrus F (5'-AGG TTC CTA ACT GGA TTA CA-3') and 2PCrus R (5'-CCC CTA TCA CTG TTA AAA CTT-3'), showed 100% homology with β -tubulin sequence of *P. crustosum*. Also, they presented homology levels lower than 91% for at least one of the primers in the annealing region with all the β -tubulin sequence of other species.

To determine the highest annealing temperature for the primers selected a temperature gradient PCR was carried out. A single PCR product of the expected length, about 86 bp, was obtained at all the annealing temperatures tested (from 51 to 60 °C) when *P. crustosum* DNA was used as template (results not shown). Thus, 60 °C was selected as the annealing temperature for the real time PCR method. Specificity of the primers was confirmed at the selected temperature since no amplification product was obtained when DNA from the other tested species (*P. commune* and *P. chrysogenum*) was used as template. Those species were selected to check specificity because their β -tubulin sequence had the highest homology with β -tubulin sequence of *P. crustosum* in the primers annealing zones.

qPCR design

The real time PCR method developed to quantify *P. crustosum* growth had a efficiency of 107%, which was calculated from the slope of the standard curve, with a

$R^2 = 0.98$ (Fig. 1a). The dynamic range obtained for the method was between 5.6 and 0.0014 ng of *P. crustosum* DNA (Fig. 1a). Melting curve analysis shows that melting temperature of the amplicon is 81.8 °C, as expected. Reactions without template (NTC) did not generate amplification products. Thus, the fluorescence measured in each case could be attributed to *P. crustosum* DNA (Fig. 1b).

The specificity of the real time PCR method was confirmed since amplification was obtained only when *P. crustosum* DNA was used as template and not when other species were assayed (*P. commune*, *P. chrysogenum*) (Fig. 2).

Effect of sponge cake matrix on real-time PCR

The effect of the sample matrix on real-time PCR determinations was evaluated by comparing standard curves generated with *P. crustosum* DNA diluted in water and in matrix. No amplicon was obtained for DNA extracted from control uninoculated cakes. Moreover, the matrix did not affect amplification by real time PCR. Difference between Ct values (ΔC_t) for the same dilutions of *P. crustosum* DNA in presence and absence of cake matrix were less than 0.5 in all cases (Table 1). These results confirmed that there was no interference of the matrix in DNA quantification in the established dynamic range (Branquinho et al. 2012).

Effect of different variables on *P. crustosum* growth in packaged cakes

In order to simultaneously evaluate the effect of four variables and to determine if there were interactions among them on the growth of *P. crustosum* on cakes at 25 °C, a FFD was performed. DNA was quantified (as an estimator of its biomass) by the real time PCR method described above. The FFD consisted in 22 trials including pH, potassium sorbate concentration, storage time (quantitative

Fig. 1 **a** Standard curve for the real time PCR assay of *P. crustosum* DNA. Bars represent confidence intervals ($\alpha = 0.05$). **b** Melting curve profile obtained by fluorescence measurement during the denaturation

of PCR amplicons of *P. crustosum* at increasing temperature. Arrow shows the melting curves of the reactions without template (NTC)

Fig. 2 Amplification curve for different *Penicillium* species. (···) *P. crustosum*, (—) *P. commune*, *P. chrysogenum* and no-template control (NTC)

Table 1 Ct values obtained for PCR real time of tenfold dilutions of *P. crustosum* DNA in water and in matrix

	Ct values			
	DNA amounts (ng)			
Diluent	5.6	0.56	0.056	0.0056
Water	15.17 ± 0.09	18.49 ± 0.06	21.97 ± 0.11	24.88 ± 0.25
Matrix	15.42 ± 0.04	18.92 ± 0.04	22.32 ± 0.20	25.32 ± 0.24

A 95% confidence interval was calculated

factors) and packaging atmosphere (qualitative factor) (Table 2). Three biological replicates were carried out at the central point of quantitative variables in air packaging and in MAP. Analysis of variance showed that the model obtained was significant (p value < 0.0001) with an adjusted R-squared of 0.9053 and it did not present lack of fit (p value = 0.5944) of fit. It Also showed that significant factors involved in *P. crustosum* growth in sponge cakes were packaging atmosphere (C, p value < 0.0001), storage time (D, p value = 0.0036) and their interaction (CD, p value < 0.0001).

Results of the FFD are shown in a Pareto chart where the main and interaction effects of factors (Fig. 3a) are described. Pareto chart clearly shows that the t value of effects associated with packaging atmosphere (C) and the interaction between packaging atmosphere and storage time (CD) exceed the Bonferroni limit of 3.44, which means that this factor (C) and the interaction (CD) certainly had significant effects on mould growth. Moreover, the effect of storage time (D) was likely to be significant since the t value of its effect was between the Bonferroni and t value limit (t Value of Effect = 2.12)(Anderson and Whitcomb 2016). The other factors involved in the assay, PS concentration and pH were below t value limit. Since a parameter with a t -value higher t value limit indicated that it had a confidence level greater than 95%, we included packaging atmosphere (C), storage time (D) and their interaction (CD) in the model. Interaction between

packaging atmosphere and storage time showed that *P. crustosum* growth rate (estimated as the slope of the interaction curves) in air was higher than in modified atmosphere packaging N₂:CO₂ 50:50(Fig. 3b).

Comparison of different preservation strategies on shelf-life extension

To compare the growth of *P. crustosum* under different storage conditions, the amount of DNA per cake was calculated in each case. DNA values within the dynamic range established in the real time PCR were transformed to obtain the corresponding amount of DNA per gram of cake. Therefore, the quantification range was established between 81,200 and 203 ng of DNA/sponge cake.

DNA concentration of *P. crustosum* in modified atmosphere packaged cakes did not change significantly after 30 days of storage at 25 °C, which means that under these conditions there was no net growth of *P. crustosum* (Fig. 4). At the end of storage, DNA concentration was slightly less than the limit of quantification of the method (203 ng of DNA/cake). The cakes packaged in air and stored at 4 °C showed a significant increase in the concentration of *P. crustosum* DNA from day 23 of storage, which means that under these conditions the mould could grow (Fig. 4). However, this growth was not detected visually throughout the incubation period.

Table 2 Full factorial design (FFD) with four factors and two levels

Trials	Factors (A-D)				Response
	A:pH	B: PS (ppm)	C: Packaging atmosphere	D:Storage time (days)	DNA (ng)
1	5.5	1000	MAP	15	306
2	6.5	0	MAP	15	378
3	5.5	0	Air	5	702
4	6	500	Air	10	6600 ^a
5	6	500	Air	10	13,331 ^a
6	6.5	1000	Air	15	5940
7	6.5	0	MAP	5	474
8	6	500	MAP	10	677 ^b
9	5.5	1000	Air	15	4826
10	5.5	0	Air	15	1865
11	5.5	1000	MAP	5	260
12	5.5	1000	Air	5	460
13	6	500	Air	10	8797 ^a
14	5.5	0	MAP	5	487
15	6.5	0	Air	15	4252
16	6.5	1000	MAP	5	660
17	5.5	0	MAP	15	312
18	6.5	0	Air	5	985
19	6	500	MAP	10	390 ^b
20	6	500	MAP	10	291 ^b
21	6.5	1000	Air	5	960
22	6.5	1000	MAP	15	258

pH (5.5–6.5), PS (0–1000 ppm), Packaging atmosphere (Air or MAP), Storage time (5–15 days). Response of the FFD is *P. crustosum* DNA in cakes

^aPS Potassium sorbate, MAP Modified atmosphere packaging N₂:CO₂ 50:50

^aCentre points in Air

^bCentre points in MAP

Discussion

In the present study the combined effect of potassium sorbate, pH and composition of the packaging atmosphere to prevent the spoilage of sponge cakes at room temperature by *Penicillium crustosum* was analysed. The packaging atmosphere was evaluated as a categorical variable with two options: air or N₂:CO₂ 50:50 (Aligal 15), a modified atmosphere recommended by Air Liquide (gas supplier) to store baked products like puddings. For the numerical variables PS concentration, pH, and storage time three values of each one were assayed. PS concentrations varied from zero to 1000 ppm which is the maximum amount legally permitted for bakery products in our country (Decreto N° 315/994 1994). pH levels, which varied from 5.5 to 6.5, were selected among those that might occur in sponge cakes. Such values are more alkaline than the pKa of sorbic acid (pKa = 4.8), restricting the concentration of the undissociated molecule, and thus the antimicrobial activity of the preservative. In fact, at the

values tested (5.5, 6 and 6.5) the undissociated form of the acid represents 15, 5 and 1% respectively. Huang et al. (2010) determined that the minimal inhibitory concentration of sodium sorbate for *P. roqueforti* and *A. flavus* in culture media was ten times lower at pH = 5 than at pH = 6, meaning an important reduction in antifungal activity at the higher pH. In the same way, Suhr and Nielsen (2004) reported that inhibitory activity of sorbate against many moulds that caused spoilage of bakery products drastically decreased at pH = 7.4. Moreover, it has been demonstrated that its inhibitory activity was also dependant on the water activity of the growing media, being higher at lower a_w values (Huang et al. 2010; Saranraj and Geetha 2012). Thus, the antifungal effect of the preservative seems to be very dependent on the chemical composition of the product to be preserved. Salts from other weak organic acids such as propionates and benzoates have also been used to lengthen shelf life of bakery products. However, potassium sorbate has been reported as the most effective for preventing fungal growth

Fig. 3 **a** Pareto charts obtained after the factorial design, showing contribution of factors v/s their main effect on *P. crustosum* growth in sponge cakes. Factors involved in the assay: **c** packaging atmosphere, **d** storage time. **b** Natural log of *P. crustosum* DNA amount per cake

v/s storage time in two different packaging conditions: Air (square) and modified atmosphere $N_2:CO_2$ 50:50 (triangle). Least significant difference ($\alpha = 0.05$) bars are showed for the predicted means

Fig. 4 DNA concentration of *P. crustosum* in cakes over time in air and MAP, at 4 °C and 25 °C respectively. Bars represent confidence intervals ($\alpha = 0.05$) and dotted line (—) shows the quantification limit of the method

in intermediate moisture weakly acidic foodstuffs (Marín et al. 2002; Suhr and Nielsen 2004; Guynot et al. 2005). According to Stratford et al. (2009) the minimum concentration (expressed in mol/L) of sorbic acid needed to inhibit conidial germination of *A. niger* was about 24 and 5 times smaller than those of acetic and propionic acids respectively, which have similar pKas. The current accepted theory of weak acid preservative suggests that antimicrobial action of weak organic acids is due to acidification of the cytoplasm. Moreover, a possible membrane-mediated mode of action of sorbic acid has also been postulated (Stratford et al. 2009). van Beilen et al. (2014) have demonstrated that sorbic acid is a potent

uncoupler of the membrane potential which may slow down the rate of ATP synthesis.

The results obtained in the present study showed that pH and PS concentration were not significant to prevent *P. crustosum* growth in the assayed ranges. Preservation of bakery products commonly involves the use of preservatives, of which sorbate is the most widely used (Marín et al. 2002). However, according to our results, the use of PS could be eliminated from sponge cakes prepared in the assayed conditions, as it did not inhibit fungal growth, not even at the maximum level permitted by our legislation. The use of lower a_w and pH values could be an alternative to prevent microbial spoilage of sponge cakes. However, since those changes could adversely affect the quality of

the product causing undesirable changes in taste and texture, other alternatives such as a modified packaging atmosphere or lower storage temperatures should be evaluated. In our study *P. crustosum* growth was evident in 15 days in sponge cakes ($a_w = 0.85$) packaged in air and stored at room temperature in the presence or absence of potassium sorbate. However, when a modified packaging atmosphere ($N_2:CO_2$ 50:50) was used, no fungal growth could be registered at 25 °C, at least for 30 days. That strategy resulted more successful than the storage in air at low temperature, in which case *P. crustosum* growth was detected after 20 days. Moreover, the use of MAP avoids the costs of cold chain maintenance. The utility of MAP to prevent fungal growth on cakes has been previously reported by (Guynot et al. 2003), who determined that inhibition of fungal growth on sponge cakes in a MAP of $N_2:CO_2$ 50:50 depended on the fungi and the a_w of the substrate, but not on the pH in the tested range (6–7.5). Higher CO_2 concentrations in the packaging atmosphere could produce a greater inhibition on fungal growth (Hasan and Abdolgader 2012). However, since CO_2 could be solubilized in the liquid phase of the packaged product, causing a decrease in the pH, associated with sensorial changes, the concentration of that gas should be carefully selected (Guynot et al. 2003). In fact, mixtures of gases with concentrations of CO_2 higher than 70% are not recommended for biscuits (Guynot et al. 2003). In our case, the use of a MAP ($N_2:CO_2$ 50:50) did not produce changes in the pH of the packaged cakes over the storage period.

Food packaging atmospheres with low O_2 concentrations generates concerns about safety with respect to the growth of anaerobic pathogens such as *Clostridium botulinum*. This pathogen is strictly anaerobic and produces botulinum neurotoxin that presents a lethal risk to the consumer (Suppakul et al. 2003). *C. botulinum* needs a minimum water activity of 0.93 for growth (Carter and Peck 2015) and 0.96 for neurotoxin production (Dodds 1989). These studies show that this pathogen is a threat for high moisture products, not for intermediate moisture products such as sponge cakes (0.75–0.95 a_w). So, it may be conclude that the use of MAP is a safe strategy for this kind of products.

In the current study *P. crustosum* growth was evaluated by quantifying DNA corresponding to the fungal species in sponge cakes using a newly developed real time PCR method. Specific primers were designed and PCR conditions were established to ensure specificity. The selected primer pair showed a high specificity for *P. crustosm* and was successfully employed in a standardized real-time PCR for the direct quantification of this fungus in sponge cakes. The PCR method designed allowed specific and quantitative detection of *P. crustosum* in sponge cakes with a higher sensitivity level (detection limit of 0.0014 ng of

DNA) compared with other works where qPCR methods were developed to quantify the growth of other *Penicillium* species. In that sense Tannous et al. (2015) reported a detection limit of 0.1 ng of DNA for a real time PCR method designed to quantify *P. expansum* in apples. Previous works studying the inhibition of fungal growth in bakery products have been based on visual determinations of fungal growth such as measurement of colony diameter (Guynot et al. 2002, 2003). However, for food safety it is important to detect fungal presence before biodeterioration signs are visible. In the present work, a sensible and specific qPCR method was developed that allowed detection of fungal growth before moulds were visible. These kind of methods that can detect and quantify low amounts of DNA of food spoilage species would be important in predicting deterioration, and would be even more relevant if the species involved are able to produce mycotoxins. In this sense, these methods allow the early detection of potential producing fungi before reaching an unacceptable level of toxin or even prior to its synthesis (Tannous et al. 2015).

Conclusion

The use of MAP resulted a safe and effective strategy to inhibit the growth of *P. crustosum* in sponge cakes stored at room temperature. Other strategies, such as the use of potassium sorbate as preservative, proved to be useless in these products since fungal growth could not be inhibited, even when the maximum concentration allowed by our legislation was added. This leads to the conclusion that is not necessary to add sorbate to sponge cakes premixes. These results could be used by the bakery industries to reformulate their products in order to meet new consumer trends.

The qPCR method developed in this work resulted a sensitive and specific method to predict cakes deterioration by *P. crustosum*, since it could detect *P. crustosum* growth before signs of visual deterioration appeared. The method developed could be used for further studies in *P. crustosum* detection.

Acknowledgements We thank the Comisión Sectorial de Investigación Científica (CSIC, Uruguay) and the Agencia Nacional de Investigación e Innovación (ANII, Uruguay) for financial aid. We also thank OLASO (Uruguay) for providing cakes samples.

References

- Altschul SF, Gish W, Miller W et al (1990) Basic local alignment search tool. J Mol Biol 215:403–410

- Anderson MJ, Whitcomb PJ (2016) DOE simplified: practical tools for effective experimentation. CRC Press, London
- Arquiza JMRA, Hunter J (2014) The use of real-time PCR to study *Penicillium chrysogenum* growth kinetics on solid food at different water activities. *Int J Food Microbiol* 187:50–56
- Branquinho MR, Ferreira RTB, Cardarelli-Leite P (2012) Use of real-time PCR to evaluate two DNA extraction methods from food. *Food Sci Technol* 32:112–118
- Carter AT, Peck MW (2015) Genomes, neurotoxins and biology of *Clostridium botulinum* Group I and Group II. *Res Microbiol* 166:303–317
- CRL-GMFF (2008) Report on the validation of a DNA extraction method for maize seeds and grains. Community Reference Laboratory for GM Food and Feed. European Union Reference Laboratory for GM Food and Feed Publishing. http://gmo-crl.jrc.ec.europa.eu/summaries/LY038_DNAExtr_report.pdf. Accessed 10 Oct 2018
- Decreto N°315/994(1994) Reglamento Bromatológico Nacional, 3rd edn. IMPO Publisher. <https://www.imo.com.uy/bases/decretos-reglamento/315-1994>. Accessed 10 Oct 2018
- Dodds KL (1989) Combined effect of water activity and pH on inhibition of toxin production by *Clostridium botulinum* in cooked, vacuum-packed potatoes. *Appl Environ Microbiol* 55:656–660
- Dorak MT (2006) Real-time PCR. Taylor & Francis Group, UK
- Forsythe SJ, Hayes PR (2000) Food spoilage. In: Forsythe SJ, Hayes PR (eds) Food hygiene, microbiology and HACCP, 3rd edn. Springer, New York, pp 86–149
- Gonda M (2015) New technologies, alternative to preservatives, to inhibit biodeterioration of bakery products during packaging and storage at room temperature (Tecnologías alternativas para inhibir el biodeterioro en productos panificados envasados y conservados a temperatura ambiente. Master in Biotechnology. Facultad de Ciencias, Universidad de la República, Uruguay. Colibrí Udelar publishing. <https://www.colibri.udelar.edu.uy/jspui/handle/123456789/8379>. Accessed 10 October 2018
- Guynot ME, Ramos AJ, Sala D et al (2002) Combined effects of weak acid preservatives, pH and water activity on growth of *Eurotium* species on a sponge cake. *Int J Food Microbiol* 76:39–46
- Guynot ME, Marín S, Sanchis V, Ramos AJ (2003) Modified atmosphere packaging for prevention of mold spoilage of bakery products with different pH and water activity levels. *J Food Prot* 66:1864–1872
- Guynot ME, Marín S, Sanchis V, Ramos AJ (2004) An attempt to minimize potassium sorbate concentration in sponge cakes by modified atmosphere packaging combination to prevent fungal spoilage. *Food Microbiol* 21:449–457
- Guynot ME, Marín S, Sanchis V, Ramos AJ (2005) An attempt to optimize potassium sorbate use to preserve low pH (4.5–5.5) intermediate moisture bakery products by modelling *Eurotium* spp., *Aspergillus* spp. and *Penicillium corylophilum* growth. *Int J Food Microbiol* 101:169–177
- Hasan S, Abdolgader R (2012) Study of weak acid preservatives and modified atmosphere packaging (MAP) on mold growth in modal agar system. *Food Nutr Sci* 3:802–809
- Huang Y, Wilson M, Chapman B, Hocking AD (2010) Evaluation of the efficacy of four weak acids as antifungal preservatives in low-acid intermediate moisture model food systems. *Food Microbiol* 27:33–36
- Janjarasskul T, Tananuwong K, Kongpensook V et al (2016) Shelf life extension of sponge cake by active packaging as an alternative to direct addition of chemical preservatives. *LWT Food Sci Technol* 72:166–174
- Marín S, Guynot ME, Neira P et al (2002) Risk assessment of the use of sub-optimal levels of weak-acid preservatives in the control of mould growth on bakery products. *Int J Food Microbiol* 79:203–211
- Mensah-Attipoe J, Reponen T, Veijalainen A-M et al (2016) Comparison of methods for assessing temporal variation of growth of fungi on building materials. *Microbiology* 162:1895–1903
- Mohammadzadeh-Aghdash H, Sohrabi Y, Mohammadi A et al (2018) Safety assessment of sodium acetate, sodium diacetate and potassium sorbate food additives. *Food Chem* 257:211–215
- Pasukamonset P, Pumalee T, Sanguansuk N et al (2018) Physico-chemical, antioxidant and sensory characteristics of sponge cakes fortified with *Clitoria ternatea* extract. *J Food Sci Technol* 55:2881–2889
- Pitt JI, Hocking AD (2009) Spoilage of stored, processed and preserved foods. In: Pitt JI, Hocking AD (eds) *Fungi and food spoilage*, 3rd edn. Springer, New York, pp 401–421
- Qiu L, Zhang M, Tang J et al (2019) Innovative technologies for producing and preserving intermediate moisture foods: a review. *Food Res Int* 116:90–102
- Saranraj P, Geetha M (2012) Microbial spoilage of bakery products and its control by preservatives. *Int J Pharm Biol Arch* 3:38–48
- Smith JP, Daifas DP, El-Khoury W et al (2004) Shelf life and safety concerns of bakery products—a review. *Crit Rev Food Sci Nutr* 44:19–55
- Stratford M, Plumridge A, Nebe-von-Caron G, Archer DB (2009) Inhibition of spoilage mould conidia by acetic acid and sorbic acid involves different modes of action, requiring modification of the classical weak-acid theory. *Int J Food Microbiol* 136:37–43
- Suhr KI, Nielsen PV (2004) Effect of weak acid preservatives on growth of bakery product spoilage fungi at different water activities and pH values. *Int J Food Microbiol* 95:67–78
- Suppakul P, Miltz J, Sonneveld K (2003) Bigger SW (2003) Active packaging technologies with an emphasis on antimicrobial packaging and its applications. *J Food Sci* 68:408–420
- Tancinová D, Barbořáková Z, Masková Z et al (2012) The occurrence of micromycetes in the bread samples and their potential ability produce mycotoxins. *J Microbiol Biotechnol Food Sci* 1:813–818
- Taniwaki MH, Pitt JI, Hocking AD, Fleet GH (2006) Comparison of hyphal length, ergosterol, mycelium dry weight, and colony diameter for quantifying growth of fungi from foods. In: Hocking AD, Pitt JI, Samson RA, Thrane U (eds) *Advances in Food Mycology*. Springer, New York, pp 49–67
- Tannous J, Atoui A, El Khoury A et al (2015) Development of a real-time PCR assay for *Penicillium expansum* quantification and patulin estimation in apples. *Food Microbiol* 50:28–37
- Valle Garcia M, Sonnenstrahl Bregão A, Parussolo G et al (2019) Incidence of spoilage fungi in the air of bakeries with different hygienic status. *Int J Food Microbiol* 290:254–261
- van Beilen JWA, Teixeira de Mattos MJ, Hellingwerf KJ, Brul S (2014) Distinct effects of sorbic acid and acetic acid on the electrophysiology and metabolism of *Bacillus subtilis*. *Appl Environ Microbiol* 80:5918–5926
- Ye J, Coulouris G, Zaretskaya I et al (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 13:134–144

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.