International Journal of Food Microbiology 138 (2010) 100–107

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

# Quantification of *Penicillium camemberti* and *P. roqueforti* mycelium by real-time PCR to assess their growth dynamics during ripening cheese

G. Le Dréan *, J. Mounier [1], V. Vasseur [1], D. Arzur [1], O. Habrylo [1], G. Barbier [1]

*Université Européenne de Bretagne, France*
*Université de Brest, EA 3882 Laboratoire Universitaire de Biodiversité et Ecologie Microbienne, IFR148 ScInBioS, Ecole de Microbiologie et Sécurité Alimentaire de Brest, Technopôle Brest-Iroise, 29280 Plouzané, France*

ARTICLE INFO

*Article history:*
Received 4 September 2009
Received in revised form 13 November 2009
Accepted 10 December 2009

*Keywords:*
Mould-ripened cheeses
*Penicillium camemberti*
*Penicillium roqueforti*
Mycelial biomass quantification
Quantitative real-time PCR

ABSTRACT

Real-time PCR has been applied to quantify mycelium of *Penicillium camemberti* and *Penicillium roqueforti* during ripening of model cheese curd and surface mould-ripened cheeses. Total fungal DNA was first validated as an indicator of mycelial biomass in pure liquid culture and then in model curds at different stages of ripening. To imitate cheese matrix effects, DNA was extracted from curd mixed with known amounts of fresh mycelium of *P. camemberti* or *P. roqueforti* and was used as biomass standards for further quantitative real-time PCR. Mycelial mass per cheese (mg/g) was then directly obtained from fluorescence data. In model cheese curd, mycelial mass of *P. camemberti* increased from 2.8 at d4 to 596 mg/g at d11 whereas *P. roqueforti* increased from 0.3 to 6.3 mg/g during the same period. *P. camemberti* showed a fast development in Coulommiers from d2 to d9 (66 to 119 mg/g) and a 100-fold increase in Carré (0.85 to 85 mg/g). While mycelial biomass reached a maximum at d9 in Coulommiers, it still developed in Carré until d45. For the first time, cheese manufacturers have a powerful technique to monitor mycelial growth dynamics of their fungal cultures, which represents an important step for controlling cheese making.


## 1. Introduction

*P. camemberti* and *P. roqueforti* play a fundamental role in the ripening process of surface mould-ripened and blue-veined cheeses. For cheese manufacture, controlling the growth of fungal ripening cultures is crucial, for example, to ensure that cheeses are rapidly colonized, which avoids the implantation of contaminants (Nielsen et al., 1998) or to monitor aroma production by the metabolically active biomass. To better define the quantitative relationship between microbiological and biochemical changes during ripening, each component of the cheese microbiota needs to be quantified, including the mycelium (hyphae) of fungal populations. Traditional methods used to quantify the viable biomass are based on colony forming units (CFU) counting after seeding on convenient solid medium. However, hyphal filaments cannot be quantified by using this enumeration technique because viable counts usually reflect spore numbers rather than mycelium. In young colonies or inside food particles, fungal growth consists predominantly of hyphae and viable counts are usually low. In contrast when sporulation occurs, CFU counts increase without a real increase in fungal biomass (Taniwaki et al., 2006).

An alternative to the quantification of viable biomass is the measurement of total biomass. Estimation of mycelium dry weight is commonly used to assess fungal growth in liquid medium but appears much less suited to complex matrices such as cheeses in which hyphal filaments interpenetrate. Indirect methods involving the measurement of substrate consumption are being developed to counteract these difficulties (Aldarf et al., 2002) but this approach may not be easily applicable in industry. Another approach based on measurement of hyphal length by direct microscopy is particularly laborious and cannot be used to estimate fungal biomass in foods (Taniwaki et al., 2006). Ergosterol content is a quite sensitive indicator of biomass but intraspecific variations are observed depending on the strains, growth medium, and cultivation time. Nevertheless, measurement of ergosterol is currently employed to characterise fungal contamination of solid substrates such as cereals (Dong et al., 2006) or soils (Montgomery et al., 2000) and also in dust, buildings materials (Reeslev et al., 2003) or indoor air (Robine et al., 2005). Data are also available in plant tissue (Gessner and Schmitt, 1996). Measurements of ergosterol concentration in fungal biomass need a prior extraction by organic solvents. In lipid-rich matrix such as cheese, this step represents an important limitation in the sensitivity of the technique. Moreover, yeasts are found within the microflora of Camembert-type (Corsetti et al., 2001) and blue-veined cheeses (Roostita and Fleet, 1995) and can represent an important part

* Corresponding author. Ecole Supérieure de Microbiologie et Sécurité Alimentaire de Brest, Technopôle Brest-Iroise, 29280 Plouzané, France. Tel.: +33 6 62 37 91 28; fax: +33 2 98 05 61 01.
*E-mail address:* gwenola.ledrean@univ-brest.fr (G. Le Dréan).
[1] Postal address: Ecole Supérieure de Microbiologie et Sécurité Alimentaire de Brest, Technopôle Brest-Iroise, 29280 Plouzané, France.

0168-1605/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2009.12.013

of the total biomass of these products. Thus, ergosterol measurement would lead to overestimation of the mycelial biomass in cheeses.

In recent years, the Polymerase Chain Reaction (PCR) method has been used successfully to detect and identify fungi. In food mycology, application of PCR-based methods for identification of filamentous fungi has been extensively developed notably for mycotoxin producing species (for review, Niessen, 2008). In cheese, the culture-independent methods available for the description of both bacterial and fungal communities have been recently reviewed (Jany and Barbier, 2008). Quantitative real-time PCR (qRT-PCR) has proven to be a simple yet reliable technique to quantify microorganisms as bacteria, for example lactic acid bacteria in milk products (Furet et al., 2004) or yeasts, for example total yeasts in wine (Hierro et al., 2006), *Candida* spp. cells in tap water (Brinkman et al., 2003) or *Geotrichum candidum* in red-smear cheese (Larpin et al., 2006). In such single cells, one molecule of DNA is representative of one cell and conversion of DNA amount to biomass is possible if copy number of the target sequence used for qRT-PCR is known. In filamentous fungi, this approach is more complex since these microorganisms evolved in their environment as conidia and multinuclear hyphae. Filamentous fungi differ in cell length, cell volume and amount of DNA per cell and it is therefore not possible to determine a universal conversion factor to convert DNA quantities into fungal biomass (Guidot et al., 2002). Consequently, in fungi, only conidia (Roe et al., 2001) or germinating conidia (Fredricks et al., 2005) are quantified. As previously considered in soils (Guidot et al., 2002; Landeweert et al., 2003), expressing the results as DNA amount per gram of substrate may be sufficient for a relative comparison of fungal abundance in samples. Such an approach has been used for quantitative detection of *Aspergillus ochraceus* in green coffee (Schmidt et al., 2004) or *Aspergillus carbonarius* in grapes (Mulè et al., 2006) to evidence a positive correlation between ochratoxin A content and DNA quantity. However, in a cheese manufacture context, such a relative comparison remains unsatisfactory and growth dynamics of mycelium expressed as quantity of biomass per gram of cheese is easier to use and then rather expected.

The aim of the present work was to develop a SYBR green-based qRT-PCR assay to quantify *P. roqueforti* and *P. camemberti* biomass in curd and to monitor their growth during ripening. The use of DNA as a marker of biomass of *P. roqueforti* and *P. camemberti* was first validated in liquid cultures. Fungal mycelial mass was then monitored and quantified during ripening in model cheese curd and in industrial Camembert-type cheeses using qRT-PCR.

## 2. Materials and methods

### 2.1. Fungal strains

Freeze-dried spores of *P. camemberti* (Degussa, France) and *P. roqueforti* TT PR5 (Chr Hansen, France) were stored at 4 °C. Each lyophilisate (1 g) was hydrated in 10 mL distilled water containing 0.01% Tween 80 (Sigma, France) prior to inoculation of Potato Dextrose Agar (PDA) (Difco, Becton Dickinson and Co., MA, USA) slants.

### 2.2. Liquid culture conditions

First, an initial spore suspension of *P. camemberti* and *P. roqueforti* was prepared by washing respectively PDA slants (incubated for 7 d at 25 °C) using 1–2 ml of distilled water containing 0.01% Tween 80. The spores were enumerated with a Malassez counting chamber. The spore suspension was diluted using sterile distilled water containing 0.01% Tween 80 to get a concentration of $10^7$ spore/mL. Fungal cultures were prepared in 250 mL Erlenmeyer flasks containing 50 mL Potato Dextrose Broth medium (Difco). Each flask was inoculated with 0.5 mL of the conidial suspension. The flasks were incubated in shaken cultures (150 rpm) at 25 °C or 14 °C for kinetic analysis during 1 month. These two different temperatures of growth (25 °C and 14 °C) were tested to describe the evolution of the DNA per biomass ratio during the mycelial development: 25 °C is considered as the optimal temperature of growth in *Penicillium* spp. (Pitt and Hocking, 1997) and 14 °C is the one used for cheese ripening. At the end of the incubation period, each fungal culture was filtered through Whatman N° 2 filter paper and rinsed with distilled water, frozen in liquid nitrogen, lyophilized to constant weight (Christ, Fisher Bioblock Scientific, Illkirch, France) and weighted. The fungal biomass of *P. camemberti* and *P. roqueforti* was estimated in triplicate.

### 2.3. Model curd and cheese manufacture

Preliminary assays were made on model curd inoculated with *P. camemberti* or *P. roqueforti*. Under sterile conditions, 23 g milk protein concentrate, 20 g anhydrous milk fat, 50 mL sterile distilled water, 1.5 g NaCl and 1.26 mL lactate were mixed to prepare 100 g of curd. Milk protein concentrate and anhydrous milk fat were provided by Lactalis (Retiers, France). The desired quantity of model curd was prepared and inoculated with a conidial suspension ($10^7$/mL) of *P. camemberti* or *P. roqueforti* to yield a conidial concentration of $10^5$/g of cheese. Fifty grams of model curd were then transferred to sterile crystallizing dishes with a diameter of 5.6 cm and incubated at 14 °C for 28 d. Samples were taken at different time intervals during ripening.

Cheese samples *e.g.* Coulommiers and Carré were directly provided by a cheese manufacturer, from a same batch, at different stages of ripening. Cheeses were ripened at 12 °C for 9 d, then packed and stored at 4 °C for 36 d.

### 2.4. Model curd and cheese sampling

The surface of each model curd or cheese was marked with the rim of a sterile plastic container (32-mm diameter) and a layer (32-mm diameter and ~5 mm in depth) was removed with a sterile scalpel. Sufficient sterile 2% (wt/v) trisodium citrate was added to yield a 1:10 dilution and the resulting suspension was homogenized with an Ultra Turrax® (Labortechnik, Rungis, France) at 24,000 rpm/min for 1 min. Each sample of curd or cheese was analyzed in triplicate for *P. camemberti* or *P. roqueforti* mycelium DNA quantification using qRT-PCR as described below.

### 2.5. CFU counting

Conidia from Coulommiers and Carré cheeses samples were enumerated by surface-plating on M2Lev agar (20 g/L malt extract, 3 g/L yeast extract and 15 g/L agar) containing 50 mg/L penicillin and streptomycin after 7 d-incubation at 25 °C.

### 2.6. DNA extraction and quantification

#### 2.6.1. From pure culture samples

About 10 mg of lyophilized mycelium from each strain were used for total genomic DNA extraction using the BIO 101 FastDNA® Kit (QBiogene, MP Biomedicals, Illkirch, France), according to the manufacturer's instructions. DNA was recovered and dissolved in sterile water. DNA concentrations were determined by measuring the ultra-violet fluorescence (GelDoc, Bio-Rad, Hercules, CA, USA) emitted by ethidium bromide molecules intercalated into DNA after migration on gel electrophoresis (Sambrooks and Russel, 2001). The fluorescent yield of the samples was compared to DNA standards (Calf thymus DNA, Sigma, St Louis, MO, USA).

#### 2.6.2. From model curd and cheeses samples

One mL of the trisodium citrate-cheese suspension (see above) was delipidated by adding 67 µl of isoamylic alcohol, followed by 10 min-incubation at 45 °C and centrifugation at 10,000*g* for 10 min at room temperature. The fat layer was gently removed using a pipetman tip and the supernatant was discarded. The remaining pellets were dried by lyophilisation, weighted and resuspended in 1 mL of the buffer lysis

provided in the FastDNA® Kit. DNA extraction from each cheese sample was made in triplicate.

### 2.7. Primer design

Primers were designed using available internal transcribed spacer (ITS) and beta-tubulin sequences of *P. camemberti* and *P. roqueforti* from Genbank database. Sequences were first aligned using Mega4 software (Tamura et al., 2007) and the PCR primers were designed from this alignment using the Primer3 software (Rozen and Skaletsky, 2000), in accordance with the criteria required for qRT-PCR primer design. Specificity of the primers was then tested *in silico* on a large number of sequences of yeasts and fungal species that may be encountered during cheese making. The primers were purchased from Sigma-Proligo (Evry, France). For *P. camemberti*, specific primers BETcam1F 5′-CGATGGC-GATGGACAGTAAG-3′ and BETcam1R 5′-TACTTGTCACCGCTGGCCTA-3′ targeting the beta-tubulin gene were designed to amplify a 207 bp fragment. For *P. roqueforti*, a 106 bp fragment from the ITS 1 region of the rRNA genes was amplified using the following primers: ITSroq1F 5′-ACCCCGAACTCTGTCTGAAG-3′ and ITSroq1R 5′-ATTTCGCTGCGTTCTT-CATC-3′.

### 2.8. qRT-PCR assay

qRT-PCR assay was performed using a Mini Opticon thermocycler on 48-well reaction plates (Bio-Rad, Hercules, CA, USA). Each well contained a 15-µL reaction mixture that included 7.50 µL of 2× SYBR Green PCR Master Mix (Bio-Rad,), 0.3 µM each of forward and reverse primers, 1 µL of DNA template at convenient dilution. For pure mycelium samples, a standard curve was constructed using 10-fold dilutions of a known amount of *P. camemberti* ($10^{-1}$ to $10^{-5}$) or *P. roqueforti* ($10^{-2}$ to $10^{-6}$) purified and quantified DNA (Nanodrop Technology®, Labtech, Palaiseau, France). The following thermocycling pattern was used: 95 °C for 3 min; 95 °C for 15 s, 60 °C for 1 min (40 cycles); and 72 °C for 15 s. A melting-curve analysis was performed at the end of each PCR assay to control that a single PCR-product was amplified.

To imitate cheese matrix effects, known amounts of *Penicillium* spp. mycelium (from 20 to 600 mg fresh weight) were mixed to model cheese curd to yield a final weight of 1 g. Fresh mycelium was obtained from 10 day-old colony, growing on cellophane sheet placed over curd. Sufficient sterile 2% (wt/v) trisodium citrate was added to yield a 1:10 dilution and the resulting suspension was homogenized with an Ultra Turrax® at 24,000 rpm/min for 1 min. Total DNA from each mixture was extracted as described above for model curd and cheese samples. Then, qRT-PCR was carried out on appropriate DNA dilution in triplicate using DNA extracted from *P. camemberti* or *P. roqueforti* grown in liquid culture as a standard.

To construct a standard curve for direct calculation of the fungal biomass in model curd and cheese, one of the previous standards (400 and 250 mg fresh mycelia mixed with 600 and 750 mg of model cheese curd for *P. camemberti* and *P. roqueforti*, respectively) was 10-fold diluted from $10^{-1}$ to $10^{-6}$. Direct quantification of mycelium biomass of the target sample was determined by interpolation of the threshold cycle values (Ct) of each sample in the corresponding standard curve, as previously described (Parladé et al., 2008).

### 2.9. Statistical analysis

All the statistical analyses were performed with the Statgraphics Plus software (Statpoint Technologies Inc., Herdon, VA, USA). One-way analysis of variance (ANOVA) was carried out to detect significant differences among means. Student–Newman–Keuls or Scheffe tests were applied to compare the mean values. When ANOVA was not applicable because of heterogeneous variances (Bartlett test), the Kruskall–Wallis test was used. Statistical significance was set at $P$ value $\leq 0.05$.

## 3. Results

### 3.1. Growth time-course of P. camemberti and P. roqueforti and DNA per biomass ratio in pure culture at 25 °C

For *P. camemberti*, mycelial biomass increased from 0.1 g at d2 to 0.3 g of dry matter (DM) at d10 (Fig. 1A). Then, mycelial biomass decreased to reach 0.21 g DM after 28 d. The DNA per biomass ratio (ng/µg) showed two significantly distinct levels: until the 12th day of growth, this ratio had an average of 0.83. At d18, this value was lower (0.29) but remained nearly constant till the end of growth (d27).

For *P. roqueforti*, the maximum amount of mycelial biomass was reached as soon as d6 (0.15 g DM) and remained constant further (Fig. 1B). Variations of the DNA per biomass ratio were not significant from d3 to d17 (average of 1.27 ng/µg) while at d25, a significant decrease was observed (0.56 ng/µg).

### 3.2. Growth time-course of P. camemberti and P. roqueforti and DNA per biomass ratio in pure culture at 14 °C

For *P. camemberti*, the time-course of growth was slower (Fig. 2A), than that observed at 25 °C. The amount of biomass increased from 0.16 g at d5 to 0.3 g at d20. A slight decrease of the total biomass was then observed at d28 with 0.24 g DM. The variations of the mean DNA per biomass ratio (0.62 ng/µg) were not significant during growth.

For *P. roqueforti*, the time-course of growth at 14 °C was different from that observed at 25 °C (Fig. 2B) since total biomass significantly increased from 0.14 g DM at d5 to 0.24 g DM at d20 and then remained constant at d28. The mean ratios of DNA per biomass were not significantly different all over the time-course of growth, with a mean value of 0.49 ng/µg.

### 3.3. qRT-PCR to quantify known amounts of mycelium in curd (standards)

From data obtained above, standards were constructed with known amounts of fresh mycelium mixed with cheese curd. DNA extraction from those standards was quantified by qRT-PCR (Fig. 3). For *P. camemberti* (A), a linear relationship ($R^2 = 0.9678$) was established between DNA concentrations and initial biomass until 400 mg of fresh mycelium per gram of model cheese curd. Over this range, DNA recovery saturated. For *P. roqueforti* (B), a linear relationship was described between 0 and 250 mg of fresh mycelium per gram of model cheese curd ($R^2 = 0.9614$). Over this range, DNA extraction saturated. Absolute values of DNA amounts were approximately 100-fold higher in *P. roqueforti* standards (amplified DNA using multi-copy ITS region primers) as compared to *P. camemberti* ones (amplified DNA using single copy beta-tubulin gene primers).

Standard curves for further real-time PCR analysis of model cheese curd or cheese samples were constructed, using different dilutions ranging from $10^1$ to $10^5$ of *P. camemberti* or from $10^2$ to $10^6$ of *P. roqueforti* genomic DNA extracted from respectively 400 and 250 mg of fresh biomass mixed to 1 g of curd. For both species, quantification showed a linear relation between log values of fungal genomic DNA and real-time PCR threshold cycles over the range of DNA concentrations examined (Fig. 4).

### 3.4. qRT-PCR in model cheese curds

Mycelial biomass of *P. camemberti* dramatically increased in model cheese curd from d4 to d21 (360-fold increase) to reach values around 1 g/g (mycelial biomass per curd) at the end of ripening (Fig. 5A).

For *P. roqueforti*, growth of mycelium was particularly fast from d4 to d8 (15-fold increase) and from d11 to d13 (6-fold increase) (Fig. 5B). Then, growth was slower until d21 (2-fold increase) and remained stable until d28 (average of 67 mg/g).

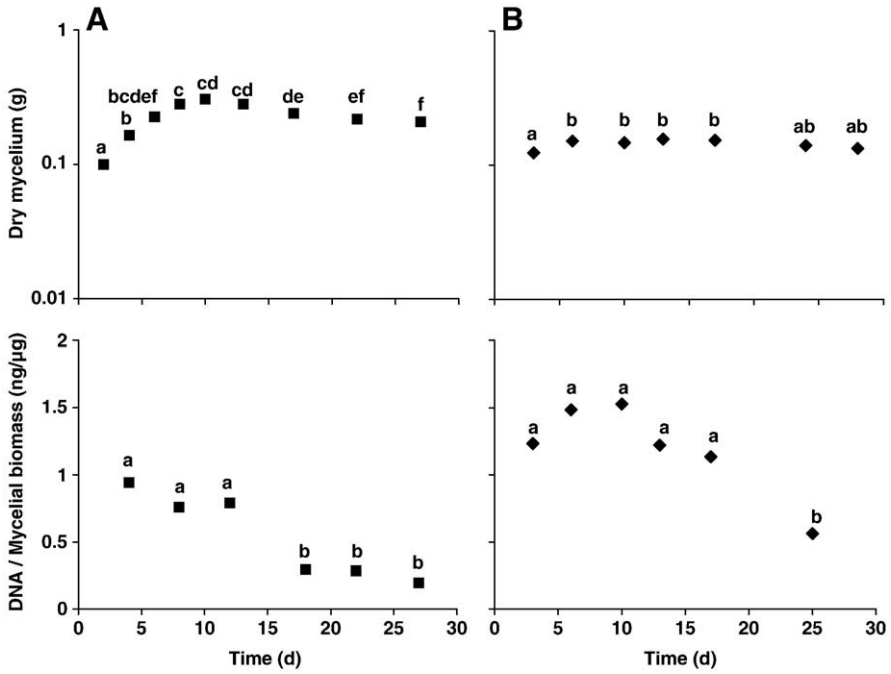


**Fig. 1.** Time-course of growth (upper two graphs) and DNA per mycelial mass ratio (lower two graphs) for (A) *Penicillium camemberti* and (B) *P. roqueforti* cultivated at 25 °C in an agitated liquid medium. Extracted genomic DNA was quantified by agarose-gel electrophoresis. Mean values ($n=3$) bearing different letters are significantly different ($p<0.05$).

### *3.5. qRT-PCR and CFU counting in ripening Camembert-type cheeses (Coulommiers and Carré)*

After 2 d of ripening, *P. camemberti* biomass was 10-fold higher in Carré cheese compared to Coulommiers. From d2 to d9, mycelial development in the both cheeses was exponential: hyphal biomass reached 119 and 86 mg/g at d9, respectively (Fig. 6A). Then, the biomass of *P. camemberti* hyphae remained constant till the end of ripening in Coulommiers cheese whereas a prolonged increase (ten-fold increase compared with d9) was observed until d45 in Carré cheese. In the both cheeses, CFU counts slightly decreased from d2 to d6 and suddenly increased at d9 (Fig. 6B).

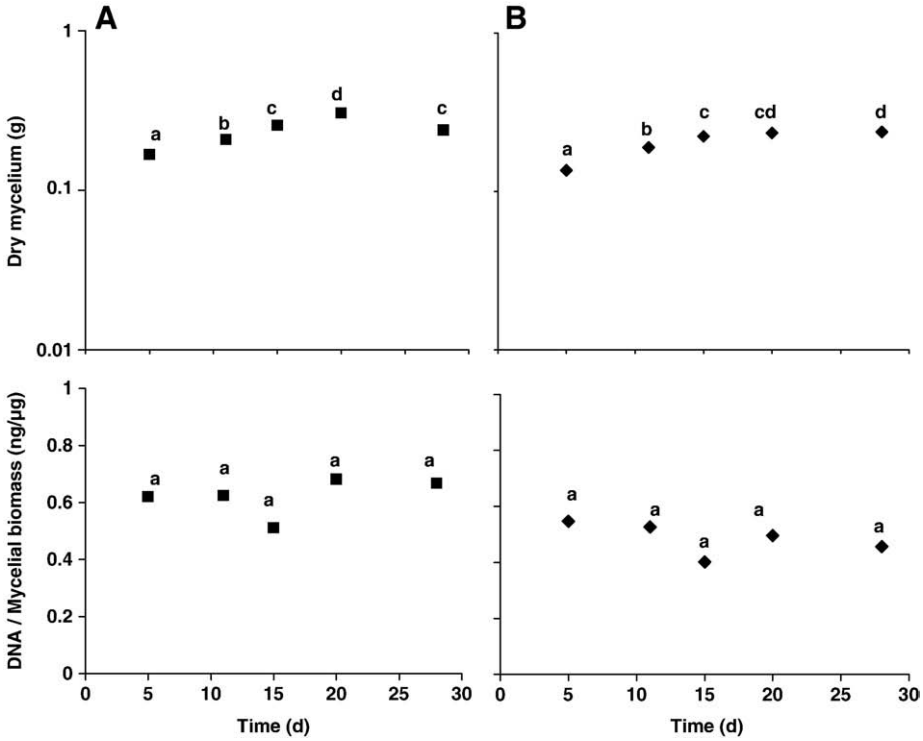


**Fig. 2.** Time-course of growth (upper two graphs) and DNA per mycelial mass ratio (lower two graphs) for (A) *P. camemberti* and (B) *P. roqueforti* cultivated at 14 °C in an agitated liquid medium. Extracted genomic DNA was quantified by agarose-gel electrophoresis. Mean values ($n=3$) bearing distinct letters are significantly different ($p<0.05$).

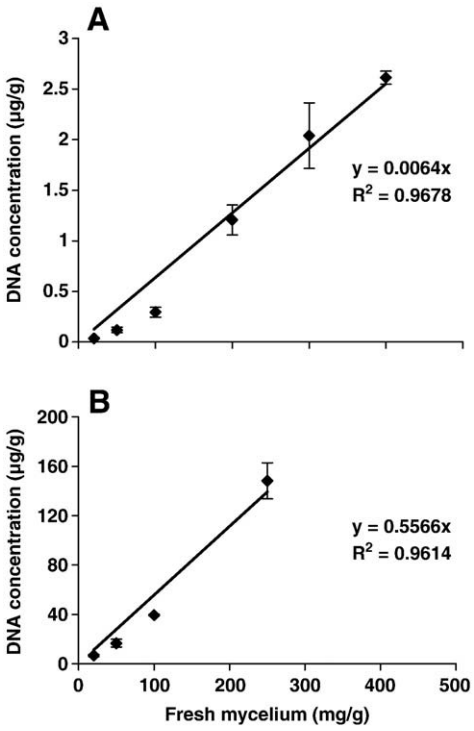


**Fig. 3.** Amounts of DNA quantified by qRT-PCR after extraction from different known amounts of fresh mycelium of (A) *P. camemberti* or (B) *P. roqueforti* mixed to fresh curd (qsp 1 g). Standard curves for qRT-PCR were constructed with purified genomic DNA of each species. *P. camemberti* DNA was amplified using specific primers targeting the β-tubulin gene (single copy gene) whereas *P. roqueforti* DNA was amplified in the ITS1 region of the rRNA genes (multi-copy genes) (100-fold difference in the *y*-axis between the two species).

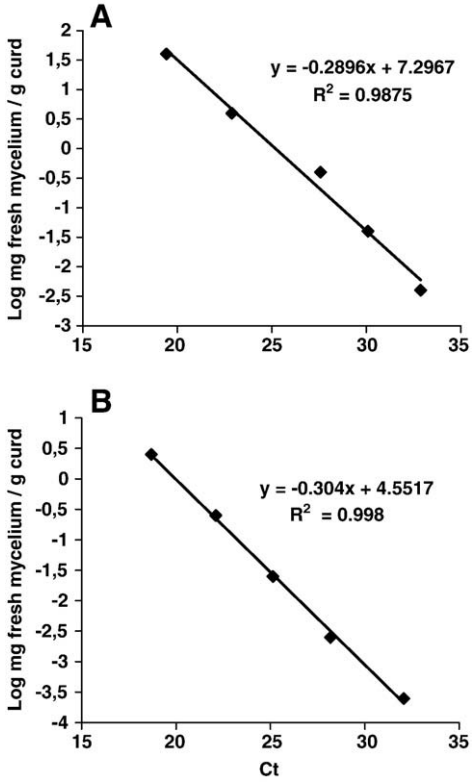


**Fig. 4.** Standard curves generated with the Ct values plotted against the logarithm of the amount of (A) *P. camemberti* or (B) *P. roqueforti* mycelium mixed to milk curd. DNA extracted from one standard mix described in Fig. 3 (400 and 250 mg/g for *P. camemberti* and *P. roqueforti*, respectively) was serially ten-fold diluted to carry out qRT-PCR.

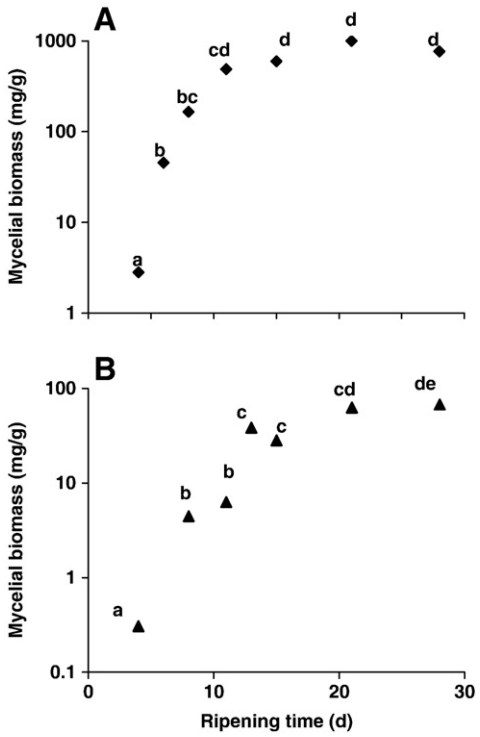


**Fig. 5.** Estimated mycelial biomass quantified by qRT-PCR (using standard curves described in Fig. 4, so that data are directly expressed as biomass concentration in curd) during ripening of model cheeses curd inoculated by (A) *P. camemberti* or (B) *P. roqueforti*. Mean values bearing distinct letters are significantly different ($p<0.05$).

## 4. Discussion

The first part of this study aimed at testing the use of DNA as a biomass indicator for *P. camemberti* and *P. roqueforti* hyphae, first in pure culture and then in cheese matrix. This step was important to check before proceeding to qRT-PCR. In filamentous fungi, the DNA quantity measured cannot be directly converted into cell numbers, because such a conversion factor appears meaningful only in the case of unicellular microorganisms (Guidot et al., 2002). Anyway, cheese manufacturers expect that qRT-PCR data should allow expressing dynamic growth of molds in biomass units and not only in relative amounts of DNA. Some authors have proposed a calibration and conversion of ITS amounts quantified by qRT-PCR into absolute biomass of the extramatrical mycelium of *Piloderma croceum*, an ectomycorrhizal fungus (Schubert et al., 2003; Raidl et al., 2005). However, in cheese, this approach would be limited by the difficulty to measure length of hyphae that are intrinsic or in close relationship with milk matrix. Moreover, even though the number of ITS copies is supposed to be constant for each species, it is still unknown in *Penicillium* spp.

To first validate the use of DNA as a biomass indicator, we studied the evolution of the amounts of DNA recovered from different amounts of dry pure mycelium in liquid culture. *P. camemberti* and *P. roqueforti* are known to produce mainly mycelium when cultivated in liquid agitated media (Boualem et al., 2008). To optimize the efficiency of mycelial DNA extraction and to limit variability throughout the procedure, we used a commercial kit. The FastDNA kit was validated as a reliable tool for DNA extraction in our experimental condition. Indeed, DNA amounts quantified by densitometry (to avoid RNA and protein interference when quantifying by UV spectrometry) were proportional to the dry mycelium mass up to 20 mg (data not shown). Over this value, column provided in the kit saturated.

In mycelium cultivated in liquid medium, the DNA per biomass ratio evolved differently with temperature. At 25 °C, for *P. camemberti*,

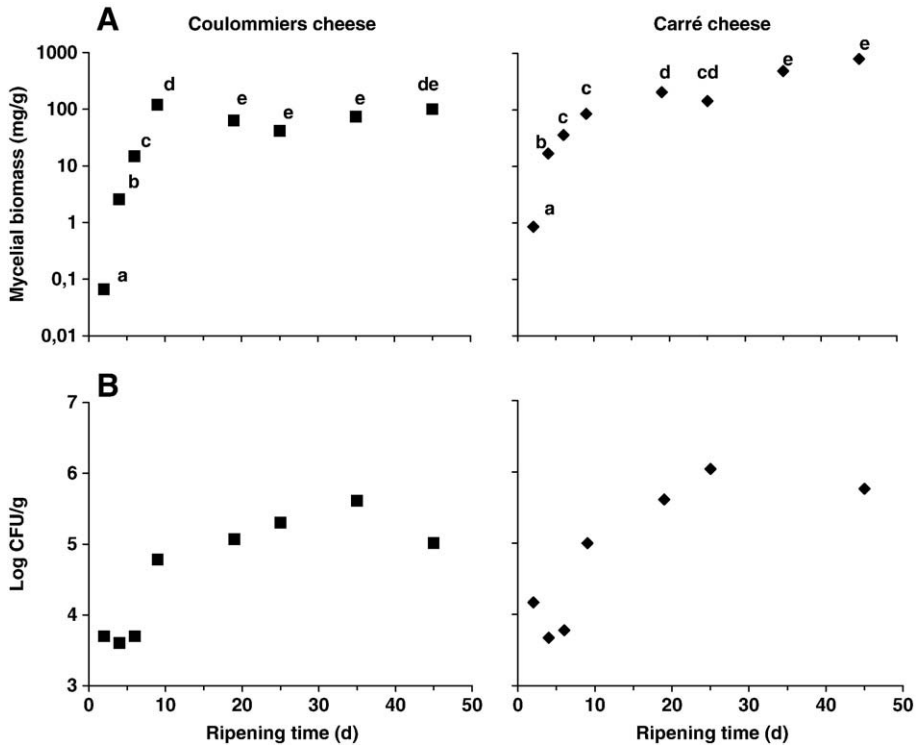


**Fig. 6.** (A) Estimated mycelial biomass of *P. camemberti* quantified by qRT-PCR (using standard curves described in Fig. 4, so that data are directly expressed as biomass concentration in curd) during ripening of two Camembert-type cheeses: Coulommiers and Carré. Mean values bearing distinct letters are significantly different ($p<0.05$). (B). Corresponding CFU counts ($n=1$) are represented below each graph.

this ratio evolved into two phases, directly in relation with the decreasing growth after d12. At 14 °C, the growth was slower than at 25 °C and the ratio appeared constant all over the time-course of growth. For *P. roqueforti* at 25 °C, the ratio was significantly lower at d25 as compared to d3. This could be explained by a diminution in the number of nuclei in senescent hyphae or by a partial degradation of cellular DNA as shown during yeasts autolysis (Zhao and Fleet, 2003). However, cheese maturation frequently operates at 14 °C and, at this temperature the DNA per biomass ratio appeared constant, which validated the use of DNA as a marker of biomass in these experimental conditions.

One can argue that conidia that are obviously present in cheese samples (but not in agitated liquid media culture) would influence this ratio. To check this, we quantified DNA extracted from spore suspension only by the FastDNA kit (data not shown). For spore concentrations ranging from 10 to $10^8$ spores per mL, the amount of DNA detected by densitometry was near the threshold of detection (3 ng/µL). Using qRT-PCR, DNA amounts were at least 50-fold lower as compared to those obtained from dry mycelium. We consequently concluded that DNA extracted from spores using the FastDNA kit was in negligible amount.

The second phase of this study was to check that the DNA per biomass ratio was also constant in cheese curd. The presence of compounds such as milk proteins, lipids and salts in cheese may decrease DNA extraction yields and/or inhibits PCR reaction (Bleve et al., 2003). To test these potent effects of the cheese matrix, known amounts of fresh mycelium were mixed to 1 g of curd (standards). A large scale of fungal biomass quantity (from 5 to 150 mg DM which roughly correspond to 20 and 600 mg fresh mycelium, respectively) was covered. A linear relationship could be established between fresh biomass and DNA recovery for the two species, although a lower yield of extraction could be observed for the lowest mycelium per cheese curd ratio (20–100 mg/g). The 100-fold difference observed between DNA contents of the two *Penicillium* species seems related to the different primers targets. Actually, a recent qRT-PCR study in *Stachybotrys chartarum* showed that the multi-copy rRNA region was at least 2 $\log_{10}$ greater in number than the single copy target (beta-tubulin) (Black and Foarde, 2007).

The good relationship observed between fresh biomass and recovered DNA amounts in standards not only validated the use of DNA as a marker of fungal biomass in model cheese curd but also validated our protocol for eliminating the cheese matrix and extracting DNA. However, since biomass values are preferred by cheese manufacturers, one of these standards was used for qRT-PCR calibration: fluorescence data were then directly converted into fungal biomass, as previously described for *Lactarius deliciosus* extraradical soil mycelium (Parladé et al., 2008).

The qRT-PCR assays allowed specific and quantitative detection of *P. camemberti* and *P. roqueforti* in model cheese curds with a high sensitivity level (detection limit of 0.25 and 4 µg mycelium per gram of curd, respectively). The efficiency of DNA extraction was considered similar for standards and samples since they were both prepared with a similar procedure (cheese matrix dissolution and delipidation, DNA extraction, qRT-PCR). Nevertheless, a bias may still exist because the composition of the model cheese curd differed slightly from that of cheese. Moreover, the standard consisting in fresh mycelium (10-d-old cultures) mixed with curd does not reflect the spatial organization of *Penicillium* spp. in the cheese matrix or aging hyphae during the 45 d-ripening period in cheeses. However, this qRT-PCR method allowed representing the growth dynamics of *P. camemberti* and *P. roqueforti* during ripening.

During ripening of model cheeses, *P. roqueforti* grew into two phases of rapid growth and then remained stable. To our knowledge, this is the first time that the growth dynamic of *P. roqueforti* in model curd is described. However, further experiments are needed to describe the growth of *P. roqueforti* in blue-veined cheeses. For *P. camemberti*, growth rate was high till d11 and then a stationary phase was reached. These data fit well with those of Aldarf et al.

(2002) who reported a similar growth dynamic of *P. camemberti* estimated by dry weight measurements and biomass reconstruction from substrate consumption (peptone, lactic acid) and ammonia release. In the present study, similar growth dynamics were observed in model cheeses and in Coulommiers and Carré cheeses using qRT-PCR. Taken together, these data confirmed the high growth rate of *P. camemberti* during the first days of ripening. For cheese manufacturers, this description of mycelial growth is of great relevance since CFU counts remained constant or decreased slightly during the same period. Actually, using a plate count method, Leclercq-Perlat et al. (2004) reported no variation of *P. camemberti* growth the first 14 d of ripening whereas mycelial growth was visible from d7 to d12. In a second study (Leclercq-Perlat et al., 2006), *P. camemberti* spore counts were constant from d1 to d6 followed by a maximal sporulation rate from d8 to d12. Our data obtained in Coulommiers and Carré cheeses showed not only that a mycelial growth occurred as soon as d2 but also that the highest growth rate occurred from d2 to d4. During the same period, CFU counts decreased, which confirmed the inaccuracy of CFU counting for measuring fungal growth in cheese.

*P. camemberti* biomass reached 1 g/g of cheese (model and Carré cheeses) at the end of ripening. This high level suggests that the samples collected at the later stages of ripening were exclusively composed of mycelium, as previously reported by Engel et al. (2001). This result should be taken with caution since DNA extraction yields were not anymore linear above 400 mg mycelium per gram of cheese. At this stage of ripening, senescent and unviable hyphae, e.g. DNA from dead cells was also probably quantified, leading to an overestimation of the viable biomass. To overcome this bias, an mRNA-based qRT-PCR method may be used to solely quantify viable biomass. Such an approach was published by Bleve et al. (2003) using actin mRNA amplification to detect and quantify viable yeasts and molds in milk product. However, in this study, the calibration curves were still built using CFU counts. During ripening, estimated biomass concentration of *P. camemberti* was 10-fold higher in Carré cheese than in Coulommiers cheese. The slower colonization of *P. camemberti* on the surface of Coulommiers cheese was also confirmed visually throughout the ripening process (data not shown). This difference may be explained by a competition between *P. camemberti* and *Geotrichum candidum,* as previously reported for other *Penicillium* spp. (van den Temple and Nielsen, 2000; Decker and Nielsen, 2005). Indeed *G. candidum* counts were ~2 $\log_{10}$ CFU/g higher in Coulommiers cheese than in Carré cheese (data not shown). Further work would be necessary to confirm if such a competition really occurs.

The qRT-PCR method developed in this study provides a simple and specific tool for cheese manufacturer to better understand the relationship between microbiological and biochemical changes in cheese with special regards to *P. camemberti* and *P. roqueforti* growth dynamics. This method may also be useful in order to evaluate and compare the competitiveness and colonization capacity of *P. camemberti* and *P. roqueforti* commercial ripening cultures directly in cheeses in the presence of a complex microbiota.

## Acknowledgements

The authors wish to acknowledge the financial support provided by the Lactalis Research and Development laboratory (Retiers, France).

## References

Aldarf, M., Amrane, A., Prigent, Y., 2002. Reconstruction of the biomass history from carbon and nitrogen substrate consumption, ammonia release and proton transfer during solid cultures of *Geotrichum candidum* and *Penicillium camembertii*. Applied Microbiology and Biotechnology 58, 823–829.

Black, J.A., Foarde, K.K., 2007. Comparison of four different methods for extraction of *Stachybotrys chartarum* spore DNA and verification by real-time PCR. Journal of Microbiological Methods 70, 75–81.

Bleve, G., Rizzotti, L., Dellaglio, F., Torriani, S., 2003. Development of reverse transcription (RT)-PCR and real-time RT-PCR assays for rapid detection and quantification of viable yeasts and molds contaminating yogurts and pasteurized food products. Applied and Environmental Microbiology 69, 4116–4122.

Boualem, K., Waché, Y., Garmy, D., Karbowiak, T., Durand, A., Gervais, P., Cavin, J.F., 2008. Cloning and expression of genes involved in conidiation and surface properties of *Penicillium camemberti* grown in liquid and solid cultures. Research in Microbiology 159, 110–117.

Brinkman, N.E., Haugland, R.A., Wymer, L.J., Byappanahalli, M., Whitman, R.L., Vesper, S. J., 2003. Evaluation of a rapid, quantitative real-time PCR method for enumeration of pathogenic *Candida* cells in water. Applied and Environmental Microbiology 69, 1775–1782.

Corsetti, A., Rossi, J., Gobbetti, M., 2001. Interactions between yeasts and bacteria in the smear surface-ripened cheeses. International Journal of Food Microbiology 69, 1–10.

Decker, M., Nielsen, P.V., 2005. The inhibitory effect of *Penicillium camemberti* and *Geotrichum candidum* on the associated funga of white mould cheese. International Journal of Food Microbiology 104, 51–60.

Dong, Y., Steffenson, B.J., Mirocha, C.J., 2006. Analysis of ergosterol in single kernel and ground grain by gas chromatography-mass spectrometry. Journal of Agricultural and Food Chemistry 54, 4121–4125.

Engel, E., Tournier, C., Salles, C., Le Quéré, J.L., 2001. Evolution of the composition of a selected bitter Camembert cheese during ripening: release and migration of taste-active compounds. Journal of Agricultural and Food Chemistry 49, 2940–2947.

Fredricks, D.N., Smith, C., Meier, A., 2005. Comparison of six DNA extraction methods for recovery of fungal DNA assessed by quantitative PCR. Journal of Clinical Microbiology 43, 5122–5128.

Furet, J.P., Quénée, P., Tailliez, P., 2004. Molecular quantification of lactic acid bacteria in fermented milk products using real-time quantitative PCR. International Journal of Food Microbiology 97, 197–207.

Gessner, M.O., Schmitt, A.L., 1996. Use of solid-phase extraction to determine ergosterol concentration in plant tissue colonized by fungi. Applied and Environmental Microbiology 62, 415–419.

Guidot, A., Debaud, J.P., Marmeisse, J.C., 2002. Spatial distribution of the below-ground mycelia of an ectomycorrhizal fungus inferred from specific quantification of its DNA in soil samples. FEMS Microbiology Ecology 42, 477–486.

Hierro, N., Esteve-Zarzoso, B., González, Á., Mas, A., Guillamón, J.M., 2006. Real-Time Quantitative PCR (QPCR) and Reverse Transcription-QPCR for detection and enumeration of total yeasts in wine. Applied and Environmental Microbiology 72, 7148–7155.

Jany, J.L., Barbier, G., 2008. Culture-independent methods for identifying microbial communities in cheese. Food Microbiology 25, 839–848.

Landeweert, R., Veenman, C., Kuyper, T.W., Fritze, H., Wernars, K., Smit, E., 2003. Quantification of ectomycorrhizal mycelium in soil by real-time PCR compared to conventional quantification techniques. FEMS Microbiology Ecology 45, 283–292.

Larpin, S., Mondoloni, C., Goerges, S., Vernoux, J.P., Guéguen, M., Desmasures, N., 2006. *Geotrichum candidum* dominates in yeast population dynamics in Livarot, a French red-smear cheese. FEMS Yeast Research 6, 1243–1253.

Leclercq-Perlat, M.N., Buono, F., Lambert, D., Latrille, E., Spinnler, H.E., Corrieu, G., 2004. Controlled production of Camembert-type cheeses. Part I: microbiological and physicochemical evolutions. Journal of Dairy Research 71, 346–354.

Leclercq-Perlat, M.N., Picque, D., Riahi, H., Corrieu, G., 2006. Microbiological and biochemical aspects of Camembert-type cheeses depend on atmospheric composition in the ripening chamber. Journal of Dairy Science 89, 3260–3273.

Montgomery, H.J., Monreal, C.M., Young, J.C., Seifert, K.A., 2000. Determination of soil fungal biomass from soil ergosterol analyses. Soil Biology and Biochemistry 32, 1207–1217.

Mulè, G., Susca, A., Logrieco, G., Stea, A., Visconti, A., 2006. Development of a quantitative real-time PCR assay for the detection of *Aspergillus carbonarius* in grapes. International Journal of Food Microbiology 111, S28–S34.

Nielsen, M.S., Frisvad, J.C., Nielsen, P.V., 1998. Protection by fungal starters against growth and secondary metabolite production of fungal spoilers of cheese. International Journal of Food Microbiology 42, 91–99.

Niessen, L., 2008. PCR-based diagnosis and quantification of mycotoxin-producing fungi. Advances in Food and Nutrition Research 54, 81–138.

Parladé, J., Hortal, S., Pera, L., Galipienso, L., 2008. Quantitative detection of *Lactarius deliciosus* extraradical soil mycelium by real-time PCR and its application in the study of fungal persistence and interspecific competition. Journal of Biotechnology 128, 14–28.

Pitt, J.I., Hocking, A.D., 1997. Fungi and Food Spoilage. Blackie Academic and Professional, London.

Raidl, S., Bonfigli, R., Agerer, R., 2005. Calibration of quantitative real-time Taqman PCR by correlation with hyphal biomass and ITS copies in mycelia of *Piloderma croceum*. Plant Biology 7, 713–717.

Reeslev, M., Miller, M., Nielsen, K.F., 2003. Quantifying mold biomass on gypsum board: comparison of ergosterol and beta-N-acetylhexosaminidase as mold biomass parameters. Applied and Environmental Microbiology 69, 3996–3998.

Robine, E., Lacaze, I., Moularat, S., Ritoux, S., Boissier, M., 2005. Characterisation of exposure to airborne fungi: measurement of ergosterol. Journal of Microbiological Methods 63, 185–192.

Roe, J.D., Haugland, R.A., Vesper, S.J., Wymer, L.J., 2001. Quantification of *Stachybotrys chartarum* conidia in indoor dust using real time, fluorescent probe-based detection of PCR products. Journal of Exposure Analysis and Environmental Epidemiology 11, 12–20.

Roostita, R., Fleet, G.H., 1995. The occurrence and growth of yeasts in Camembert and Blue-veined cheeses. International Journal of Food Microbiology 28, 393–404.
Rozen, S., Skaletsky, H.J., 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, S., Misener, S. (Eds.), Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 38–365.
Sambrooks, J., Russel, D.W., 2001. Molecular Cloning, a laboratory manual, vol. 1, 3rd edition. Cold Spring Harbor Laboratory Press, New York, 5.14.
Schmidt, H., Bannier, M., Vogel, R.F., Niessen, L., 2004. Detection and quantification of *Aspergillus ochraceus* in green coffee by PCR. Letters in Applied Microbiology 38, 464–469.
Schubert, R., Raidl, S., Funk, R., Bahnweg, G., Müller-Starck, G., Agerer, R., 2003. Quantitative detection of agar-cultivated and rhizotron-grown *Piloderma croceum* Erikss. & Hjortst. by ITS1-based fluorescent PCR. Mycorrhiza 13, 159–165.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24, 1596–1599.
Taniwaki, M.H., Pitt, J.I., Hocking, A.D., Fleet, G.H., 2006. Comparison of hyphal length, ergosterol, mycelium dry weight and colony diameter for quantifying growth of fungi from foods. Advances in Experimental Medicine and Biology 571, 49–67.
van den Temple, T., Nielsen, M.S., 2000. Effects of atmospheric conditions, NaCl and pH on growth and interactions between moulds and yeasts related to blue cheese production. International Journal of Food Microbiology 57, 193–199.
Zhao, J., Fleet, G.H., 2003. Degradation of DNA during the autolysis of *Saccharomyces cerevisiae*. Journal of Industrial Microbiology and Biotechnology 30, 175–182.