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Modelling the sporulation of some fungi associated with cheese, at different temperature and water activity regimes

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ABSTRACT

The objectives of this study were to determine, *in-vitro,* the influence of temperature (T; 10–30 °C, step 5°), water activity (a_w, 0.83–0.99; step 0.04) and time on sporulation (SPO) of some cheese-related fungi belonging to *Penicillium* spp. and *A. versicolor*. Overall, sporulation started rapidly (8 h in optimal conditions); it was significantly influenced by T and a_w and the fungi studied were clearly distinguished based on their thermo-hydro adaptation. Boundary conditions for sporulation were defined for all the fungi considered and the sporulation rate was successfully modelled, especially based on T and time regimes. *Penicillium crustosum, P. nordicum* and *P. verrucosum* showed optimum for SPO at T between 20 and 25 °C and their sporulation continued up to $a_w = 0.87$ ($a_w = 0.83$ for *P. nordicum*). They resulted the fungi best adapted to the environmental conditions of ripening grana cheese storehouses; therefore, it is expected they dominate on the grana cheese surface. Studies on cheese are necessary to validate these results obtained on artificial media and without fungi co-occurrence.

1. Introduction

Sporulation and spore dispersal affects the successful colonization of potential hosts by fungi; therefore, they are important steps in agricultural and industrial processes where beneficial fungi are involved. Abundant sporulation is required, for instance, in fungi applied as biocontrol agents, such as *Penicillium oxalicum* to control soil-borne diseases (Pascual et al., 1997) or as starter inocula in cheese-making, like *P. camemberti* and *P. roqueforti* in Camembert (Bockelmann et al., 1999), Roquefort and Gorgonzola cheese production (Samson and Frisvad, 2004). However, in some cases, moulds can also act as spoiling agents in food processing, badly affecting the sensory quality and possibly leading to mycotoxin contamination (Ropars et al., 2012). Efficient sporulation is unwanted in this case, as an enhancing factor for spoilage.

Driving variables for sporulation of *Penicillium* spp., commonly dominant and frequently co-occurring with some *Aspergillus* spp. in cheese and cheese working environments (Hymery et al., 2014), have been considered by some authors. Basu and Bhattacharyya (1962), Mossini et al. (2009) and Sharma and Pandey (2010) tested the effect of different carbon sources (xylose, glucose, fructose, sucrose, maltose, dextrin, starch and cellulose) on the sporulation of various *Penicillium* spp. They found maltose was more effective in promoting sporulation than simple sugars, but less suitable compared to polysaccharides (starch, cellulose). Carbon and nitrogen sources were also considered by Trique (1968) who found that *A. versicolor* sporulation was promoted at low carbohydrate (glucose and sucrose) concentrations.

Other works have focused on reducing the sporulation of Penicillia by using various control strategies. Yamaga et al. (2015) found that blue light-emitting diodes (LED) significantly reduced *P. italicum* growth and spore production. Cinammon (*Cynnamomum zeylanicum*) oil can significantly reduce/stop *P. expansum* sporulation (Landero Valenzuela et al., 2016), while pennyroyal (*Mentha pulegium L.*) essential oil inhibits growth and the sporulation of *Aspergillus* sp., *Penicillium* sp. and *Rhizopus* sp. (Amalich et al., 2016). Finally, Matei et al. (2015) highlighted how selected strains of lactic acid bacteria may be profitably used in controlling the growth and sporulation of potential mycotoxigenic fungal species belonging to the genera *Aspergillus* and *Penicillium*.

However, the effect of ecological conditions on conidia production by Penicillia has been poorly considered in published studies, as also claimed by Canteri and Ghoul (2015) that reviewed the effect of nutritional sources and operating conditions on some Penicillia. The only extensive work on the influence of temperature, water potential and pH on *in-vitro* spore production was undertaken by Pascual et al. (1997) on *P. oxalicum*.

Past works reported that the development of uncontrolled moulds on the surface of different types of cheese, including grana cheese, may occur during ripening and ageing (Decontardi et al., 2017; Ropars et al., 2012) possibly leading to mycotoxin contamination (Bailly et al., 2002;

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Cooper et al., 1982; Dall'Asta et al., 2008; Jarvis, 1983; Ostry et al., 2013; Pugazhenthi et al., 2000). Grana cheese is produced using partially skimmed milk added with natural whey starter along with calf rennet. The resulting curdle is cooked at 55–56 °C and put in a special frame called "fascera" which gives the typical wheel-shape. After two days, the cheese is dunked into brine for 15–30 days and then moved to ripening chambers.

During grana cheese ripening, temperature must stay at 15–22 °C, but water activity (a_w) changes significantly. Measurements on fresh-prepared cheese wheels show a_w = 0.97 (Pietri A., personal communication). Then, salt penetrates into the matrix till to an equilibrium with a_w values of 0.91–0.92 in the whole cheese wheel, but a_w = 0.85–0.88 can be measured in the peripheral part (Pellegrino and Resmini, 2001). As a general trend, however, humidity of the cheese matrix decrease over the course of time in both the external and central part (Tosi et al., 2008).

As shown by Camardo Leggieri et al. (2017), mycotoxin producing fungi find suitable a_w regimes to grow and produce toxins during cheese ripening. Therefore, a clear understanding on sporulation as the main inoculum source for cheese and environments colonization is crucial.

For all these reasons, the present study aimed to: i) produce quantitative data on how the sporulation of some cheese-related fungi, belonging to *Penicillium* spp. and *A. versicolor* is affected by T and water activity (a_w) regimes, ii) model sporulation data obtained as basic knowledge for predictive modelling. These are crucial tassels to optimise cheese making management and minimise mould contamination of Italian grana cheese since early ripening stages.

2. Materials and methods

2.1. Chemicals

Mycological peptone, microbiological grade agar, and malt extract were purchased from Himedia Laboratories (Mumbai, India); D(+) glucose was purchased from Sigma-Aldrich (Merck K.G.a.A., Darmstadt, Germany); salts and absolute anhydrous ethanol absolute were purchased form Carlo Erba Reagents S.r.l. (Milano, Italy).

2.2. Fungal strains

Seven *Penicillium* species and *Aspergillus versicolor* (later identified as *A. jensenii* by Decontardi et al., 2018) were used in this study, previously characterised for growth and mycotoxin production in different T and a_w regimes (Table 1; Camardo Leggieri et al., 2017).

Table 1
Fungal species, matrix of isolation and fungal collection code of strains used in this study.

Species	Matrix	Code
A. versicolor[a]	Cheese	CBS 108959[b]
P. camemberti	–[c]	CBS 122399
P. citrinum	–	CBS 122396
P. crustosum	Lemon	CBS 115503
P. nalgiovense	Cheese	CBS 109609
P. nordicum	Salami sausage	CBS 112573
P. roqueforti	Roquefort cheese	CBS 221.30
P. verrucosum	Italian salami sausage	CBS 325.92

[a] *A. versicolor* CBS108959 was later identified as *A. jensenii* by Decontardi et al. (2018) but *A. versicolor* will be kept in the text to remain consistent with the CBS website database labellin.

[b] CBS: CBS-KNAW Fungal Biodiversity Centre Utrecht, The Netherlands.

[c] Not reported.

2.3. Culture media

Malt Extract Autolysate Agar (MEA, Samson and Frisvad, 2004; malt extract 30 g; mycological peptone 1 g; glucose 20 g; agar, 20 g; $CuSO_4 \cdot 5H_2O$ 0.005 g; $ZnSO_4 \cdot 7H_2O$ 0.01 g; bidistilled water, 1 L), was used to perform both the inoculum preparation and sporulation trials. In order to test the influence of different a_w regimes, NaCl was added to MEA medium (a_w = 0.99) to modify a_w according to Dallyn and Fox (1980), to simulate the natural cheese conditions. NaCl was selected being the best additive to simulate the real cheese condition, being salt added at early cheese making steps. Medium a_w levels were confirmed using Aqualab LITE (version 1.3 © Decagon devices Inc., WA, USA).

2.4. Inoculum preparation and inoculation

Petri plates (9 cm diameter) were three-point inoculated (Pitt, 1979) using 10 µL of a stock spore suspension (10^7–10^8 spores/mL concentration), then incubated at 25 °C in the dark for 7 days. At the end of incubation, plates were added with 20 mL of bidistilled sterile water and gently scratched with a sterilized metal spatula to remove conidia. The resulting spore suspension was filtered on double layer sterilized gauze, adjusted to 10^6 spores/mL using a haemocytometer and used as inoculum for the study.

2.5. Study conditions and measurements

Two independent experiments were performed to assess the influence of T and a_w on fungal growth and sporulation. MEA plates (9 cm diameter), adjusted for the a_w when appropriate, were centrally inoculated with 10 µL of spore suspension, incubated in the dark for 24 h at 25° ± 1 °C, to allow a common starting point of colony growth, and then moved to the proper T condition fixed in the study; 1 day at 25 °C was necessary to allow all fungi to start growing, bringing them to a common starting point (Rossi et al., 2009).

2.5.1. T regime experiment

Inoculated MEA plates (a_w = 0.99) were incubated between 10 °C and 30 °C, 5 ± 1 °C step, each condition in triplicate.

2.5.2. a_w regime experiment

Water activity modified plates (a_w = 0.83, 0.87, 0.91, 0.95, 0.99) after inoculation were incubated at 20 ± 1 °C, each condition in triplicate. 20 °C T regime was chosen because it is a common environmental condition occurring in Italian grana cheese ripening chambers (Camardo Leggieri et al., 2017).

Two perpendicular diameters (cm) of each colony were measured at scheduled time: 0, 8, 24, 48, 72, 96, 168 and 240 h and the colony area was calculated for each fungus. Then, at each incubation time, the colonies were washed with bi-distilled sterile water added with some drops of absolute anhydrous ethanol; the amount of water used varied between 5 and 20 mL. The spore suspension was filtered with single layer sterile gauze and used for spore counting with a haemocytometer. Dilution 1:10 was also performed when needed. Spore counts were expressed as total spores produced per colony.

2.6. Data analysis

Statistical analyses were performed using IBM SPSS v.24 (SPSS Inc., 2013). Quantitative relationships between spore numbers, T/a_w and incubation time were then analysed using non-linear regression analysis. The sporulation rate (SPO, 0–1 scale) was calculated by dividing the average numbers of spores counted in each "T/a_w × incubation time" combination by the maximum number counted in each T/a_w regime.

The equation parameters were estimated using the non-linear regression procedure of SPSS, which minimises the residual sums of