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Modelling the effect of water activity reduction by sodium chloride or glycerol on conidial germination and radial growth of filamentous fungi encountered in dairy foods

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ABSTRACT

Water activity (a_w) is one of the most influential abiotic factors affecting fungal development in foods. The effects of a_w reduction on conidial germination and radial growth are generally studied by supplementing culture medium with the non-ionic solute glycerol despite food a_w can also depend on the concentration of ionic solutes such as sodium chloride (NaCl). The present study aimed at modelling and comparing the effects of a_w, either modified using NaCl or glycerol, on radial growth and/or conidial germination parameters for five fungal species occurring in the dairy environment. The estimated cardinal values were then used for growth prediction and compared to growth kinetics observed on commercial fresh cheese. Overall, as compared to glycerol, NaCl significantly increased the fungistatic effect resulting from a_w reduction by extending latency and/or reducing radial growth rates of *Paecilomyces niveus*, *Penicillium brevicompactum*, *Penicillium expansum* and *Penicillium roqueforti* but not of *Mucor lanceolatus*. Besides, NaCl significantly reduced a_w range for conidial germination and delayed median germination time of *P. expansum* but not of *P. roqueforti*. Despite these observations, cardinal a_w values obtained on glycerol-medium yielded similar predictions of radial growth and germination time in commercial fresh cheese as those obtained with NaCl. Thus, it indicates that, for the studied species and a_w range used for model validation, the use of NaCl instead of glycerol as a a_w depressor had only limited impact for fungal behavior prediction.

1. Introduction

Salt, sodium chloride (NaCl), has been added to foods as a flavoring agent and food preservative since Antiquity. Bacteriostatic and fungistatic effects of NaCl mainly result from the combination of (i) a toxic effect of Na^+ and/or Cl^- ions which affect membranes and cytosolic systems above a certain threshold and (ii) an hyperosmotic stress in response to water activity (a_w) reduction (Morita et al., 2004; Bois et al., 2006; Plemenitas et al., 2014). The water fraction available to microorganisms is represented by the a_w which can limit microbial development (Scott, 1957). Indeed, a_w reduction causes rapid water efflux from the cell, affecting its volume, turgor and metabolic activities (Deacon, 2006; Plemenitas et al., 2014).

Filamentous fungi are generally tolerant to lower a_w levels than yeast and bacteria and thus are the major spoilage agents of low and intermediate a_w foods (Pitt and Hocking, 2009). To counteract the water efflux, fungal cells accumulate compatible solutes, mainly glycerol, trehalose, erythritol, arabitol and mannitol (Brown, 1974; Hallsworth et al., 2003; Wyatt et al., 2013). Fungal development in foods can be controlled by physico-chemical factors, including food extrinsic (temperature, humidity and atmosphere composition of the storage environment) and intrinsic parameters, mainly a_w, pH, redox potential, texture, available nutrients and antimicrobial substances. These factors, also called hurdle in "Hurdle technologies concept", may be combined and represent an effective tool for food safety and quality management (Leistner and Gorris,

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The influence of these hurdles on microbial development is quantifiable through predictive modelling approaches, initially developed to predict bacterial growth and more recently applied to predict fungal development (predictive mycology) and mycotoxin contamination in foods (Dantigny et al., 2005). In predictive mycology, use of Gamma-type models provides cardinal values which are relevant for food safety and quality management systems (Dagnas and Membré, 2013). To date, cardinal models have been successfully used to describe the influence of temperature, pH, a_w and their combination on conidial germination or radial growth, as recently reviewed by Dagnas and Membré (2013). Concerning a_w, its effect on fungal germination and growth has been mainly studied with culture media containing the non-ionic solute glycerol. In contrast, only few studies have been conducted with NaCl (Cuppers et al., 1997) because NaCl, in addition to its a_w depressor effect, possess a fungistatic effect (Hocking and Pitt, 1979). However, in several food products containing salt, NaCl is the main a_w depressor and thus, cardinal a_w values only estimated with media containing glycerol may not correctly represent the fungal response to a_w in such products.

The present study aimed at comparing the effect of NaCl and glycerol as an a_w depressor in the culture medium on radial growth and growth limit of five fungal species encountered in the dairy environment. The estimated cardinal values were then used for growth prediction and compared to growth kinetics observed on commercial fresh cheese to evaluate the robustness of our observations. In addition, two fungal species were selected to compare the effect of a_w reduction by NaCl and glycerol on conidial germination.

2. Material and methods

2.1. Fungal strains

Paecilomyces niveus (anamorphic form of *Byssochlamys nivea* UBOCC-A-11024), *Mucor lanceolatus* UBOCC-A-109153, *Penicillium brevicompactum* UBOCC-A-110007, *Penicillium expansum* UBOCC-A-110032 and *Penicillium roqueforti* UBOCC-A-113022 respectively isolated from cow's milk, cheese, fresh dairy product, fresh cheese and Roquefort were obtained from the Université de Bretagne Occidentale Culture Collection (UBOCC, Plouzané, France). Concerning *P. niveus*, the anamorphic name was used because only asexual development was studied. These fungi were routinely cultured on potato dextrose agar (PDA, Difco, Becton Dickinson, Sparks, MD, USA) at 25 °C.

2.2. Culture media

The culture medium used throughout the experiments was PDA either supplemented with NaCl (Sigma-Aldrich, Saint-Louis, MO, USA) (NaCl-PDA) or glycerol (Thermo Fischer Scientific, Waltham, MA, USA) (glycerol-PDA) for a_w adjustments. Eleven NaCl and glycerol concentrations respectively ranging from 0 to 20% (w/w) (a_w ranging from 0.995 to 0.767 a_w) and 0–50% (w/w) (a_w ranging from 0.995 to 0.758 a_w) were tested. These NaCl and glycerol levels were chosen to establish the a_w limits of conidial germination and radial growth of the tested fungi.

Media were buffered at pH 4.2 using a 3:2 (v/v) mixture of citric acid monohydrate (0.1 M) and dibasic sodium phosphate (0.2 M) solutions (Sigma-Aldrich, Saint-Louis, MO, USA). These solutions and double concentrated agar medium were prepared and autoclaved separately before mixing and pouring 25 mL into 90-mm Petri dishes. The chosen pH level had no effect on the growth of the tested fungi as required for modelling the effect of a_w with a monofactorial design.

Besides, 25 g of commercial pasteurized fresh cheese (pH 4.9) containing 1.3% NaCl (w/w) (0.982 a_w) or supplemented with saturated NaCl solution to reach a 5.0% NaCl (w/w) concentration (0.956 a_w) was spread into Petri dishes using a sterile L-shaped spreader and used for model validation. For each culture medium batch, pH and a_w were controlled in triplicate at 20 °C using a pH surface-electrode (SF 113, VWR, Radnor, PA, USA) with an accuracy of 0.01 pH unit and an a_w-meter (Tunable Diode Laser a_w-meter Aqualab, Decagon Devices, Pullman, WA, USA) with an accuracy of 0.005 a_w unit. The a_w apparatus was calibrated according to the manufacturer's instructions using salt solutions of known a_w.

2.3. Conidia production

Fungal conidia were produced and harvested as previously described (Nguyen Van Long et al., 2017). Briefly, conidia were harvested from cultures incubated for 10 days at 25 °C on PDA medium at 0.980 a_w and pH 4.2. Conidial concentrations in suspensions were determined using a haemocytometer (Malassez, Preciss, Paris, France) and diluted in glycerol or NaCl solutions adjusted to the a_w and pH values of the inoculated medium. Conidia suspensions were standardized at 1.10^6 conidia/mL or 1.10^5 conidia/mL for measurements of radial growth or conidial germination, respectively.

2.4. Assessment of radial growth of *P. niveus*, *M. lanceolatus*, *P. brevicompactum*, *P. expansum* and *P. roqueforti* on PDA and fresh cheese

Ten microliters of a fresh conidia suspension (1.10^6 conidia/mL) were deposited in the center of PDA or fresh cheese plates. The plates were then placed in plastic boxes (34 × 25 × 12 cm) containing 200 mL of NaCl solution adjusted to the corresponding a_w of the culture medium in order to avoid a_w fluctuation (Sautour et al., 2001) followed by incubation at 25 °C in temperature-controlled incubators (KB 240, Binder GmbH, Tuttlingen, Germany). Thallus radius was daily measured in two perpendicular directions for a maximum of 30 days. Three biological replicates were made for each condition.

2.5. Assessment of conidial germination of *P. expansum* and *P. roqueforti* on PDA

Conidial germination on solid medium was followed as previously described (Nguyen Van Long et al., 2017). Immediately after harvesting the conidia, 100 µl of a fresh conidia suspension (1.10^5 conida/mL) prepared as described above were surface-plated on PDA filled to the brim of Petri dishes (50 mL). Plates were incubated at 25 °C in plastic boxes containing 200 mL of NaCl adjusted to the a_w of the corresponding medium. In order to monitor the same conidia population throughout the experiments, a reading frame was taped on the top lid of each Petri dish. These frames were handmade with 80-mm-diameter circular plastic sheets (A4 laminating pouches 80 microns, Fellowes, Itasca, IL, USA) in which three rectangular slots (1 × 25 mm) were cut. Conidial germination was followed without opening the Petri dish by phase-contrast microscopy at 10X magnitude directly through the reading frames. A minimum of 100 conidia was counted for each reading frame to determine the germination kinetic (expressed as the percentage of germinated conidia as a function of time). Conidia were considered germinated when the length of the germ tube was greater or equal to the greatest dimension of the swollen conidia (Dantigny et al., 2006). The conidial germination was assessed for a maximum of 30 days. Three biological replicates were made for each condition.