LWT - Food Science and Technology 90 (2018) 145–151

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

Effects of fungal growth on the firmness of a cheese analogue formulated with different casein-to-fat ratios

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ARTICLE INFO

Keywords:
Cheese
Fungi
Mould
Casein
Penetrometer

ABSTRACT

The growth rates of nine fungal species inoculated on a cheese analogue formulated with different casein-to-fat ratios (0.6, 0.8, 1.0 and 1.2) and incubated at 10 and 20 °C was evaluated. The species belonged to the genera *Penicillium*, *Aspergillus*, *Mucor* and *Cladosporium*. It was found that, in general, the species tended to grow faster at 20 °C, and at casein-to-fat ratios of 1.0 and 1.2. In order to determine the impact of fungal presence on loss in firmness, the texture of cheese analogues was analyzed with a penetrometer ball probe. When incubation was done at 20 °C, loss in firmness was higher (17.56–64.85%) than at 10 °C (11.12–58.03%), but it was not affected by the casein-to fat ratio. The species differed on their ability to reduce the firmness of the cheese analogues, although this was not strictly correlated with their growth rate. Thus, our results indicate that whereas fungal growth in cheese is affected by temperature and casein-to-fat ratio, the amount of visible mycelium on the rind would not be a good parameter to predict the extent of textural modifications, which would be mostly influenced by the temperature of ripening and the species involved.

1. Introduction

Moulds are common inhabitants of cheese and play an important role on its quality. On one hand, their presence is required in some varieties such as blue veined cheeses (e.g., Roquefort, Gorgonzola) and surface mould-ripened cheeses (e.g., Camembert, Brie), where they are responsible of their typical aspect and flavour (Larsen & Jensen, 1999; Le Dréan et al., 2010). On the other hand, moulds might also be responsible of cheese spoilage, causing defects such as anomalous odors and flavors, pigmentations and accumulation of mycotoxins (Sengun, Yaman, & Gonul, 2008).

Many fungal species are also able to break down casein through secretion of proteases (Zhang & Zhao, 2010), contributing to changes in texture, since this quality attribute is influenced by the total amount of intact casein present at the end of the ripening period (Lawrence, Creamer, & Gilles, 1987; O'Callaghan & Guinee, 2004). Changes in texture due to proteolysis caused by mould growth have been reported in some varieties of cheese where fungal species are either added intentionally, or deliberately encouraged to grow under particular ripening conditions of temperature and humidity (Button & Dutton, 2012; Fernández-Bodega, Mauriz, Gómez, & Martin, 2009; Fox & McSweeney, 2004). However, and although it has been frequently pointed that contaminating fungi could also cause changes in texture (Hymery et al., 2012; Montagna et al., 2004), there is scarce information about the precise consequences of their presence on cheese firmness.

In addition to the total amount of intact casein, the casein-to-fat ratio (CFR) of cheese is a major factor influencing texture, and it is directly determined by the CFR of the milk used in its production (Faber, Jaishankar, & McKinley, 2017; Guinee, Mulholland, Kelly, & O'Callaghan, 2007; Guinee & O'Callaghan, 2013a, 2013b). It is important to note that variations in fat and casein concentration of milk occur and are due to factors such as the species of dairy animal, breed, health status, stage of lactation and animal-feeding practices, among others (Amenu & Deeth, 2007). Because of these natural variations, milk is frequently standardized prior to cheese manufacturing to a predetermined CFR. This step of standardization is crucial to obtain a product of consistent composition and texture (Johnson & Law, 2010). Therefore, CFR differs from one variety of cheese to another. Several studies have suggested that variability on composition of cheese could have an influence on the distribution and dynamics of fungal populations (Marín, Palmero, & Jurado, 2014; Montagna et al., 2004). In fact, it is known that other compositional factors of cheese such as pH, salt concentration and water activity exert a critical influence on spore germination and growth of fungi associated to cheese (Bekada, Benakriche, Hamadi, & Bensoltane, 2008; Marín et al., 2014; Morin-Sardin, Rigalma, Coroller, & Jany, 2016; Valik, Baranyy, & Görner, 1999). In this context, this research was aimed to explore the

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https://doi.org/10.1016/j.lwt.2017.12.016
Received 7 September 2017; Received in revised form 5 December 2017; Accepted 6 December 2017
Available online 09 December 2017

relationships among fungal growth, cheese composition, and changes on textural properties. Cheese analogues containing different CFR were inoculated with fungal species typically associated to cheese with two purposes: (i) to investigate the effect of temperature and cheese matrix composition on fungal growth and spore production, and (ii) to evaluate the subsequent loss in firmness of the cheese analogue by means of penetrometer tests.

2. Material and methods

2.1. Fungal isolates

Nine fungal strains isolated from cheese and previously identified by molecular and/or phenotypic methods (Marín et al., 2014) were used for this study. The species comprised *Penicillium discolor, Penicllium commune, Penicillium olsonii, Penicillium roqueforti, Penicillium solitum, Aspergillus pseudoglaucus, Cladosporium cladosporoides, Mucor racemosus* and *Mucor circinelloides*. Cultures were maintained on Sabouraud dextrose agar (Oxoid, Madrid, Spain) at 4 °C and stored as spore suspensions in 15% glycerol at −20 °C.

2.2. Cheese analogue

A cheese analogue was prepared based on a cheese medium developed by Nielsen, Frisvad, and Nielsen (1998). Acid casein (Manuel Riesgo, Madrid, Spain) and unsalted butter (own production) were used as source of milk protein and milk fat, respectively, to achieve the CFR needed. The total amount of protein and fat was 36.24%, and four different CFR were used (0.6, 0.8, 1.0 and 1.2). The CFR values were selected according to those typically found in cheeses (Guinee & O'Callaghan, 2010). The casein and butter used to prepare the cheese analogues contained respectively 10% and 16.7% of water, so their final concentration in the media were adjusted as follows: 0.6 (15.73% of casein and 27.62% of butter), 0.8 (18.64% of casein and 24.55% of butter), 1.0 (20.97% of casein and 22.10% of butter) and 1.2 (22.87% of casein and 20.09% of butter). The cheese analogue also contained 1.5% of bacteriological agar to favor emulsification (Pronadisa, Madrid, Spain), NaCl (2.9%), KH_2PO_4 (0.5%), $MgSO_4$ (0.2%), $CaCl_2$ (0.138%), $CuSO_4$ (4.10^{-3}%) and $Cl_3Fe{\cdot}6H_2O$ (0.02%). The pH was adjusted to 5.5 with a solution of NaOH 10M and all blends were adjusted to 58.5% moisture, taking into account the water contents already provided by acid casein, butter, and NaOH solution.

The cheese analogues were prepared by placing the butter, agar, water and salts solution on a bottle, which was then heated to 50 °C in order to melt the butter. Casein was finally added on this suspension, and the mix was homogenized manually before being autoclaved. The cheese analogue was autoclaved (110 °C, 10 min) and 20 mL were poured into 90 mm diameter Petri dishes for growth experiments, whereas 10 mL were poured into 50 mL falcon tubes (30 mm of diameter, 115 mm of height) for spore count and textural analysis.

2.3. Inoculation and incubation

A 5-mm-diameter agar disk from the margin of a 7-day-old growing colony of each fungal isolate grown on Sabouraud dextrose agar (Oxoid) at 20 °C was used to centrally inoculate Petri plates and falcon tubes containing the cheese analogue. Petri plates were closed with parafilm. The plates and falcon tubes were incubated at 10 °C and 20 °C for 10 days, and the experiment consisted of a fully replicated set of treatments with three replicates per treatment. The experiments included non-inoculated falcon tubes as controls. All of the falcon tubes were used for penetrometer tests after 10 days of incubation.

2.4. Growth assessment

Assessment of growth was made daily during the 10-day incubation period, or until the colony reached the edge of the Petri plate. Two diameters of the growing colonies were measured at right angles and the radii of the colonies were plotted against time. Linear regressions were used to obtain growth rates from the slope of the line.

2.5. Spore counting

Spores were harvested from 10-day old cultures on cheese analogue contained in falcon tubes using 25 mL of sterile distilled water with 0.1% Tween 80. The resulting suspensions were then filtered through cotton wool plugs to remove any hyphal fragments present. The number of spores was counted using a Neubauer chamber.

2.6. Penetrometer assays

Mechanical strength tests were performed using a Stevens LFRA texture analyzer, (Brookfield Engineering Laboratories, Middleboro, MA, USA). A 6 mm ball probe was used to penetrate the cheese analogue contained in falcon tubes after 10 days of incubation (inoculated and non-inoculated) at a constant speed of 0.5 mm/s to a depth of 13.5 mm beneath the suspension surface. The probe diameter was chosen so as to avoid contributions from wall effects. Uhlherr, Guo, Fang, and Tiu (2002) found that a $D/d > 4$–where D and d correspond to the diameter of the container and the probe respectively – was suitable to avoid wall effects. This criterion is exceeded in this study since $D/d = 5$. Cheese analogue was allowed to equilibrate at room temperature (20 °C) prior to testing. The mass component of force (M, g) was recorded and the respective force–distance curves were generated using Excel (Microsoft).

The parameter 'firmness' (F), defined as the work of penetration, was converted to single values of mechanical strength by calculating the area under the force-displacement curve with trapezoidal integration technique using Excel (Version 2010; Microsoft, Redmond, USA). Loss in firmness (LF), expressed as percentage, was then calculated according to the formula: $\%\ LF = [(F_c\text{-}F_i)/F_c] \times 100$, where F_c is the firmness of the cheese analogue control at a given CFR (non-inoculated) and F_i is the firmness of the cheese analogue inoculated with a fungal isolate at the same CFR.

2.7. Statistical analysis of results

For each fungal strain, temperature and CFR, growth rates, number of spores and loss in firmness were compared by single factor ANOVA. The growth rates, number of spores and loss in firmness were also compared among the 9 fungal species for the same conditions by single factor ANOVA. Subsequent post hoc analyses (Tukey's HDS tests) were carried out at a 95% confidence level. In all cases, Statgraphics Centurion XVI (Statistical Graphics Corp. Herndon, VA) software was used and P values of $< .05$ were regarded as significantly different.

3. Results

3.1. Fungal growth in relation to temperature and casein-to-fat ratio

Fungal growth rates in response to different temperatures (10 or 20 °C) and CFR values (0.6, 0.8. 1.0 or 1.2) are shown in Fig. 1. Temperature was the factor that most affected fungal growth. At 20 °C, growth rate was higher than at 10 °C in all the species tested. The fastest growing species were *M. racemosus* and *M. circinelloides*, followed by *A. pseudoglaucus*, *Penicillium* species and *C. cladosporoides*.

With the exception of the species *P. commune,* CFR also affected fungal growth. However, differences of growth rates as influenced by the CFR were much less pronounced than for temperature. As a general trend, growth rates were higher at a CFR of 1.0 and/or 1.2 at both temperatures. The exceptions were *P. commune*, *P. olsonii*, *P. discolor* (at 20 °C) and *C. cladosporoides* (at 10 °C).