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Mycotoxin-Forming Ability of Two *Penicillium roqueforti* Strains in Blue Moldy Tulum Cheese Ripened at Various Temperatures

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ABSTRACT

Isolated and identified toxigenic and nontoxigenic *Penicillium roqueforti* (PR) strains from moldy tulum cheeses were inoculated into tulum cheeses made in the laboratory and ripened at 5 and 12°C. Mycotoxin (patulin, penicillic acid, PR toxin, and roquefortine) formation in the control and mold-inoculated cheeses were detected by thin-layer chromatography on the first through fourth months of ripening. Patulin, penicillic acid, and PR toxin were not detected in the experimental cheeses. Only roquefortine was detected in cheese inoculated with the toxigenic strain of the mold and ripened at 5 and 12°C on the third and first months of ripening, respectively. Toxin in cheeses ripened at 5 and 12°C was 2.1 to 2.4 and 2.1 to 3.8 mg/kg cheese, respectively.

Tulum cheese is a special cheese type produced in small-scale dairy plants in Turkey. Of the two types of tulum cheese, one is made directly from cheese curd and the other is produced from a mixture of civil and lor (whey curd made by high heat treatment) cheeses. Civil cheese, an acid curd cheese with rennet added, is a popular cheese variety. It is similar to pasta filata cheeses, having a fibrous structure and similar melting and stretching properties. The mixture of civil and lor cheeses is pressed into goatskins or plastic bags and then ripened. This mixture, known as blue moldy tulum cheese, is molded spontaneously by natural contaminating molds that contribute to the ripening process. The cheese is ripened at 10°C for 2 months and is preferred for its characteristically natural moldy taste and flavor.

Cheese is a suitable medium for mold growth that could cause serious health risks *(5).* Some researchers have studied the mold flora and aflatoxin formation of tulum cheese. Alperden *(1)* isolated 167 mold species from 85 samples composed of different cheese types collected from the Marmara region. The molds were 54% *Penicillium* spp., 14% *Aspergillus* spp., 15% *Mucor* spp., and 16% various other mold genera. Erdogan et al. *(8)* isolated and identified 16 molds from 12 moldy tulum cheeses; 12 were *Penicillium roqueforti* and the others were *Geotrichum candidum. Penicillium* spp. were also reported to be dominant in some cheese varieties, including civil, kashar, tulum, and white cheeses collected in Erzurum, Turkey *(8, 9, 14),* 75 to 95% of which were reported to be *P. roqueforti (9, 14).*

P. roqueforti (PR) grown on cheese produces carcinogenic toxins such as patulin, penicillic acid, and PR toxin *(17).* Research has revealed that penicillic acid and patulin were found in 2 and 3.7% of cheddar cheese and 2.7 and 2.2% of Swiss cheese, respectively *(6).* Scott and Kennedy *(13)* found roquefortine in all 16 of the blue cheese samples. Roquefortine also is produced by *P. roqueforti (17),* and it is known as a neurotoxin. It was reported that 1 of 10 Valdeon cheese samples collected from Spain contained roquefortine *(10).* The LD_{50} of the toxin was determined to be 15 to 20 mg/kg in vivo *(18).* It was also reported that *P. roqueforti* grown on blue moldy tulum cheese had toxin-producing ability at 5, 12, and 25°C; eight samples produced only at 5 and 12°C and six samples could not produce toxin at 5°C *(8).*

The purpose of this study was to establish the mycotoxin-forming (patulin, penicillic acid, PR toxin, and roquefortine) capacity of two *P. roqueforti* strains in tulum cheese that had been isolated from blue moldy tulum cheese *(8).* One of the strains could produce mycotoxins such as patulin, penicillic acid, PR toxin, and roquefortine in yeast extract sucrose medium at 5 to 12°C, whereas the other could not produce mycotoxins.

MATERIALS AND METHODS

Tulum cheese making. Civil cheese was sliced into 2- to 3-cm samples, and a mixture (1/1, wt/wt) of civil and lor cheeses was prepared. The mixture was divided into three portions, the first of which was used as control and stored in two plastic cases (traditional method). Two mold strains isolated from blue moldy cheeses *(8)* were inoculated separately into potato dextrose agar and incubated at 24°C until intensive mold spore formations appeared *(2).* Spores from these formations were transferred into sterile distilled water. The second and the third portions were inoculated with 0.1 ml of the spore suspension of either a toxigenic or nontoxigenic *P. roqueforti* strain. Then, each portion was divided into two plastic cases (~1 kg), covered tightly with sterile thick cloths, and turned upside down on sterile sand. A cheese case of each batch was ripened at 5°C, and the others were stored at 12°C for 4 months. Three replicates were performed. At the first, second, third, and fourth months of ripening, all cheese samples were analyzed for patulin, penicillic acid, PR toxin, and roquefortine.

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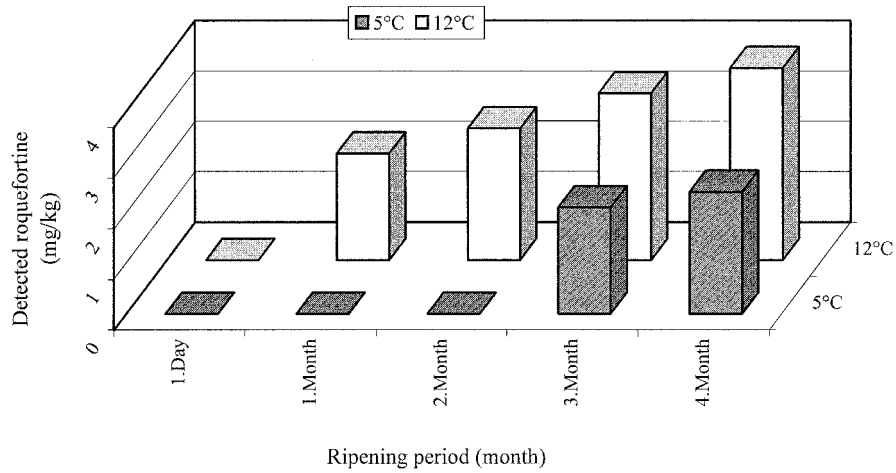

FIGURE 1. *The effect of temperature and ripening period on roquefortine formation in cheeses inoculated with the toxigenic strain of* Penicillium roqueforti.

Analytical methods: penicillic acid and patulin analysis. A 25-g sample of cheese was blended with 100 ml of 5% NaCl solution for 2 min in a Warring blender. The pH of the solution was adjusted to 6 by addition of 1 N acetic acid, 200 ml of methanol-acetone (50/50, vol/vol) was added, and the mixture was blended for an additional 3 min. The mixture was titrated, and the filtrate was held at −20°C for 5 to 6 h. The casein precipitate formed by the action of cold was centrifuged at 2,000 rpm for 20 min. Supernatant liquid (200 ml) was transferred to a 1-liter round-bottom flask and evaporated to about 75 ml in vacuum. The remaining solution was transferred quantitatively to a separatory funnel and washed three times with 100 ml of *n*-hexane. The defatted solution was extracted twice with 100 ml of chloroform: once with 100 ml of chloroform–ethyl acetate (50/50, vol/vol), then once with 100 ml of ethyl acetate. These extracts were combined, passed through a filter containing anhydrous sodium sulfate into a round-bottom flask, and evaporated to about 5 ml. The combination was transferred to a small glass tube and evaporated to about 1 ml in a heating block under a stream of nitrogen. The volume was adjusted to 5 ml with chloroform-hexane (50/50, vol/vol) and applied to a column of silica gel.

The column material was prepared by blending 10 g of Kieselgel H (Merck, type 60) with 50 ml of *n*-hexane–benzene (50/50, vol/vol). The silica gel was deactivated previously with 3% water. The slurry was added to the chromatographic tube (22 by 400 ml) and firmly packed under pressure (500 ml Hg). Then, 100 ml of *n*-hexane–benzene (50/50, vol/vol) was added to the column to elute some of the impurities, and the additional extraneous matter was eliminated by washing with another 100 ml of benzene. Patulin and penicillic acid were eluted with 300 ml of ether:*n*-hexane–formic acid (60/20/0.5, vol/vol/vol) under pressure. The two fractions were evaporated separately in a heating block under a stream of nitrogen and resolved in 200 μl of chloroform. Two-dimensional thin-layer chromatography (TLC) was used. The plates first were developed in chloroform-acetone-water (93/7/1, vol/vol/vol) and sprayed with diethylamine. To reveal patulin and penicillic acid, the plates were heated at 100°C for 15 min. Patulin appeared as a blue-gray fluorescent spot, and penicillic acid appeared as a light blue fluorescent spot under long-wavelength ultraviolet light. They were quantified by visual comparison with known standards *(16)*.

Analytical methods: roquefortine analysis. Fifty grams of cheese was blended in a Warring blender with 50 ml of chloroform, 100 ml of methanol, and 20 ml of water for 2 min at high speed and then for two additional 1-min periods after successive additions of 50 ml of chloroform and 50 ml of water. The mixture was filtered under reduced pressure through a pad of Celite filter aid (AW/545) together with 10 ml of chloroform used to rinse the blender jar. The filter cake was rinsed with an additional 10 ml of chloroform. The chloroform layer was separated and evaporated on a steam bath under a gentle stream of nitrogen to an oily residue, which was dissolved in 50 ml of ethyl acetate and extracted with two 50-ml portions of 0.5 N HCl. The combined acid layers were washed with 50 ml of *n*-hexane, made alkaline with 10 ml of 28% ammonium hydroxide solution, and re-extracted with 50 ml of chloroform. The extract was evaporated nearly to dryness under nitrogen, transferred to a 16-ml vial for complete evaporation, and dissolved in 0.5 ml of chloroform.

Silica gel F 1500/LS254 thin-layer sheets (Schleicher and Schuell) were used for analytical and preparative TLC of cheese extracts. One to 10 μl of extract was spotted for analysis. Chromatograms were developed for a distance of 13 to 15 cm with chloroform–methanol–28% ammonium hydroxide (90/10/1, vol/vol/vol) in a lined, equilibrated tank. The solvent was replaced at least every 2 days. Analytical TLC plates were sprayed with 50% sulfuric acid and heated at 110°C for 10 min. Roquefortine formed a light blue spot *(13)*.

Analytical methods: PR toxin analysis. Cheese samples (10 g) were homogenized in a Warring blender with 65 ml of methanol-water (55/45, vol/vol) and 40 ml of hexane for 5 min at high speed. The mixture was centrifuged at 1,000 × *g* at 4°C for 10 min. After removal of the hexane layer, the methanol-water portion was extracted twice with 20-ml volumes of chloroform. The two chloroform extracts were pooled and concentrated with a rotary evaporator. The sample was then evaporated to dryness under a stream of nitrogen and dissolved in 1 ml of chloroform.

Thin-layer sheets and spotted extracts were prepared as previously described for roquefortine analysis, and the chromatograms were developed in benzene–methanol–acetic acid (24/2/1, vol/vol/vol). PR toxin formed a dark blue spot *(15)*.

Statistical analysis. Data were analyzed by two-way analysis of variance. Means with significant differences were compared by Duncan's multiple range test.

RESULTS AND DISCUSSION

Mycotoxin formation (patulin, penicillic acid, PR toxin, and roquefortine) was not observed in the control or in the cheeses inoculated with the nontoxigenic strain. However, it was interesting that patulin, penicillic acid, and PR toxin also were not found, even at trace levels, in the cheeses inocu-

lated with the toxigenic strain. This finding was in agreement with Beuchat *(4),* who reported that *P. roqueforti* produced patulin, penicillic acid, and PR toxin in broth medium but only at trace levels or not at all in cheese. Bullerman *(6),* Mahfoud et al. *(11),* Bando et al. *(3),* and Chang et al. *(7)* reported that these toxins were inhibited in foods rich in proteins by compounds that contain sulfhydryl, such as sistein and glutathione. It also was reported that PR toxin reacts with ammonia and ammonia salts, amino acids, amines, casein, and their decomposition products, resulting in a less toxic PR imine, which is unstable in cheese. However, patulin and penicillic acid that react with a mixture of neutral, basic, and acidic amino acids; glutathione; casein; and peptones are not a potential carcinogenic risk in moldy cheese *(12).*

As opposed to the control and cheeses inoculated with a nontoxigenic strain, roquefortine was formed in the cheeses inoculated with a toxigenic strain of the mold and ripened at 5 and 12°C (Fig. 1). The effects of repetition, ripening period, ripening temperature, and their interactions had significant effects on roquefortine formation ($P < 0.05$). The means of replicates explain the differences in chemical composition of cheese samples, as well as the effects of different amounts of spores inoculated. The effect of temperature and ripening period on roquefortine formation is shown in Figure 1. As expected, the amount of roquefortine in cheese increased during ripening as a result of mold growth. The significant effect of ripening temperature on roquefortine formation can be attributed to the restrictive effect of temperature on mold growth. It was reported that the higher the rate of mold growth and temperature, the more the roquefortine that formed during ripening *(12).* As the ripening temperature increased, the mold growth increased, resulting in more mycelia and more roquefortine *(17).*

In cheeses inoculated with the toxin-forming strain and ripened at 12°C, a considerable amount of roquefortine appeared in the first month and increased during ripening. On the other hand, roquefortine formation was apparent in the third month and increased slightly in the fourth month in cheeses ripened at 5°C. The amount of roquefortine found in cheese ripened at 5 and 12°C was 2.1 to 2.4 mg/kg cheese and 2.1 to 3.8 mg/kg cheese, respectively. Roquefortine formation increased in parallel with higher ripening temperature. The effect of temperature on toxin formation can be explained by its effect on mycelium development. Because higher temperatures cause an increase in mold growth and the amount of mold mass, the increased amount of mycelial mass results in an increase in the formation of toxin.

The formation of patulin, penicillic acid, and PR toxin was not observed in the experimental cheeses. However, roquefortine was detected in cheeses inoculated with the toxigenic strain of the mold. This study illustrates that molded tulum cheeses should be produced under controlled conditions and inoculated with a nontoxigenic *P. roqueforti* strain and that spontaneously molded cheeses should not be consumed.

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