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ORIGINAL ARTICLE

Packaging fortified with Natamycin nanoparticles for hindering the growth of toxigenic *Aspergillus flavus* and aflatoxin production in Romy cheese

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ABSTRACT

Objective: This study assessed the effect of cellulose sheets fortified with Natamycin-loaded alginate nanoparticles on the growth of toxigenic *Aspergillus flavus* and aflatoxin production on the superficial layer of Egyptian Romy cheese after 12 weeks of maturation.

Materials and Methods: Toxigenic *A. flavus* (GenBank accession No. MT645073) was inoculated into the outer surface of Egyptian Romy cheese (at 5 log CFU/gm) and wrapped with a cellulose sheet fortified with Natamycin-loaded alginate nanoparticles. Unwrapped control contaminated Romy wheels were made as well as non-contaminated wrapped cheese wheels for sensory evaluation. Romy cheese wheels were stored at a temperature similar to commercial methods for 12 weeks. Fungal counts were enumerated during this time, and enzyme-linked immune sorbent assay detected aflatoxin after the 4th week of maturation storage.

Results: In cheese samples covered with cellulose sheets containing Natamycin-loaded alginate nanoparticles, the fungal count was reduced by 2 log approximately in contrast to control samples after the 2nd week of storage. However, within the 8th week of storage, the greatest significant reduction ($p < 0.05$) was seen where fungal growth was hindered entirely to the end of the ripening period. The mean values for taste, color, flavor, and overall acceptability were 4, 4.7, 4.09, and 4.3, respectively. Furthermore, in the treated samples, the total aflatoxin concentration was decreased by 78.6% relative to the untreated control one.

Conclusion: Using cellulose sheets fortified with Natamycin-loaded alginate nanoparticles in Egyptian Romy cheese wrapping could be an effective way of controlling *A. flavus* and subsequent aflatoxin production without influencing the typical taste, color, flavor, and overall appearance of traditional Romy cheese.

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Introduction

Ras (Romy) cheese is an Egyptian hard cheese made from bovine and buffalo milk. It takes an extended time to produce the distinctive flavor and texture, where maturation takes place for 3 months at a nearly constant relative humidity and temperature [1,2].

Fungus contamination is common in dairy products. They are accountable for visible or unseen imperfections, such as off-odor and off-flavor, resulting in considerable food waste and losses, leading to economic losses [3]. The production of toxigenic fungi during maturation of Ras cheese, as some *Aspergillus* spp., is considered a safety concern for human consumption. They are xerophilic fungi and are responsible for many cases of contamination of foods and feeds [4].

Aflatoxins are unavoidable, common food pollutants with a significant effect on well-being and food safety [5]. Dietary exposure to aflatoxin-polluted food has been correlated with considerable health complications such as liver cancer, impediment to growth, immune reduction, and doom [6]. Hindering fungal growth might be a severe concern for industrials and scientists seeking efficient

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solutions to prevent and cut-off fungal decay in dairy products [3].

One of the innovative practices for preserving food products is active packaging technology, and antimicrobial packaging films could be a serious field, encouraging this innovation [7]. Active packaging technology could also be described as a packaging technique which, through the coating of packaging materials, adds antimicrobials, antioxidants or other quality enhancing agents, allowing the active packaging agent to be released in minutes into the packaged food to ensure the safety of packaged foods. The great attention paid to active packaging with antimicrobial substances has been significantly increased among many active packaging applications [8].

Natamycin is produced by aerobic fermentation of *Streptomyces natalensis* [9]. After being approved by the Food and Drug Administration and the European Community as Generally Recognized as Safe, it is being used as a natural preservative in foods [10,11]. Natamycin carries out its antifungal function by binding to fungal membrane sterols, leading to cell lysis [12]. Natamycin can bind to ergosterol within the fungal plasma membrane, leading to a loss of membrane function [13]. While Natamycin is widely used as a food preservative, some factors can compromise its application effectiveness on the food surface, such as poor water solubility, inactivation by food matrix components, and rapid migration [14]. Natamycin's low aqueous dissolvability restricts its availability for antimicrobial activity in the molecular state and its diffusion rate to the place of antifungal action. The low solubility of Natamycin in water, however, results in a non-uniform distribution on the coated surface and decreases its fungal efficacy, so Natamycin inclusion complexes have been developed for various applications [15,16]. Nanotechnology is one of these applications which could provide Natamycin with greater availability and enhanced antifungal performance [17].

According to our knowledge, active packaging is not applied to the outer surface of the Egyptian Romy cheese to overcome fungal spoilage, thus the development of aflatoxin occurs. The purpose of this study was to investigate the inhibitory effect of Natamycin nanoparticles on the growth of *Aspergillus flavus* and its production of toxins in the maturation of the Egyptian Ras (Romy) cheese.

Materials and Methods

Natamycin nanoparticles

Natamycin-alginate nanoparticles were prepared with some modifications using the strategy defined by Lertsutthiwong et al. [18]. In brief, 0.6 ml Natamycin solution (20 mg/ml ethanol) (Sigma, Chicago, IL No: 32417-50MG) was regularly descended into 20 ml sodium alginate (0.3 mg/ml) (Sigma, Chicago, IL No: W201502) incorporated with 10% (*w/v*) pluronic F-127 (Sigma, Chicago, IL No: P2443). The solution was subjected to sonication for 15 minutes, then 4 ml of 0.67 mg/ml $CaCl_2$ was added with stirring for 30 minutes. Finally, the external structures and morphology of the Natamycin-loaded alginate nanoparticles were assessed using a scanning electron microscope (SEM).

Packaging fortified with Natamycin

As indicated by Dias et al. [19], the antifungal sheets were set up; 30 gm of cellulose acetate (Rhodia Co., Courbevoie, France) were dissolved into 500 ml acetone. The suspension of Natamycin-loaded alginate nanoparticles was applied at a concentration of 10% (*v/w*) to the sheet solution and was well blended. The sheet approaches were distributed with the aid of the thin layer chromatography spreader apparatus. The acetone was evaporated at 25°C. Sheets measured 30 × 20 cm with 0.5 mm thickness and were yellowish in color.

Manufacture of Romy cheese

As per the method described by Abou-Donia [20], cow milk (with 3% fat) was utilized and enough rennet was included with heating at 50°C to complete the coagulation and was molded in the Romy cheese molds lined with cloth sheets. Whey was then drained out from cheese curd by manual pressure to expel excess whey overnight. Cheese wheels were then removed from molds at room temperature for extra drainage.

Preparation of inoculated strain

In the Reference Lab for Safety Analysis of Food of Animal Origin, Animal Health Research Institute (AHRI), an Egyptian field isolate of toxigenic *A. flavus* (Asp. Food Hygiene Department.1; GenBank accession No. MT645073) was used. The frozen culture was triggered in 9 ml of Potato Dextrose Broth. Mycelia were centrifuged and washed by phosphate buffer solution, with a final concentration of approximately10^6 colony forming unit (CFU)/ml.

Inoculation and packaging

Cheese disks were divided into three groups; the first was packed without dipping into the contamination solution with a Natamycin nanoparticle sheet (for sensory examination). In the contamination solution, the second and third groups were immersed for 10 minutes, then left to dry. Meanwhile, the second group was packed with a sheet of Natamycin nanoparticles, while the third group was left unpackaged. All the cheese disks were ripened for 3 months at 25°C.

Aspergillus flavus enumeration

Samples of cheese were taken for fungus count on 0 day (after 2 hours) and then every week within the 1st month and every 2 weeks within the 2nd and 3rd months. The sheet was removed from cheese, and fungal enumeration was carried out as per the method described previously [21] using the spread method on Rose Bengal chloramphenicol agar and incubated for 7 days at 25°C. The plates with 15–150 characteristic fungal colonies (green colored and 15 mm in diameter) were counted. All the tests were implemented in triplicate.

Determination of total aflatoxin

According to manufacture, aflatoxin concentrations were set on using enzyme-linked immune sorbent assay (Ridascreen®, aflatoxin Total -r-biofarm- R4701). Triple determinations were carried out, then the mean was calculated. Aflatoxin concentrations were expressed as µg/kg of the sample.

Sensory analysis

The analysis was carried out by using a numeric descriptive 5-point scale, ranging from 5 to 1, with 5 = very much like, 4 = like, 3 = approved, 2 = dislike, and 1 = very much dislike [22]. Cheese samples were evaluated for taste, color, flavor, and the overall acceptability. The sensory panel team comprised 15 qualified assessors by the Reference Lab for Safety Analysis of Food of Animal Origin, AHRI. Samples with a ranking below three were considered rejected.

Data analysis

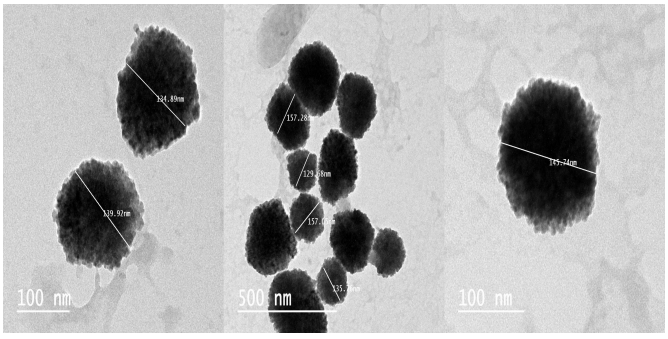
Three repetitions of the experiment were carried out. The data were analyzed using the Statistical Package for the Social Sciences software mixed procedure (production 20, IBM CO) after logarithmic fungal count transformation. To detect significance ($p < 0.05$), the *t*-test was used.

Results and Discussion

Most of the dairy products favor the undesirable growth of spoilage as well as toxigenic fungi [3]. *Penicillium* and *Aspergillus* species are often isolated from cheese and are typically associated with poor hygiene requirements and resistance to antimicrobials or heat treatment [3,23]. New approaches were used to control fungal spoilage of cheese as packaging fortified with Natamycin (Pimaricin), which is a potent antifungal compound that can effectively prevent mold growth and inhibit the development of aflatoxin [24]. In the current study, Natamycin-loaded alginate nanoparticles were studied to determine their ability to control fungal growth and aflatoxin production on the outer surface of Romy cheese and their impact on the sensory quality of Romy cheese during ripening.

An SEM was used to determine the morphology and shape of the nanoparticles. The SEM images (Graph 1) revealed that the particles of Natamycin-loaded alginate nanoparticles presented a uniform appearance with spherical-shaped, characteristic shell and core appearance, and the absence of significant agglomeration. The particle size ranged from 129 to157 nm. Uniformity in size resulted in stronger interaction between the Natamycin-nanoparticles and microorganism, also resulted in better distribution and sustained release of Natamycin on the surface of the examined product [25]. The loading of Natamycin on alginate nanoparticles resulted in higher availability, improved efficiency of Natamycin, and being easier incorporated into food. This phenomenon could be attributed to the fact that some oligosaccharides as alginate containing functional groups show biological activities as antibacterial, antiviral, and antioxidant activities [26].

The impacts of Natamycin-loaded alginate nanoparticles incorporated cellulose sheet on the development of *A. flavus* inoculated in Egyptian Ras cheese were studied and shown in Table 1 and Figure 1. The count of *A. flavus* on non-wrapped inoculated cheese samples (control samples in Table 1 and Figure 3) developed from 5.3 to 5.9 CFU gm^{-1} over 4 weeks of maturation, then reduced to 5.74 CFU gm^{-1} at the end of maturation. Almost, fungal count significantly decreased ($p < 0.05$) in cheese samples wrapped with Natamycin sheet than control samples after the 1st week of ripening. This decrease reached 2 log after the 2nd week of storage. However, the greatest significant reductions ($p < 0.05$) were seen in the 8th week of storage, where the fungal growth was completely inhibited till the end of the ripening period. The same outcomes were acquired by Medina et al. [27], who found that Natamycin at 50–100 ng ml^{-1} can inhibit the growth of *Aspergillus carbonarius*. Similar findings were also described by Ture et al. [28] revealing that wheat gluten sheets and methyl-cellulose incorporated with Natamycin have potent antifungal capacity upon *Aspergillus niger* on fresh kashar cheese for a whole month.



Graph 1. Scanning electron micrograph (SEM) of an alginate-Natamycin nanocapsule, size between 129 and 157 nm.

Table 1. Count of *Aspergillus flavus* (log_{10} CFU/gm ± SD) in contaminated Romy cheese samples during ripening.

Period (weeks)	Zero	1st	2nd	3rd	4th	6th	8th	10th	12th
Control	5.30^{a} ± 0.09	5.60^{a} ± 0.11	5.84^{a} ± 0.09	5.95^{a} ± 0.097	5.92^{a} ± 0.11	5.84^{a} ± 0.1	5.79^{a} ± 0.11	5.69^{a} ± 0.12	5.74^{a} ± 0.11
Treated	5.47^{a} ± 0.1	4.62^{b} ± 0.10	3.90^{b} ± 0.09	3.17^{b} ± 0.09	2.47^{b} ± 0.095	1.17^{b} ± 0.096	$<1^{b}$	$<1^{b}$	$<1^{b}$

Values are three replicate means. Mean values marked with different small letters for each column are significantly different ($p < 0.05$).

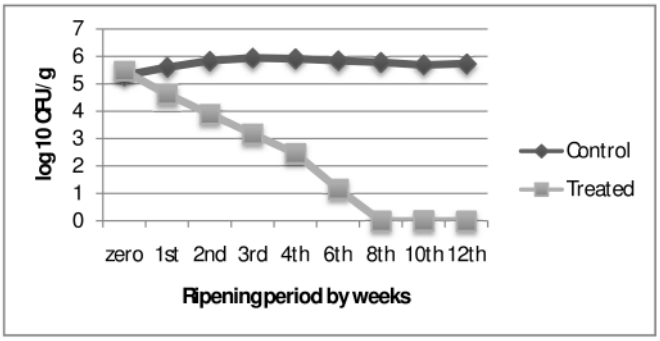


Figure 1. Count of *A. flavus* (log_{10} CFU/gm) in contaminated Romy cheese samples during ripening.

Moreover, Kallinteri et al. [29] stated that Natamycin was able to effectively reduce the fungal growth within the Galotyri soft cheese and resulted in doubling its shelf-life (>28 days), when compared to the control untreated sample. Santonicola et al. [30] also showed that the complex of Natamycin-chitosan significantly lowered the yeasts and molds in cheese stored at 20°C for 7 days. However, Gonzalez-Forte et al. [31] showed that Natamycin has an antifungal effect against fungi isolated from cheese planets. These findings were consistent with Shah et al. [32], who stated that Natamycin significantly ($p < 0.05$) reduced the growth of yeast, aerobic bacteria, and enterobacteria, while lactic acid bacteria were not influenced. Natamycin's inhibitory action against *A. flavus* can be contributed to that it can inhibit fungal growth and causes cell lysis by binding to cell membrane sterols [12].

Regarding results of the sensory attributes (Fig. 2) showed that Natamycin did not influence the original taste, color, flavor, and the overall appearance of traditional Romy cheese, where the mean values were 4, 4.7, 4.09, and 4.3, respectively. This result was similar to the finding recorded previously [33-37], stating that wrapped cheese was better than the non-wrapped ones. Nottagh et al. [38] also pointed out that the edible coating supported chitosan and Natamycin significantly prevented microorganisms' growth that causing cheese spoilage and did not affect starters that had a role in cheese ripening and sensory characters.

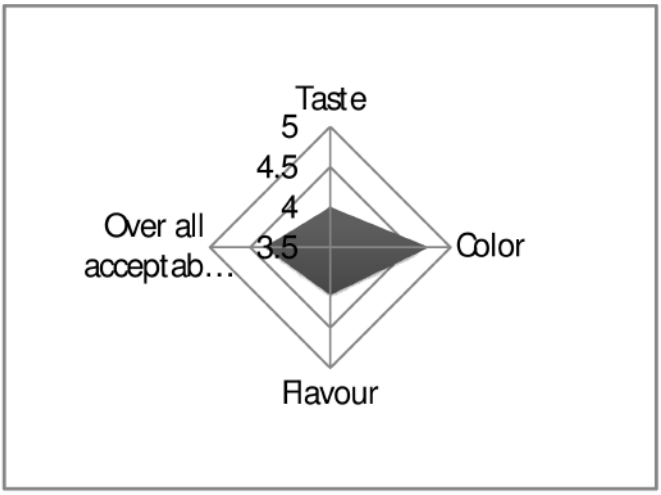


Figure 2. The average score of taste, color, flavor, and the overall acceptability of packed cheese.

As summarized in Table 2 and Figure 3, the control samples were higher in aflatoxin concentrations than those treated with Natamycin by 78.6% at the end of maturation (12th week). The mean of aflatoxin concentration in control samples was 2.75 ± 0.01 at 4th week of maturation and increased throughout the period to reach 8.52 ± 0.15. However, the mean of aflatoxin concentration in Natamycin-treated samples was 1.83 ± 0.03 at 4th week of maturation and remained almost constant throughout the maturation period. These results are agreed with those of Rusul and Marth [39], who found that increasing the concentration of Natamycin in glucose yeast extract-salt medium reduced the amounts of aflatoxin produced after 3 days of incubation. However, the presence of 20 ppm Natamycin inhibited toxin production after 7 days. Also, Medina et al. [27] have demonstrated that at 20°C, ochratoxin production by *A. carbonarius* in the fresh red grape medium was only significantly inhibited by 10 ppm Natamycin.

In this study, these results demonstrate that using an active packaging containing Natamycin nanoparticles may provide the prevention and regulate the growth of fungi in hard cheese. Other dairy products, including various soft, semi-soft, and semi-hard cheeses, can be used to conduct future studies on the efficacy of Natamycin nanoparticles.

Table 2. The effect of Natamycin nanoparticles on total aflatoxin (µg/kg ± SD) by *A. flavous* after 4 weeks of Romy cheese maturation at 25°C (mean of three trials).

Storage period (weeks)	4th	6th	8th	10th	12th
Control	2.75 ± 0.01[a]	4.07 ± 0.05[a]	4.32 ± 0.04[a]	6.71 ± 0.01[a]	8.52 ± 0.15[a]
Treated	1.83 ± 0.03[b]	1.82 ± 0.03[b]	1.81 ± 0.05[b]	1.8 ± 0.05[b]	1.82 ± 0.05[b]

Values are three replicate means. Mean values marked with different small letters for each column are significantly different ($p < 0.05$).

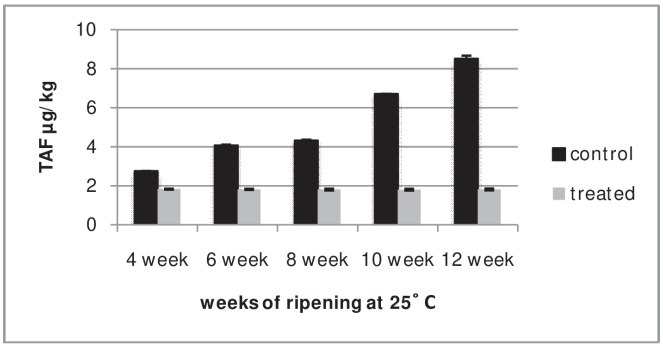


Figure 3. Total aflatoxin concentrations (µg/kg).

Conclusion

Cellulose sheets fortified with Natamycin-loaded alginate nanoparticles were sufficient to complete the elimination of log 5 CFU gm^{-1} *A. flavus* after 8 weeks of ripening. Here, cheese storage also reduced aflatoxin production by 78.6% without affecting the original taste, color, flavor, and overall appearance of traditional Romy cheese.

List of abbreviations

AHRI = Animal Health Research Institute; SEM = Scanning Electron Micrograph; CFU = Colony forming unit, FHD = Food Hygiene Department.

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Conflict of interest

The authors shall certify that they have NO affiliations with or participation in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this Manuscript.

Authors' contributions

All the authors made substantial contributions to the conception, design, analysis, interpretation of data. The authors read the final version and approved it for publication.

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