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Control of fungal prevalence through manipulation stages of soft cheese, using chitosan and essential oils

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Abstract
The current study aimed to determine the fungal population in two-hundred soft kareish cheese and processed cheese samples, 100 of each, collected from Gharbiya governorate's groceries; followed by estimation of aflatoxin M1 (AFM1) in some of the examined samples. Moreover, an experimental study was conducted for reduction of the fungal count in cheese. Results revealed positive contamination of 36.0% of the total examined samples; where kareish cheese showed higher fungal prevalence than processed cheese. Although, Aspergillus species revealed the highest prevalence ratio, *A. niger* particularly, in the examined samples, Penicillium, Fusarium, Phoma, Alternaria and Geotrichum species were also detected in lower prevalence. Moreover, 32.0% and 22.2% of kareish and processed cheese samples had AFM1, with mean values of 0.49 and 0.36 µg/kg, respectively. On the other hand, addition of chitosan, marjoram essential oil and mustard essential oil showed significant antifungal effect; which appeared to be dose dependent, where higher concentration gave more reduction effect. In addition, chitosan had the superiority as an antifungal substrate, where it revealed reduction in the *A. niger* count by 97.7% within two days of incubation, followed by mustard EO and marjoram EO, respectively. Food safety and quality are the main topics concerning with the consumer, therefore, continuous searching for natural food additives of antimicrobial effect has been demanded.

Keywords: Antimicrobial, cheese, food safety, mould species

Introduction

Soft cheese occupies a significant nutrition part of wide range of people around the world, offering a unique blend of flavors, textures, and nutritional benefits. Rich in calcium and protein, soft cheeses are not only versatile ingredients but also contribute positive impacts to bone health and dental wellness (Aly *et al.*, 2016) [4]. The high moisture content in these cheeses makes them susceptible to microbial growth, which necessitates careful handling and storage to prevent foodborne illnesses (Nájera *et al.*, 2021) [39].

Mould contamination in soft cheese is a significant concern in the dairy industry, primarily stemming from various environmental sources including airborne spores; which has been recognized as a major source of mold contamination in cheese production facilities, surrounding environment, especially in cheese ripening rooms and during storage, including high humidity and suitable temperatures. Contamination can also occur from improperly cleaned equipment, utensils, and surfaces that come into contact with cheese. In addition, the quality of raw ingredients used in cheese production plays a crucial role in mold contamination (El-Badry and Raslan, 2016) [19].

Presence of such harmful mould contamination of cheese may lead to spoilage and production of mycotoxins with potential health hazards to human due to their carcinogenic effects, liver diseases and organ damage (Darwish *et al.*, 2014) [16].

In recent years, there has been growing interest in natural preservatives such as chitosan, mustard oil, and marjoram oil for enhancing the shelf life and safety of soft cheeses (Baptista *et al.*, 2020) [7]. Chitosan, derived from chitin found in crustacean shells, exhibits notable antifungal properties that can inhibit the growth of mould on cheese surfaces (Barik *et al.*, 2024) [8] Similarly, essential oils from mustard and marjoram have demonstrated antimicrobial effects that can further protect against spoilage organisms while contributing unique flavors (Chouhan *et al.*, 2017) [13]. Therefore, the current study aimed to investigate the microbiological quality of kareish and processed soft cheese samples, in relation to the prevalence and mould profile of the examined samples. Besides that, an experimental study was conducted to assess the inhibitory effect of chitosan and essential oils of mustard and marjoram against mould growth in cheese samples.

Materials and Methods

1. Collection of cheese samples

A total of two-hundred random samples of soft kareish cheese and processed cheese, 100 of each, were purchased from different groceries and supermarkets in Gharbia Governorate, Egypt. Samples were collected and transferred to the laboratory as soon as possible in sterile polyethylene bags.

2. Samples preparation

The examined cheese samples were prepared according to the technique recommended by ISO (2017) [28]; where, one gram was diluted with 10 ml sterile dist. water, then incubated with sabouraud dextrose broth aerobically at 25ºC for 3 days.

3. Isolation of mould species (ISO, 2008) [27]

0.1 ml of each incubated broth sample was spread over Sabouraud’s dextrose agar and incubated at 25ºC for 5-7 days aerobically. Each colony of the raised fungi was picked up aseptically for enrichment in Sabouraud’s dextrose broth for obtaining of pure culture.

4. Identification of isolated moulds (Pitt and Hocking, 2009)

Careful observation of the growth pattern, color and texture of basal and surface mycelia were observed and recorded. The examination was carried out by using a magnifying hand lens.

In microscopical examination, lactophenol stain and suitable lens power was used to differentiate various structures of the examined isolated mould strain.

5. Detection of aflatoxin residues in examined cheese samples by using Thin Layer Chromatography "TLC"

The same two mentioned cheese samples which had been examined microbiologically were screened for aflatoxin. Extraction, cleanup, quantification and confirmation by thin layer chromatography was conducted according to Van Egmond *et al.* (1986) [48]. In addition, known volumes of the extracted sample and standards were applied in the chromate-plates for the quantification. All calculations were done according to the Manual of Official methods of Analysis (AOAC, 2000) [5].

The aflatoxin concentration in the sample extracts determined by matching the fluorescence intensities of the toxin spots in the sample with those of the standard.

The calculation of the concentration of aflatoxin in the sample was made by using the following formula:

$$ug/kg = S.Y.V/X.W.$$

Where:

S= µL of aflatoxin standard equal to unknown.

Y= Concentration of aflatoxin standard in µg/ml.

V= µL of final dilution of final extract.

X= µL of sample extract giving a spot intensity equal to S.

W= Mass (weight) of the sample, represented by the final extract in gram.

6. Experimental estimation of the anti-fungal effect of chitosan and essential oils against the most common fungus

6.1. Preparation of chitosan solution (Zuhannisa *et al.*, 2017) [50]

The chitosan (1.0 and 2.0%) was prepared by mixing chitosan powder as much as 1 and 2 g in 100 mL of dist. water mixed with 2 mL acetic acid. The solution was stirred using a magnetic stirrer for 1 h. The prepared solution was kept in opaque bottle until the application.

6.2. Preparation of the used essential oils

The crude essential oils of marjoram and mustard were obtained from Captin Pharma Co. – Shebin Elkom, Elmonoufiya governorate; from which, 0.1, 0.5 and 1.0 mL of the raw marjoram and mustard oils were mixed in 100 mL of tween-80 and mixed well to obtain 0.1, 0.5 and 1.0% concentration, respectively. The prepared solutions were kept in amber bottle until application.

6.3. Preparation of fungal inoculum

Mycological enrichment was performed according to ISO (2008) [27] on sabouraud dextrose broth, followed by incubation at 25ºC for 3 days aerobically. From which, 0.1 mL was spread on SDA for determination of the original count (CFU/mL), from which cheese samples were inoculated experimentally.

6.4. Experimental design

One kg and 440 g of kareish cheese were made from high-quality raw milk in the Lab. for more hygienic condition, followed by culturing on SDA to confirm it is free from contamination.

Cheese was equally distributed into eleven sterile containers, followed by experimental inoculation of nearly 10^3 CFU/g. After that, 1st group was kept without treatment representing control positive group (G1). The other groups were treated by addition of the prepared chitosan (1.0%, and 2.0%), marjoram EO (0.1%, 0.5% and 1.0%), mustard EO (0.1%, 0.5% and 1.0%) representing the treated groups named as (G2 – G9). Moreover, chitosan (1.0%) was combined with marjoram EO (1.0%) and mustard EO (1.0%) in separate groups representing (G10 and G11). Furthermore, control negative group was kept without fungal inoculation for confirming hygienic conditions of cheese preparation (G12).

After 30 minutes of incubation, the fungal count was estimated according to the preparation procedures (ISO, 2017) [28], (ISO, 2008) [27] and recorded as initial count (zero time). Aspergillus counting was repeated every 72h, and the reduction percent was calculated in relation to the initial count according to the following equation:

$$R\% = \frac{R1 - R2}{R1} \; x \; 100$$

Where, R1: original count (control count), R2: Count of each treated group's plate, R%: Reduction percent

7. Statistical analysis

SPSS version 20 was used to analyze the obtained data. The significance of the differences in the mean values of the groups under investigation was determined using ANOVA analysis and the Duncan posthoc value. A significance level of $p < 0.05$ was deemed significant. NB. Statistical analysis of less than three groups was performed using independent T test; where the significance was symbolled by superscript star sign (*).

Results

1. Incidence of Mould isolated from the examined cheese samples

The results recorded in **Table (1)** showed that the samples of soft kareish samples recorded higher contamination rate with mould than the processed cheese samples; where 40/100 (40%) and 32/100 (32%) of the examined kareish and processed cheese samples were positive for mould growth.

2. Prevalence of mould genera isolated from different examined samples

Referring to the recorded results in **Table (1),** *Aspergillus, Penicillium* and *Fusarium* spp. were the predominant species detected in the examined samples; whereas, other genera were detected in lower prevalence. In details, regarding to kareish cheese samples, 48 strains were isolated; where *Aspergillus spp., Penicillium spp., Fusarium spp., Alternaria spp.* and *Geotrichum spp.* were detected in the prevalence (%) of 52.1, 25.0, 12.5, 6.25 and 4.2%, respectively. On the other hand, out of 41 isolated strains, *Aspergillus spp., Penicillium spp., Fusarium spp.* and *Phoma spp.* were detected in the prevalence (%) of 43.9, 29.3, 19.5 and 7.3%, respectively in the processed cheese samples. It is worth noted that *Phoma spp.* were not detected in kareish cheese samples, while *Alternaria* and *Geotrichum spp.* were, also, not detected in processed cheese samples.

3. **Frequency of Aspergillus species in the examined samples**

Regarding with the identification of the isolated aspergillus species, as was illustrated in Table (2), *A. niger* revealed the highest prevalence in both kareish and processed cheese samples, 48 and 50%, respectively. On the other hand, *A. fumigatus* and *A. terreus* were not detected in processed cheese samples.

4. **Frequency of Penicillium species in the examined samples**

Regarding with the identification of the isolated Penicillium species, as was illustrated in Table (3), *P. aethiopicum* revealed the highest prevalence in both kareish and processed cheese samples, 33.3 and 25.0%, respectively.

5. **Frequency of other mould species in the examined samples**

Regarding with the identification of the mould other than Aspergillus and Penicillium spp., *Fusarium spp.* revealed higher prevalence than *Phoma spp., Alernaria spp*, and *Geotrichum spp.* processed cheese samples recorded higher *Fusarium spp.* contamination then kareish samples. On the other hand, *Phoma spp.* was not detected in kareish samples, as well as *Alternaria* and *Geotrichum spp.* were not detected in processed cheese samples.

6. **Incidence of aflatoxin M1 in the examined cheese samples**

Out of the examined *Aspergillus*-positive cheese samples, Table (4) recorded higher prevalence of aflatoxin in kareish cheese samples with incidence of 32.0%; whereas, 22.2% of the *Aspergillus*-positive processed samples had aflatoxin.

7. **Quantitative detection of aflatoxin M1 (AFM1) in the examined cheese samples**

Table (4) revealed that kareish samples had significant ($P \leq 0.05$) higher AFM1 concentration than processed cheese samples. Mean values of AFM1 in the examined kareish and processed cheese samples were 0.49 and 0.36 µg/kg, respectively.

8. **Antifungal effect of chitosan, marjoram oil and mustard oil against A. niger**

Estimation of the antifungal effect of the used additives against *A. niger* in cheese samples revealed that all of the used additives showed significant antifungal effect; which appeared to be dose-time dependent, where higher concentration with longer time of storage gave more reduction effect. In addition, chitosan had the superiority as an antifungal compound, where it revealed reduction in the *A. niger* count by 97.7% at the end of the experiment, followed by mustard EO and marjoram EO, respectively. Moreover, the combined groups revealed synergistic antifungal effect of the EOs by the powerful effect of chitosan, that was proven by higher reduction %; 97.0 and 98.0% for chitosan-marjoram and chitosan-mustard combination, respectively; revealing that the chitosan-mustard combination had some higher antifungal characteristics (Table 5).

Discussion

The detection of yeast and mold in any quantity within milk and dairy products is considered unacceptable due to the adverse alterations they cause, which significantly compromise product quality (Abd El-Hameed, 2011) [2]. It is estimated that fungal contamination is responsible for a loss of about 5 to 10% of global food production (Pitt and Hocking, 1999) [44]. Mainly, the hot climate and poor cooling processes are the main factors causing a heavy contamination of milk products by fungi that leads to undesirable quality attributes such as color, texture, and aroma (Pal, 2014) [41].

The present data illustrated the level of mould contamination in Kareish cheese was 40%, this was lower than that recorded by ELbagory *et al.* (2014) [20], El-Leboudy *et al.* (2015) [23], Elsharawy *et al.* (2019) [25], Mohammed (2020) [28] and Elbassiony *et al.* (2021) [21] who found the mould level in examined kareish cheese were 100%,76.6%,100%,70%, and 89.5%, respectively. While the recorded mould level for the examined processed cheese was 32%, and this is lower than that reported by ELbagory *et al.* (2014) [20], El-Kest *et al.* (2015) [22], El-Shafei *et al.* (2017) [24] and Abd El Tawab *et al.* (2020) [1] who revealed that moulds were isolated from 77.1%, 90%, 100%, and 90% of processed cheese.

The present analysis of 48 mould strains isolated from kareish cheese samples showed the occurrence rates as follows: *Aspergillus spp.* was found to be the most prevalent (52.1%), followed by *Penicillium spp.* (25%), *Fusarium spp.* (12.5%), *Alternaria spp.* (6.25%), and *Geotrichum spp.* (4.2%); which came nearly similar to the findings of El-Asuoty (2011) [18] and Elsharawy *et al.* (2019) [25], who found that *Aspergillus spp., Geotrichum* and *Penicillium spp.* were the most predominant mould species isolated from kariesh cheese samples. In contrast, processed cheese samples showed a different distribution of species, with *Aspergillus spp.* (43.9%), *Penicillium spp.* (29.3%), *Fusarium spp.* (19.5%), and *Phoma spp.* (7.3%). This came in accordance with El-Kest *et al.* (2015) [22] and Abd El Tawab *et al.* (2020) [1].

The current records indicated the prevalence of various aspergillus species in the two types of cheese samples. In kareish cheese, *A. niger* showed the highest frequency at 48%, followed by *A. flavus* (32%). The presence of *A. fumigatus, A. terreus*, and *A. versicolor* are significantly lower. This is nearly agreed to ELbagory *et al.* (2014) [20] who found that *A. fumigatus*, and *A. terreus*, were present in percentages of 28.5 and 2.8 % of examined kareish cheese, respectively; and Elsharawy *et al.* (2019) [25] who observed that *A. niger, A. flavus, A. fumigatus,* and *A. parasiticus* were found in kareish samples with 39.6%, 37%, 8.1% and 15.3%, respectively. In contrast, processed cheese has a higher percentage of *A. flavus* at 38.9% and *A. niger* at 50%, with no detection of *A. fumigatus* and *A. terreus*. The occurrence of *A. versicolor* is slightly higher in processed cheese compared to kareish cheese. This came in the same line of the results described by Abd El Tawab *et al.* (2020) [1] where the most isolated strains were *A. flavus* at 66.7% followed by *A. niger* 16.7% then *A. versicolor* 5.5% and unable to isolate *A. fumigatus* and *A. terreus.* The absence of *A. fumigatus* and *A. terreus* in processed cheese could be due to the different manufacturing processes or preservatives used, which may inhibit the growth of these species. Understanding the distribution of aspergillus species is crucial for food safety and quality control, as some of these species can produce mycotoxins that are harmful to consumers (Banjara *et al.*, 2015) [6].

Regarding our results that demonstrated the different species of *Penicillium* that was detected in the examined cheese samples, several species of *Penicillium* were isolated from kareish cheese and this nearly resembles the data mentioned by Elsharawy *et al.* (2019) [25]; whereas, the isolated strains from processed cheese also previously isolated by El-Shafei *et al.* (2017) [24] and Abd El Tawab *et al.* (2020) [1]. Several other mould species may isolated from the examined cheese samples like *Fusarium spp., Phoma spp., Alternaria spp.,* and *Geotrichum spp.* within different percentages; which came nearly similar to Elsharawy *et al.* (2019) [25].

Aflatoxins, a group of mycotoxin, are secondary metabolites synthesized by *Aspergillus species*, predominantly *A. flavus, A. parasiticus,* and *A. nomius*. These toxins are produced while the fungi grow on various food and feed products (Wochner *et al.*, 2018) [49]. Aflatoxins pose significant health risks and are a serious concern in food safety. It led to a range of harmful effects including liver toxicity, genetic mutations, birth defects, weakened immune system, and increased risk of cancer (Kumar *et al.*, 2017; Wochner *et al.*, 2018) [33, 49].

The most prominent and highest carcinogenic aflatoxin is AFB1, which mainly converted in the animal's liver into the hydroxylated aflatoxin M1 (AFM1), that excreted mainly within milk and biological fluid (Min *et al.*, 2021) [27]. The presence of AFM1 in dairy products poses a significant risk to public health, it is mainly classified as group 1 (human carcinogens) by the International Agency for Research on Cancer (IARC) (Marchese *et al.*, 2018; Schrenk *et al.*, 2020) [34, 45].

According to the results of prevalence of AFM1 in the examined cheese samples (Table, 6), it seems that it presents in 32% from examined kariesh cheese and 22.2% from processed cheese. This is lower than the level reported by Aiad and Abo El-makarem (2013) [3] and El-Shafei *et al.* (2017) [24].

Aflatoxin M1 in cheese occurs either due to the use of contaminated milk (Campagnollo *et al.*, 2016) [12], or due to the flourishing and growth of *Aspergillus spp.* in cheese sample. Aflatoxin M1 (AFM1) has a high affinity for casein present in milk, leading to its increased presence in protein-rich dairy items, notably cheeses. Additionally, conventional dairy processing methods like pasteurization and sterilization are ineffective in eliminating AFM1 (Peña-Rodas *et al.*, 2020) [42].

The mean values of AFM1 in the examined kareish and processed cheese samples were 0.49 and 0.36 µg/kg, respectively. This is lower than that detected by Aiad and Abo El-makarem (2013) [3] and El-Kest *et al.* (2015) [22], while exceeding the Egyptian regulations which stated that milk and dairy products should be free from AFM1, but all of them were within the US regulations (0.50 µg/kg) (Aiad and Abo El-makarem, 2013) [3].

Enhancing food quality and safety remains a priority, with a focus on managing the proliferation of pathogens and spoilage organisms by minimizing the ordinary usage of chemical preservatives that may associated with microbial resistance and potential health hazards (Pisoschi *et al.*, 2018) [43]. So, a notable shift towards using essential oils as a natural substitute for these traditional preservatives increased recently (Burt, 2004) [11].

Natural and biodegradable substances such as chitosan are considered top-tier antimicrobial and fungicidal agents suitable for food preservation (Benelli *et al.*, 2010; Ke *et al.*, 2021a) [10, 30]. Its antifungal properties are mainly related to the interaction of chitosan with the cell wall or cell membrane, leading to the inhibition of DNA/RNA and protein synthesis (Kravanja *et al.*, 2019; Shih *et al.*, 2019) [47] [32], or even affects in mitochondrial activity (Ke *et al.*, 2021b) [31].

Several previous authors also demonstrated the inhibitory effect of mustard on moulds (Dimic *et al.*, 2015; Horvath *et al.*, 2016) [17, 26]; and marjoram essential oils against numerous fungal and bacterial disease agents (Šernaitė *et al.*, 2020; Bellumori *et al.*, 2021) [9, 46]. In the present study, the assessment of the antifungal efficacy of various additives against *A. niger* in cheese samples indicated that all tested additives exhibited a significant antifungal effect. This effect was found to be dependent on both dosage and duration, with higher concentrations and longer storage times resulting in greater reductions in fungal counts.

Notably, chitosan emerged as the most effective antifungal agent, demonstrating a remarkable (97.7%) reduction in *A. niger* counts by the end of the study. This is similar to the findings of Meng *et al.* (2020) [36] who observed that treating *A. ochraceus* with chitosan led to the development of atypical hyphal branching and had a significant impact on the structure of the cell walls and membranes leading to damaging the integrity of the cell wall and loss of membrane fluidity. While, using marjoram EO lead to 93.3% reduction in *A. niger* count in the examined samples. This agreed with the result of Kara (2024) [29] who recorded potent ability of marjoram EO in inhibiting mycelial growth of mould completely. In addition, using mustard oil ordered as the most effective oil used as antifungal agent, in the present study, with 96.7% reduction at the end day of experiment, this mainly came in the same line with the findings of Clemente *et al.* (2019) [14] who determined antifungal effect of mustard oil against different mould strains, Mejia-Garibay *et al.* (2015) [35] against *A. ochraceus*, and Nielsen and Rios (2000) [40] against *P. roqueforti* and *A. flavus*.

Furthermore, combinations of these additives showed a synergistic antifungal effect, particularly with chitosan. The chitosan-marjoram and chitosan-mustard combinations achieved reductions of 97.0% and 98.0%, respectively, indicating that the chitosan-mustard combination exhibited slightly superior antifungal properties. This approves the records of Clemente *et al.* (2016) [15] who possess that the mixing of essential oils with chitosan, as complementary properties, can be a potent method to achieve an antimicrobial impact while minimizing the amount of EOs required. This approach influences the enhanced effectiveness that results from the combined action of the oils, thereby allowing for a reduction in their overall usage without compromising the desired effect.

Table 1: Prevalence of different mold genera strains isolated from the examined cheese samples

Mold genera	Kareish cheese (n = 48)		Processed cheese (n = 41)	
	No.	%	No.	%
Aspergillus spp.	25	52.1*	18	43.9*
Penicillium spp.	12	25.0*	12	29.3*
Fusarium spp.	6	12.5*	8	19.5*
Phoma spp.	ND	0	3	7.3*
Alternaria spp.	3	6.25*	ND	0
Geotrichum spp.	2	4.2*	ND	0
Total No. of isolates	48	100	41	100

* Prevalence in relation to the total number of the mold isolates

Table 2: Frequency of Aspergillus species in the examined samples

Aspergillus spp.	Kareish cheese (n=25)		Processed cheese (n=18)	
	No.	%*	No.	%*
A. flavus	8	32.0	7	38.9
A. fumigatus	3	12.0	ND	0
A. niger	12	48.0	9	50.0
A. terreus	1	4.0	ND	0
A. versicolor	1	4.0	2	11.1

* Prevalence in relation to the total number of the Aspergillus spp. isolates

Table 3: Frequency of Penicillium species in the examined samples

Penicillium spp.	Kareish cheese (n=12)		Processed cheese (n=12)	
	No.	%*	No.	%*
P. aethiopicum	4	33.3	3	25.0
P. cammberti	2	16.7	3	25.0
P. citreonigrum	2	16.7	2	16.7
P. implicatium	3	25.0	1	8.3
P. simplicissimum	1	8.3	1	8.3

* Prevalence in relation to the total number of the Penicillium spp. isolates

Table 4: Incidence and quantification of aflatoxin M1 in the examined cheese samples

Samples	No. of Positive samples	%*	Min. conc. (µg/kg)	Max. conc. (µg/kg)	Mean conc. ± SE (µg/kg)
Kareish cheese (n = 25)	8	32.0	0.44	0.69	0.49 ± 0.06*
Processed cheese (n = 18)	4	22.2	0.30	0.50	0.36 ± 0.04*

* Prevalence in relation to the number of Aspergillus-positive cheese samples

Table 5: Antifungal effect of different natural products on the growth reduction of *A. niger*

	Chitosan 1.0%	Marjoram 1.0%	Mustard 1.0%	chitosan-marjoram (1:1)	chitosan-mustard (1:1)
Start	0.0	0.0	0.0	0.0	0.0
3rd day	80.0	66.7	76.7	76.7	83.3
6th day	93.3	80.0	90.0	86.7	96.7
9th day	97.7	93.3	96.7	97.0	98.0

Conclusion

The findings of this study reveal a concerning prevalence of fungal contamination in Kareish and processed cheese, particularly from Aspergillus and Penicillium species, with the detection of Aflatoxin M1 (AFM1) posing significant public health risks due to its carcinogenic nature. Although AFM1 levels were lower than in some previous studies, they still exceeded Egyptian regulatory limits, highlighting the urgent need for improved monitoring and control measures. Additionally, the investigation into antifungal agents demonstrated that natural additives such as chitosan, marjoram essential oil, and mustard oil exhibit considerable antifungal properties, with chitosan proving to be the most effective. The synergistic effects observed in combinations of these additives suggest a promising strategy for enhancing food safety and quality in dairy products. Overall, these results underscore the necessity for ongoing research into natural antifungal agents and the implementation of rigorous quality control practices in dairy production to mitigate risks associated with fungal contamination and mycotoxin presence.

Conflict of interset

There is no conflict of interest.

Authors' contributions

This work was carried out in collaboration among all authors. Authors ASE, AHE and RHE designed the research plan and wrote the manuscript. Author AHE performed the experimental work. All authors read and approved the final manuscript.

Disclaimer (Artificial Intelligence)

Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during writing or editing of manuscripts**.**

Consent
It is not applicable.

Ethical approval
No need to use experimental animals, no need for ethical approval for the present work.

Competing Interests
Authors have declared that no competing interests exist.

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