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Mitigation of microbiological hazards associated with Kareish cheese using selenium nanoparticles

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ABSTRACT:

Some traditional fermented dairy products like Egyptian Kareish cheese are manufactured in farmhouses following traditional techniques using raw milk without heat treatment or the addition of selected starter cultures, Kareish samples were examined for total aerobic plate count, Enterobacteriaceae, yeast, molds, and staphylococci. The results of the microbiological examination revealed the presence of high numbers of investigated microbes, showing the poor sanitary practice of Kareish cheese production, beginning with the use of poor raw milk quality and processing in unregulated situations. Antimicrobial nanoparticles (NPs) are an innovative method of ensuring the safety of milk and milk products. The present study investigated the effect of selenium NPs (Se-NPs) on the count of undesirable microorganisms. The concentrations of used nanoparticles were 0.5%, 1%, and 1.5% for Se-NPs, they were used to improve the microbial properties of Kareish cheese samples during storage at the refrigerated temperature of 4°C. The study found that Se-NPs had strong antibacterial efficacy against the pathogens tested at all doses. Conclusion: These NPs have the potential to be used as preservatives in milk and milk-based products like Kareish cheese. Additionally, raising the concentration of these NPs by 1.5% Se-NPS increased their effects.

INTRODUCTION

Milk and milk products constitute crucial constituents of the human diet. Several dairy products are manufactured primarily from cow's milk. In some regions of the world, milk from other animal species is also used to prepare dairy products. A diversity of dairy products such as butter, cheese, dried milk powder, ice cream, and yoghurt are available worldwide (**Pal and Jadhav, 2013).**

Cheese is a popular dairy product, a rich source of protein, vitamins, calcium, and phosphorus. Microbial contamination of cheese can occur from various sources including handler, packaging material, and environment (**Pal et al. 2014).** Cheese as ready-to-eat food should be

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considered as a potential source of food-borne pathogens. The post-processing phase is the most important food-borne pathogen in cheese. (**Vrdoljak et al. 2016).**

Kareish cheese is usually made from raw milk without heat treatment under artisanal conditions. Numerous microorganisms, including bacteria, yeasts, and molds, construct traditional Kareish cheese's complex ecosystem and were analyzed using classical methods. **(Irlinger and Mounier 2009).**

Nanotechnology has found ways to improve food quality, unique dietary supplements, additives, and nutrients **(Huang et al. 2017)**. The aim is to improve the taste, texture, and bioavailability of minerals and supplements and to extend the shelf life of the products (**Chaudhry and Castle 2011).**

Selenium nanoparticles (Se-NPs) can replace antibiotics such as ampicillin for the prevention and treatment of various bacterial diseases and infections in humans. Nano-selenium is 60 times more effective than conventional therapy against *Staphylococcus aureus*, *E. coli*, and *Pseudomonas aeruginosa* infections. **(Srivastava, 2019).**

It improves the absorption of plants, animals, humans, and microorganisms and acts as an antioxidant, with less risk of selenium toxicity. One of the most important applications of selenium nanoparticles is chemoprevention through immune activation (**Majeed et al. 2018).**

Therefore, this work aimed to identify undesirable pathogenic microbial groups (total colony count, Enterobacteriaceae*, Staphylococcus aureus* coagulase-positive, mold, and yeast,) on milk and kareish cheese, and to determine the antimicrobial effect of selenium nanoparticle on kareish cheese.

2. MATERIALS AND METHODS:

2.1. Collection of Samples:

A total of 100 random samples of marketed kareish cheese samples were collected from local markets and different shops in El Menufia Governate, cheese samples were transferred into an ice box to the laboratory and stored at refrigerator temperature (4 ± 1°C). For microbiological examination

2.2. Microbiological assay

2.2.1. Preparation of serial dilutions (APHA, 2004):

11 grams of soft cheese sample were put in a sterile plastic bag and 99 ml of 2% sterile solution of sodium citrate was added at 45±1˚C. The mixture was homogenized for 1 min. Thus, cheese samples and sodium citrate are equal to 10^{-1} dilution. Then, a tenth-fold serial dilution was prepared.

2.2.2. Determination of aerobic plate count: (Petran et al. 2015).

One ml of each dilution was transferred into duplicated labeled Petri dishes. 12 to 15 ml of liquefied sterile plate count agar (PCA) at 44°C- 46°C were poured into each plate, then incubated at 35°C for 48 hours.

2.2.3 Determination of staphylococcal count (FDA, 2001).

Baird barker is used for the isolation and differentiation of coagulase-positive staphylococci in food. Typical colonies on Baird Parker medium are black to gray, brilliant and convex and surrounded by a clear zone, which can be partially opaque and confirmed by coagulase test

2.2.4 Determination of Enterobacteriaceae count (Violet Red Bile Glucose Agar (VRBG) (Ph. Eur.) (ISO21528: 2017)

Enterobacteriaceae such as *Escherichia coli* and salmonella species are able to ferment glucose with production of acid which resulted in a pH drop indicated by neutral red resulting in pink colonies.

2.2.5 Determination of molds according to ICMSF (1996)

One ml from each of the prepared dilutions was mixed by pouring technique in duplicate plates with 10-15 ml of Sabouraud Dextrose agar containing 50 mg chloramphenicol per

liter tempered at 45 ºC. After solidification, the inoculated plates were incubated at 28-30 ºC for 5-7 days and at 37 ºC for 24 hours. molds will grow as filamentous colonies of various colors.

2.3 Preparation of Nano-materials:

The Se-NPs were prepared at the Naqaa Foundation for Scientific Research, Technology, and Development in Giza, Egypt. The Se-NPs were prepared according to the modified method of **Qian et al. (2010).**

2.4 Kareish cheese manufacturing

Low salt kareish cheese was manufactured as described by **Abou-Donia (2008)** Skimmed milk was used for the manufacture of cheese and NaCl (3%) was added to milk at 37°C. In Kareish cheese, the fat level in dry matter and the moisture content should not exceed 10% and 75%, respectively (Egyptian Standard 2005/4-1008). The cheese was divided into 4 groups followed by the addition of Se-NPs (0.5%, 1%, and 1.5%)
Tests were performed in triplicate.

2.5 Sensory evaluation:

Samples were examined for changes in color, odor, and consistency by the analyst panel (members of Animal Health Research Institute, Shibeen El- koom) according to **Gaza et al. (2015)**.

2.6 Microbiological assay:

2.6.1 Preparation of serial dilutions (APHA, 2004).

2.6.2 Determination of aerobic plate count: (Petran et al. 2015).

2.6.3 Determination of staphylococcal count (FDA 2001).

2.6.4 Determination of Enterobacteriaceae count (Violet Red Bile Glucose Agar (VRBG) (Ph. Eur.) (ISO21528: 2017).

2.6.5 Determination of molds according to ICMSF (1996)

2.7. Statistical Analysis

Microbiological data were converted into logarithms of the colony number of forming units (CFU/gm). The analysis of variance (ANOVA) was performed in SPSS software (Version 22, SPSS Inc. Chicago, IL, and USA). Means and standard deviations were calculated. Multiple mean comparisons were done by applying Duncan's Multiple Range test for measuring the specific differences between pairs of means. Values were statistically significant at the $p \leq 0.05$ level (**Feldman et al. 2003).**

3. RESULTS

Table 1. Microbial analysis (CFU/ /g) in the examined samples of kareish cheese. (n=100).

Samples	NO	%	Min	Max	Mean± SE
Total aerobic plate count	100	100	2.25	4.79	$4.1^{a} \pm 0.10$
Total staphylococcal count	80	80	2.19	3.04	$2.99^{b} \pm 0.10$
Enterobacteriaceae count	88	88	3.91	5.64	$4.87^{c} \pm 0.06$
Molds and Yeast count	92	92	4.36	5.66	$5.12^{ac} \pm 0.08$

SE = Standard error
NO =number of positive samples
% =percent of positive samples
The values are expressed as Mean ± standard error. Means within a column followed by different letters (a,b) are significantly different ($P \leq 0.05$).

Table 2. Effect of different concentrations of selenium nanoparticles on the Sensory evaluations of examined cheese samples

Parameter	Normal %	Abnormal %
Color	100	100
Odor	70	30
Consistency	100	100

Table 3. Effect of different concentrations of selenium nanoparticles on the aerobic plate count of the examined cheese samples during storage at 4ºC

Groups	First day	Third day	Sixth day	Ninth day	Twelfth day	Fifteenth day
Control	4.52 ± 0.3^{a}	4.73 ± 0.3^{b}	4.95 ± 0.3^{e}	5.36 ± 0.13^{e}	5.53 ± 0.13^{g}	5.9 ± 0.4^{g}
0.5%selenium	4.52 ± 0.3^{a}	4.41 ± 0.15^{ab}	4.29 ± 0.2^{c}	4.15 ± 0.2^{c}	4.78 ± 0.1^{c}	3.6 ± 0.13^{d}
1% selenium	4.52 ± 0.3^{a}	4.35 ± 0.1^{ab}	4.23 ± 0.1^{cd}	4.01 ± 0.3^{c}	3.66 ± 0.3^{d}	3.46 ± 0.11^{d}
1.5%selenium	4.52 ± 0.3^{a}	4.28 ± 0.2^{ab}	4.18 ± 0.1^{cd}	3.89 ± 0.2^{cd}	3.53 ± 0.1^{d}	3.25 ± 0.20^{d}

The values represent mean ±standard deviation of three experiments. $^{a, b,c, d,e, f, g}$ means superscript letters within a column are significantly different ($p \leq 0.05$)

Table 4. Effect of different concentrations of selenium nanoparticles on staphylococcal count of the examined cheese samples during storage at 4ºC

Groups	First day	Third day	Sixth day	Ninth day	Twelfth day	Fifteenth day
Control	4.42 ± 0.1^{a}	4.69 ± 0.3^{a}	4.98 ± 0.1^{e}	5.12 ± 0.13^{e}	5.34 ± 0.13^{f}	5.6 ± 0.2^{f}
0.5% selenium	4.42 ± 0.1^{a}	4.33 ± 0.2^{ab}	4.17 ± 0.2^{b}	3.99 ± 0.2^{c}	3.72 ± 0.1^{c}	3.6 ± 0.15^{cd}
1% selenium	4.42 ± 0.1^{a}	4.24 ± 0.1^{ab}	4.09 ± 0.1^{b}	3.86 ± 0.3^{c}	2.63 ± 0.1^{cd}	3.45 ± 0.21^{cd}
1.5% selenium	4.42 ± 0.1^{a}	4.12 ± 0.2^{ab}	3.99 ± 0.1^{b}	$3,68.\pm 0.2^{cd}$	3.46 ± 0.1^{cd}	3.15 ± 0.23^{d}

The values represent Mean ± Standard deviation of three experiments. $^{a, b, c, d, e, f, g}$ means superscript letters within a column are significantly different ($p \leq 0.05$).

Table 5. Effect of different concentrations of selenium nanoparticles on Enterobacteriaceae count of the examined cheese samples during storage at 4ºC

Groups	First day	Third day	Sixth day	Ninth day	Twelfth day	Fifteenth day
Control	3.38 ± 0.1^{a}	3.5 ± 0.3^{a}	3.88 ± 0.1^{e}	4.14 ± 0.13^{e}	4.12 ± 0.13^{f}	4.58 ± 0.2^{f}
0.5% selenium	3.38 ± 0.1^{a}	3.3 ± 0.2^{ab}	3.28 ± 0.2^{b}	3.15 ± 0.2^{c}	3.02 ± 0.1^{c}	2.73 ± 0.15^{cd}
1% selenium	3.38 ± 0.1^{a}	3.28 ± 0.1^{ab}	3.25 ± 0.1^{b}	2.99 ± 0.3^{c}	2.75 ± 0.1^{cd}	2.54 ± 0.21^{cd}
1.5% selenium	3.38 ± 0.1^{a}	3.25 ± 0.2^{ab}	3.2 ± 0.1^{b}	2.84 ± 0.2^{cd}	2.62 ± 0.1^{cd}	2.43 ± 0.23^{d}

The values represent Mean ± Standard deviation of three experiments. $^{a, b, c, d, e, f, g}$ means superscript letters within a column are significantly different ($p \leq 0.05$).

Table 6. Effect of different concentrations of selenium nanoparticles on mold and yeast count of the examined cheese samples during storage at 4ºC.

Groups	First day	Third day	Sixth day	Ninth day	Twelfth day	Fifteenth day
Control	3.44 ± 0.3^{a}	3.64 ± 0.2^{a}	3.95 ± 0.21^{e}	4.26 ± 0.13^{e}	4.56 ± 0.2^{f}	5.25 ± 0.3^{f}
0.5% selenium	3.44 ± 0.3^{a}	3.4 ± 0.13^{ab}	3.36 ± 0.21^{b}	3.27 ± 0.14c	2.89 ± 0.1^{c}	2.65 ± 0.11^{cd}
1% selenium	3.44 ± 0.3^{a}	3.36 ± 0.11^{ab}	3.28 ± 0.11^{b}	3.12 ± 0.20^{c}	2.82 ± 0.14^{cd}	2.58 ± 0.2^{cd}
1.5% selenium	3.44 ± 0.3^{a}	3.27 ± 0.21^{ab}	3.21 ± 0.2^{b}	2.81 ± 0.21^{d}	2.55 ± 0.1^{cd}	2.47 ± 0.13^{d}

The values represent Mean ± Standard deviation of three experiments. $^{a, b, c, d, e, f, g}$ means superscript letters within a column are significantly different ($p \leq 0.05$).

DISCUSSION

4.1. Total bacterial count:

Total bacterial count is an indicator for the microbiological status of cheese. A high count often indicates contamination of raw materials, improper sanitation, storage, and/or production. Under unsatisfactory conditions (**Kaldes 1997)**

Total colony counts of the examined Kareish recorded the highest contaminated samples with an incidence of 100%, ranging from 2.25 $\log_{10}$ to 4.79 $\log_{10}$ CFU/g with mean values $4.1^{b} \pm 0.10$ $\log_{10}$ CFU/g.

The results of kareish cheese agreed with **Hawazen et al. (2016)** who reported a total plate count of 2.28×10^{9} CFU/g in all examined Kariesh cheese samples. Results also agreed with **Mohamed et al. (2020)** who reported a total plate count of 5.9×10^{9} CFU/g with prevalence of 80% of examined samples. On the other hand, a higher value of total bacterial count in Kareish cheese $7.2 \pm 0.1 \times 10^{10}$ CFU/g detected by **Ibrahim et al. *(*2015)** and also **Marwa et al. (2017)** . **Seifu (2013)** also discovered that traditional Ethiopian soft cheese samples contained a total viable bacterial count of up to 6.9 × 107 CFU/g.

4.2 Sensory evaluations:

The results showed that the examined samples had 100% good color and texture with while 70% of the examined samples had a normal odor. That is agreed with **Mohamed et al. (2020)** who mentioned that 90% of the examined fresh kareish cheese had good flavor, 84% of the examined samples had good body and texture and 88% of the samples had good appearance and color.

On the other hand, **Tawfik et al. (2021)** found that all the samples were 100% normal in color, odor, and texture.

4.2 Staphylococcal count:

Staphylococci food poisoning resulting from contaminated milk and dairy products, especially cheese produced from raw milk in unclean conditions, causes staphylococcal intoxication **(Can and Celik, 2012)**.

Results of Staphylococcal count for kareish samples showed that the Staphylococci counts on Baird Parker agar ranged from 2.19 $\log_{10}$ to 3.04 $\log_{10}$ CFU/g with mean values $2.99^{b} \pm 0.10$ $\log_{10}$ CFU/. This is also related to inadequate hygiene practices in the cheese industry. these samples are not within **Egyptian Standards for Karish cheese (2005)** as it is recommended that the cheese should be free from coagulase-positive staphylococci, in addition, *staph aureus* count not exceed 100 cells/ml in raw milk. according to **(EOS, 2005)**

Examined Karish cheese samples results agreed with that detected by **El-Leboudy et al. (2015**) as positive samples were (80%) of the examined samples and higher than that reported by **Nazem et al. (2011)** (45%), and **Eid and El telawy (2014)** (26.6%).

Staphylococcus aureus is responsible for food poisoning as well as potentially fatal infections such as bacteremia, necrotic pneumonia in infants, and endocarditis (**Mahendra et al. 2022).** In animals, it causes mastitis in cow, botryomycosis in horses, dermatitis in dogs, septicemia and arthritis in poultry (**Zunita et al. 2008; Luzzago et al. 2014).**

4.3 Enterobacteriaceae count

Enterobacteriaceae on VRBG agar showed that Enterobacteriaceae ranged from $\log_{10}$ 3.91 CFU/g to $\log_{10}$ 5.64 CFU/g with mean values $4.87^{b}\pm0.06$ $\log_{10}$ CFU/g in kareish cheese. **The Egyptian Standard for Karish cheese (2005)** recommended that the coliform bacteria should be not more than 10 cfu/g. and free from *E. coli* and salmonella. Also, raw milk should be free from salmonella according to **The Egyptian Standard for raw milk (EOS, 2005)**

The presence of high level of Enterobacteriaceae and coliform bacteria in Karish cheese showed poor hygienic practises throughout the cheese's preparation. These results were higher than results reported by I**brahim et al. (2015), Nosir (2014)** who detected *E coli* in an incidence of (33%)**,** (31.67%) respectively. However, **Esho et al. (2013**) found that 27.3% of the analyzed soft cheese samples were positive for coliforms. On the other hand, results agreed with **Mohamed et al. (2020)** as they detected coliform bacteria in 76.7% of the total examined soft cheese**. Brien et al. *(*2009)** concluded that *E. coli* was absent in examined kareish cheese.

It is advised to follow proper manufacturing practices, as well as distribution and retail storage practices, be used to ensure cheese's microbiological safety **(Osama et al. 2014).**

The Enterobacteriaceae are among the most important causes of serious hospital-acquired and community-onset bacterial infections in humans (**Paterson 2006)**

4.4 Mold and yeast count:

It is widely recognized that fungi are an important component of the microflora of many cheese varieties. The high prevalence of fungi in cheese is due to several factors which are: the ability to ferment/assimilate lactose, produce extracellular lipolytic and proteolytic enzymes, utilize lactic and citric acid, grow at 10 ^{o}c and their relative resistance to cleaning compounds and sanitizers. (**Soliman and Aly 2011)**

The results of molds and yeast count of fresh Karish samples ranged from 3.36 to 5.66 $\log_{10}$ CFU /g with an average of $5.12^{ab}\pm0.08$ $\log_{10}$ CFU/g. These results are similar to those of **Rasha et al. (2019)** and also **Tawfik et al. (2021).**

According to **The Egyptian Standard for Karish cheese (1008/4/2005)** the yeasts and the molds count should not exceed 400 CFU/g and 10 CFU/g respectively. The high concentration of yeasts and molds was a sign of low hygiene standards used throughout the cheese-making process.

Soliman and Aly (2011) found lower total yeasts and molds count in Kareish cheese samples with a mean of 7.89 x 10 CFU/g.

The obtained results were lower than that recorded by **Gamal et al. (2019)** who reported that mean values of mold are1.83×10^{5} ± 9.25×10^{4} CFU/g. According to **EOS (2005**), kareish cheese should not contain mold counts more than 10 CFU/g.

Aflatoxin M1 is an important microbial toxin that may occur in human and animal milk and dairy products. It is a carcinogenic element for humans. It is mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus* **(IARC, 2002).**

4.5 Antimicrobial effect of selenium nanoparticles on Kareish cheese samples.

4.5.1 Aerobic bacterial count:

In Kareish cheese samples APC count significantly decreased from 4.52 to 3.25 ~1 CFU/ml in cheese samples treated with 1.5% Se-NPS (Table 2, $p \leq 0.05$). As **Hariharan et al. (2016**) stated, the antibacterial activity was related to the concentration of nanoparticles.

Se-NPs' antibacterial activity inhibits both gram-positive and gram-negative bacteria, albeit their exact mechanism is still unknown. Currently, it is believed that Se-NPs interact with the peptidoglycan layer and harm the double-stranded DNA structure in order to breach the bacterial cell wall (**Sonkusre et al. 2014).**

Table 3, on the day 6th of storage, there was a significant difference between the control group and the other treated groups ($p \leq 0.05$).

On the day 12th of storage, there were significant differences between the group treated with 0.5% selenium and the groups treated with 1%, 1.5% selenium ($p \leq 0.05$).

4.5.2. Staphylococci count:

Staphylococcal count in cheese samples treated with Se-Nps at the concentration of 0.5%, staphylococci decreased from 4.42 to 3.6~1 CFU/ml, and when the concentration reached 1.5%, staphylococci decreased from 4.42 to 3.15 ~1 CFU/ml as seen in Table 4. This is similar to the findings of **Qi et al. (2004), Ro-drigus-Nunez et al. (2012), Salmabi and Seema (2013), Van Toan et al. (2013), Younes et al. (2014), and Widnyana et al. (2021)**. Moreover, the antimicrobial effect of Se-NPs recorded by **Khiralla *&El Deep* (2015**) indicated that the inhibition zone increased with an increase in the concentration of Se-NPs. According to reports, Se-NPs are an effective antibacterial agent against *S. aureus* (**Chudobova et al., 2014).** According to **Phong *&* Thoman (2011)**, the proportion of live *S. aureus* decreased in the presence of Se-NPs at 7.8, 15.5, and 31 g/mL after 3, 4, and 5 hours. The Egyptian standards for Kareish cheese mentioned that *S. aureus* (coagulate-positive) should be absent in 1 g (**EOS, 2005).** On the day 6th of storage, there was a significant difference between the control group and the other treated groups ($p \leq 0.05$).

4.5.3 Enterobacteriaceae count:

Khiralla & El Deep (2015) found that the inhibition zone for *E. coli* and *S. aureus* was increased with increasing Se-Nps concentration. According to **Shrestha et al. (2010) and khurana et al. (2019).** Selenium NPs were highly effective against *E. faecalis* biofilm at the concentration of 1mg/ml (**Sanjay &Nobuyuki, 2021).** According to Egyptian Kareish cheese standards (No. 1008/2005), the total coliform count should be less than 10 CFU/g, E. coli should not be present in 1 g of the cheese, and Salmonella and other pathogens should not be present in 25 g. (**EOS, 2005).**

Table 5, on the day 6th of storage, there was a significant difference between the control group and the other treated groups ($p \leq 0.05$).

On the day 15th of storage, there were no significant differences between all treated groups ($p \geq 0.05$).

4.5.4 Mold and yeast count:

The antibacterial action of nanoparticles against molds was demonstrated by the fact that the counts of treated and control samples were significantly different ($p \leq 0.05$)**.** The mold count significantly decreased by ~1 CFU/ml in cheese samples at high concentrations of Se-NPs (1.5%)**.** Antifungal activity in the current study agreed with that of **Yien et al. (2012)**. **Shakibaie et al. (2015)** indicated the anti-biofilm activity of biologically generated (Se-NPs) in concentrations ranging from 10 to 200 mg/mL against the biofilm.

CONCLUSION

According to the **Egyptian standards (2005)**, the results of the present study reported that there is insufficient sanitation during the manufacture and handling of this type of cheese. Kariesh cheese is sold uncovered and without containers where the risk of contamination is high, so it is considered a good medium for the growth of different types of spoilage and pathogenic microorganisms. The implementation of ‘‘Good Manufacturing Practices’’ in the production of traditional cheese is necessary for limiting contamination. Biocompatible Se-NPs had high antimicrobial activity against pathogenic and spoilage Gram -positive and Gram-negative bacteria, as well as molds that affect Kareish cheese. According to this study, nanoparticles can be employed as a preservative in Kareish cheese to extend its shelf life. Further studies should be con-

ducted on the effectiveness of nanotechnology and nanoparticles on dairy products, their prevention of microbial contamination, and the limitation of mold excretions like aflatoxins.

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