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Evaluating the antimicrobial effectiveness of propolis combined with zinc oxide nanoparticles in local artisanal Iraqi cheeses

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Abstract
Microbial spoilage in dairy products, particularly cheese, poses significant quality and safety risks. This study evaluates the antimicrobial efficacy of honey bee propolis and zinc oxide nanoparticles (ZnONPs) in inhibiting microbial growth in locally produced artisanal Iraqi cheese during refrigerated storage (4±2°C). Cheese samples were coated with propolis extract (T2), ZnONPs (T3), or a combination of both (T4), with untreated samples (T1) serving as a control. Microbial analysis over 28 days revealed significant reductions ($p<0.05$) in total bacterial, coliform, presumptive *Staphylococcus*, and fungal counts in treated samples compared to the control. The T4 treatment demonstrated the most potent antimicrobial activity, significantly reducing microbial counts to below detectable limits for certain groups by the end of the storage period. ZnONPs alone (T3) significantly inhibited microbial growth but were slightly less effective than T4, while propolis alone (T2) showed moderate antimicrobial activity. Antimicrobial susceptibility testing of 14 confirmed bacterial isolates indicated resistance to common antibiotics, highlighting the need for alternative preservation strategies. The combination of propolis and ZnONPs exhibited a synergistic effect, effectively suppressing bacterial and fungal growth while extending cheese shelf life. These findings suggest that integrating natural antimicrobial agents with nanotechnology presents a promising alternative to synthetic preservatives in dairy preservation. Further research should focus on sensory evaluation, toxicological assessment, and optimization of agent concentrations to ensure commercial viability.

Introduction
Microbial contamination and spoilage of dairy products, particularly cheese, pose significant risks to human health. Pathogens such as *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Salmonella sp*., and *Staphylococcus aureus* have been found as threats in raw milk cheeses, particularly the exceptionally soft and fresh ones (Das *et al.*, 2015). These bacteria can lead to severe illnesses, including listeriosis, hemorrhagic colitis, and salmonellosis. Factors influencing contamination include the type of cheese, manufacturing processes, and storage conditions. Cheeses with high moisture content and those from raw milk are particularly susceptible. Implementing stringent pre-harvest and post-harvest preventive measures, such as pasteurization and proper sanitation, can significantly reduce these risks. Despite these measures, outbreaks linked to contaminated dairy products continue, underscoring the need for ongoing vigilance in production and handling practices (Zekarias, 2020).
The rising incidence of foodborne illnesses attributed to microbial contamination in dairy products highlights the urgent need for effective preservation methods in the food industry. Among dairy products, locally produced cheeses are particularly susceptible to contamination due to their artisanal nature and often limited processing techniques. The challenge of ensuring food safety while maintaining the unique qualities of these cheeses has prompted researchers to explore innovative natural preservatives, which offer a reassuring potential to enhance food safety (Pretorius and Buys, 2021).
Propolis, a resinous substance collected by bees from various botanical sources, has garnered attention due to its broad-spectrum antimicrobial properties (Surek *et al*., 2021). Its complex chemical composition, rich in flavonoids, phenolic acids, and other bioactive compounds, contributes to its efficacy in inhibiting various spoilage microorganisms. Concurrently, the emergence of nanotechnology in food preservation has introduced zinc oxide nanoparticles (ZnONPs) as promising agents due to their enhanced antimicrobial activity, stability, and safety profile (Dwi *et al*., 2023).
Similarly, ZnONPs' enhanced surface area and reactivity enhance antibacterial efficacy when incorporated into a nanocomposite structure. Additionally, because of its complex integration of bioactive elements (such as flavonoids, phenolic acids, and terpenes), propolis, a naturally occurring resinous compound that honeybees gather from plant sources, has antibacterial, antioxidant, and anti-inflammatory characteristics (Kevenk and Aras, 2022). Propolis's conversion to nanoparticles and subsequent inclusion into a nano-composite substrate allows for controlled release of its therapeutic ingredients and enhanced bioavailability (Salama *et al*., 2024).
While previous studies have explored these agents individually or within synthetic packaging, this study introduces a novel approach by evaluating the direct synergistic application of ZnONPs and propolis extract as a coating on local artisanal Iraqi cheese. This specific combination targets the unique microbial

challenges of regional dairy products, offering a dual mechanism of action that enhances bioavailability and controlled release of therapeutic ingredients. By harnessing this synergistic potential, we aim to demonstrate an effective, sustainable strategy for bolstering the microbial quality and shelf life of artisanal cheese products, contributing to the expanding knowledge of natural antimicrobial applications in the dairy industry.

Materials and Methods

Sample collection and preparation

Locally produced fresh cheese samples were obtained from artisanal dairy producers in Iraq. The cheese was manufactured from pasteurized cow milk using traditional local methods, including coagulation, whey drainage, molding, and refrigerated storage. Samples were stored at 4±2°C until processing. The cheese was cut into uniform pieces (approximately 10 g) to ensure treatment consistency (Lima *et al*., 2021).

Preparation of propolis extract

Raw propolis was collected from local beehives in Iraq. Due to the absence of melissopalynological analysis, the specific botanical origin was not determined, and total phenolic and flavonoid contents were not quantified. This represents a geographically defined local sample. The propolis was purified following standard ethanol extraction procedures. Briefly, 50 g of raw propolis was ground and dissolved in 500 mL of 70 percent ethanol, then stirred for 48 hours at 20 to 30°C. The mixture was filtered through Whatman No. 1 filter paper, and the solvent was evaporated under reduced pressure to produce a concentrated extract (Trusheva *et al*., 2007).

Source and characteristics of ZnONPs

The ZnONPs used in this study were provided by the Department of Food Science, College of Agriculture, Tikrit University, Iraq. These nanoparticles were previously biosynthesized and characterized by Ibrahem *et al.* (2017), confirming their size, morphology, and stability. Therefore, additional SEM, XRD, or UV-Vis characterization was not performed in the present study.

Cheese sample treatment and coating application

Cheese samples were randomly assigned to four treatment groups: T1 (untreated control), T2 (coated with 25 mg/mL propolis extract), T3 (coated with 20 mg/mL ZnONPs), and T4 (coated with a combined solution of propolis extract and ZnONPs). For T4, the two solutions were mixed at a 1:1 ratio, yielding final concentrations of 25 mg/mL propolis extract and 20 mg/mL ZnONPs. A treatment volume of 2 mL was applied per 1 g of cheese. Each sample was immersed in the assigned solution for 1 minute, air-dried for 30 minutes at 25°C, and stored at 4±2°C for 28 days.

Microbial counts determination and replication

Microbial contamination was assessed at five time points (days 1, 7, 14, 21, and 28). The experimental design utilized three independent cheese samples as biological replicates for each treatment at each sampling time (n=3). Each microbiological determination was performed in triplicate as analytical replicates, which were averaged to obtain one value per biological replicate. Total bacterial, coliform, presumptive *Staphylococcus*, and fungal counts were determined using standard plating methods on Plate Count Agar, MacConkey Agar, Mannitol Salt Agar, and Sabouraud Dextrose Agar, respectively.

Microbial identification and antibiotic sensitivity testing

Presumptive *Staphylococcus aureus* and *Escherichia coli* colonies were purified and identified using Gram staining and biochemical tests, including the API 20E system for *Enterobacteriaceae*. A total of 14 confirmed isolates (7 *S. aureus* and 7 *E. coli*) were obtained across the sampling periods. All 14 isolates were subjected to antimicrobial susceptibility testing using the Kirby-Bauer disk diffusion method against Amoxicillin (10 µg), Ciprofloxacin (10 µg), Vancomycin (30 µg), and Azithromycin

(15 µg). Results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines.

Statistical analysis

Data were analyzed using SPSS software (version 26.0). A one-way ANOVA followed by Tukey's post hoc test assessed differences between treatments. Statistical significance was set at $p < 0.05$. Results are presented as mean ± standard deviation.

Results

Total bacterial and coliform counts

The total bacterial and coliform counts of cheese samples over the 28-day storage period are presented in Table 1. The initial total bacterial count on day 1 was below 2 log CFU/g across all groups. This relatively low initial load can be attributed to the specific hygienic practices and rapid cooling employed by the artisanal producers from whom the samples were sourced. In the control group (T1), the total bacterial count significantly increased to 7.51 log CFU/g by day 21. Conversely, treatments T2, T3, and T4 demonstrated a marked reduction in bacterial growth. T4 showed the most promising results, with the total bacterial count remaining at 2.83 log CFU/g on day 28, highlighting a synergistic effect.

Regarding coliform counts, the control group increased significantly from 1.30 log CFU/g on day 1 to 7.52 log CFU/g by day 28. Notably, while 8 °C is generally considered the lower limit for coliform growth, the observed increase at 4 °C may also reflect the development of psychrotrophic bacteria, such as Pseudomonadaceae, which can grow on MacConkey agar and thrive at refrigeration temperatures. Treatment T4 exhibited the lowest coliform counts throughout the study, reaching only 1.98 log CFU/g by day 28.

Coliform bacterial count

Table 1 summarizes the coliform total count (CFU/g) of contaminated cheese samples coated with propolis and ZnONPs over a 28-day storage period at 4±2ºC. The results indicate significant differences in coliform counts across treatments (T1, T2, T3, and T4) and throughout the storage period, reflecting the potential efficacy of the tested antimicrobial agents in reducing coliform contamination. In the control group (T1), the coliform count began at log 1.3 CFU/g on day 1 and significantly ($p<0.05$) increased to log 7.52 CFU/g by day 28. This trend highlights the rapid proliferation of coliform bacteria in cheese samples when no antimicrobial intervention is applied, indicating a significant risk to food safety. The consistent increase in coliform counts underscores the challenge of controlling microbial populations in dairy products over time.

In contrast, the treatments containing propolis and ZnONPs exhibited varying levels of effectiveness in inhibiting coliform growth. Treatment T2 started with a coliform count of log 1.1 CFU/g, which increased to log 4.37 CFU/g by day 28. Although some growth occurred, the levels remained significantly lower than those observed in the control group (T1), suggesting that the combination of propolis and ZnONPs effectively mitigated coliform proliferation during storage.

Notably, treatment T3 demonstrated the most significant reduction in coliform counts, decreasing from log 1.5 CFU/g on day 1 to log 2.96 CFU/g by day 28. The counts on days 7, 14, 21, and 28 were statistically different from those in the control group, indicating that the antimicrobial properties of the applied agents were particularly effective in this treatment. Treatment T4 exhibited the lowest coliform counts throughout the study, starting from log 1.1 CFU/g and reaching only log 1.98 CFU/g by day 28. The significant reduction observed in T4 suggests that this combination of propolis and ZnONPs effectively prevented coliform contamination, maintaining microbial levels well below those in the control group.

When comparing these results with recent studies on using natural preservatives in dairy products, it becomes evident that the combination of propolis and ZnONPs is a promising strategy for enhancing food safety. Recent literature has highlighted the antimicrobial properties of propolis against various foodborne pathogens, including coliform bacteria. The findings in this study reinforce the notion that Propolis, particularly when combined with nanotechnology (as seen with ZnONPs), can significantly

reduce microbial counts in food products. Moreover, previous studies have reported similar trends in the effectiveness of natural antimicrobial representatives in dairy preservation. For instance, studies have shown that applying various essential oils and plant extracts can significantly reduce microbial loads in cheese. However, the current study's findings demonstrate that the synergistic effect of honey bee propolis and ZnONPs may provide a more robust and effective approach compared to other natural preservatives alone. The results from Table)1(indicate that applying propolis and ZnONPs significantly reduces coliform contamination in locally produced cheeses during storage. The effectiveness of these treatments, especially in T3 and T4, highlights their potential as natural preservatives, offering a viable alternative to synthetic additives in the dairy industry.

Presumptive Staphylococcus and fungal counts

Enumeration of *Staphylococcus* spp. is considered presumptive in this study. Given that *S. aureus* has a minimum growth temperature of approximately 7°C, the colonies observed at 4°C may partially reflect the presence of Micrococcaceae, which have lower minimum growth temperatures and are common in dairy environments. Despite this, the T4 treatment significantly suppressed the growth of these presumptive colonies, reducing the count to 0.32 log CFU/g by day 28 (Table 2). Similarly, fungal contamination was completely eradicated in the T4 group by day 14 and remained undetectable through day 28, demonstrating potent antifungal synergy.

Antimicrobial susceptibility of bacterial isolates

A total of 14 confirmed bacterial isolates (7 *S. aureus* and 7 *E. coli*) were recovered and tested for antimicrobial susceptibility (Table 3). *S. aureus* isolates were sensitive to most tested antibiotics but exhibited resistance to Amoxicillin. *E. coli* isolates showed resistance to Vancomycin and Amoxicillin. The resistance of *E. coli* to Vancomycin is an intrinsic characteristic of Gram-negative bacteria due to the impermeability of their outer membrane to this large glycopeptide molecule, rather than an acquired resistance mechanism.

Antibacterial activity (inhibition zones)

The *in vitro* antibacterial activity of propolis and ZnONPs against the isolated *S. aureus* and *E. coli* strains demonstrated a clear dose-dependent response, with larger inhibition zones observed at higher concentrations (Table 4). *S. aureus* consistently showed larger inhibition zones than *E. coli*, which aligns with the known higher susceptibility of Gram-positive bacteria to these agents due to their simpler cell wall structure.

Discussion

The primary objective of this research was to evaluate the antimicrobial efficacy of honey bee propolis, ZnONPs, and their combination on the microbial quality of local artisanal Iraqi cheese during refrigerated storage. The results demonstrated that the combined treatment exhibited the most potent synergistic effect, significantly reducing total bacterial, coliform, presumptive staphylococcal, and fungal counts compared to the control and individual treatments. These findings provide a comprehensive understanding of how natural antimicrobial agents can be integrated with nanotechnology to extend the shelf life of regional dairy products.

The initial total bacterial load in the cheese samples was relatively low, which aligns with the specific hygienic practices and rapid cooling employed by the local artisanal producers. This observation is consistent with findings from other regional artisanal cheeses where formal microenterprises maintain lower microbial counts compared to informal ones (Castellanos-Rozo *et al*., 2021). However, the rapid proliferation of bacteria in the untreated control group highlights the inherent risk of spoilage in fresh dairy products stored without active preservation (Lima et al., 2021). The significant reduction in the combined treatment group indicates that the integration of propolis and ZnONPs effectively delays microbial spoilage and maintains product quality over an extended period.

An interesting observation in the present study was the increase in coliform counts on MacConkey agar at a refrigeration temperature of 4 degrees Celsius. Since the minimum growth temperature for typical

coliforms is generally around 8 degrees Celsius, the growth observed in this study likely reflects the development of psychrotrophic bacteria, such as Pseudomonadaceae, which are known to thrive at low temperatures and can grow on MacConkey agar (Gram *et al*., 2002). The combined treatment successfully suppressed this psychrotrophic growth, maintaining counts at minimal levels and demonstrating efficacy against cold-tolerant spoilage organisms.

The enumeration of *staphylococci* in this study was presumptive. It is well documented that Staphylococcus aureus has a minimum growth temperature of approximately 7 degrees Celsius. Therefore, the colonies observed on Mannitol Salt Agar at 4 degrees Celsius may partially represent Micrococcaceae, which possess lower minimum growth temperatures and are common residents of the dairy environment (Risson, 2023). Despite this taxonomic overlap, the combined treatment significantly suppressed the growth of these presumptive colonies, demonstrating broad efficacy against Gram-positive spoilage organisms commonly found in cheese matrices.

Fungal spoilage is a major concern in refrigerated cheese, and the complete eradication of fungal counts in the combined treatment group by day 14 demonstrates potent antifungal synergy. Recent research has demonstrated the efficacy of propolis extracts in reducing spoilage microorganisms in semi-hard cheeses without compromising product quality (Paula *et al*., 2024). Furthermore, the use of natural extracts combined with nano zinc oxide has been shown to enhance efficacy against common dairy spoilage organisms compared to either agent alone (Korany *et al*., 2024, Al-Nabulsi *et al*., 2020). The current findings strongly support these previous observations and extend them to the specific context of artisanal Iraqi cheese.

The antimicrobial susceptibility testing revealed resistance to Amoxicillin in both isolated *Staphylococcus aureus* and *Escherichia coli*, which is a common finding in dairy isolates due to the historical use of beta-lactam antibiotics in livestock. The observed resistance of *Escherichia coli* to Vancomycin is an intrinsic characteristic of Gram-negative bacteria, attributed to the impermeability of their outer membrane to large glycopeptide molecules, rather than an acquired resistance mechanism. The in vitro inhibition zone assays further confirmed that Gram-positive bacteria were more susceptible to the treatments than Gram-negative bacteria. This differential susceptibility is primarily due to the complex outer membrane of Gram-negative bacteria, which acts as a permeability barrier against antimicrobial agents (Sirelkhatim *et al*., 2015).

From a scientific perspective, the present study contributes to the existing body of knowledge by demonstrating the direct synergistic application of green-synthesized ZnONPs and local propolis extract as an edible coating on regional artisanal cheese. Unlike previous studies that incorporated these agents into synthetic packaging films or evaluated them in different food matrices such as meat or starch-based films, this research addresses a specific gap in local dairy preservation . The proposed dual mechanism, wherein the bioactive flavonoids in propolis enhance cell membrane permeability to facilitate the penetration of zinc ions and reactive oxygen species, provides a theoretical basis for the observed synergistic efficacy. This combination offers a viable, natural alternative to synthetic preservatives, potentially reducing the reliance on chemical additives in the dairy industry.

Despite the promising findings, this study has several limitations that must be acknowledged. First, the propolis used was not botanically standardized, and its total phenolic and flavonoid contents were not quantified, which limits the chemical reproducibility of the extract. Second, while the ZnONPs were previously characterized, their specific physical behavior and potential aggregation within this specific cheese matrix were not re-evaluated. Most importantly, sensory evaluations, including taste, odor, texture, and color, as well as toxicological assessments and chemical migration analyses of zinc into the food matrix, were not conducted. Therefore, claims regarding absolute food safety and commercial applicability must be interpreted with caution. Future research must prioritize sensory profiling and rigorous toxicological safety evaluations before this combined treatment can be recommended for commercial food use.

Conclusions

These findings highlight the potential for propolis and ZnONPs to replace synthetic preservatives, offering a safe, natural, and effective strategy for enhancing food safety. Incorporating these

antimicrobial agents into cheese processing and packaging could significantly reduce bacterial contamination, prolong shelf life, and provide consumers with healthier and safer dairy products.

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Table 1. Changes in total bacterial and coliform counts (log CFU/g) of contaminated cheese samples coated with honey bee propolis and ZnO nanoparticles during storage at 4±2ºC.

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28
Total bacterial count					
T1 (Control)	1.81±0.5^{a}	6.35±1.2^{a}	7.51±1.6^{a}	HG	HG
T2 (Propolis)	1.84±0.4^{a}	3.26±0.7^{b}	3.42±0.9^{b}	3.84±0.7^{a}	4.37±0.9^{a}
T3 (ZnO-NPs)	1.80±0.6^{a}	2.12±0.7^{c}	2.48±0.7^{c}	2.63±0.8^{b}	3.15±0.5^{b}
T4 (Propolis + ZnO-NPs)	1.81±0.5^{a}	2.03±0.8^{c}	2.20±0.8^{c}	2.51±0.5^{c}	2.83±0.7^{c}
Coliform count					
T1 (Control)	1.30±0.02^{a}	4.24±0.5^{a}	6.31±1.0^{a}	6.85±1.1^{a}	7.52±1.2^{a}
T2 (Propolis)	1.10±0.01^{a}	2.11±0.2^{b}	3.05±0.5^{b}	3.64±0.4^{b}	4.37±0.4^{b}
T3 (ZnO-NPs)	1.50±0.01^{a}	1.34±0.3^{c}	2.12±0.2^{c}	2.83±0.5^{c}	2.96±0.1^{c}
T4 (Propolis + ZnO-NPs)	1.10±0.02^{a}	1.15±0.8^{c}	1.41±0.4^{d}	1.93±0.2^{d}	1.98±0.1^{d}

Data are presented as mean ± standard deviation. Different letters within each column indicate significant differences ($p<0.05$). T1=control, T2=propolis, T3=ZnO-NPs, T4=propolis + ZnO-NPs.

Table 2. Presumptive Staphylococcus and total fungal counts (log CFU/g) of cheese samples coated with honey bee propolis and ZnO nanoparticles during storage at 4±2.

Treatment	Day 1	Day 7	Day 14	Day 21	Day 28
Presumptive *Staphylococcus*					
T1 (Control)	1.80±0.3^{a}	5.37±0.8^{a}	6.50±1.1^{a}	7.10±1.3^{a}	7.83±1.5^{a}
T2 (Propolis)	1.75±0.4^{a}	2.01±0.5^{b}	2.65±0.6^{b}	3.20±0.7^{b}	3.81±0.8^{b}
T3 (ZnONPs)	1.60±0.3^{a}	0.11±0.05^{c}	0.45±0.1^{c}	0.80±0.2^{c}	1.15±0.3^{c}
T4 (Propolis + ZnONPs)	1.90±0.4^{a}	1.13±0.3^{c}	0.75±0.2^{c}	0.50±0.1^{c}	0.32±0.1^{c}
Total fungal count					
T1 (Control)	2.00±0.1^{a}	2.18±0.1^{a}	2.75±0.2^{a}	3.15±0.3^{a}	3.64±0.8^{a}
T2 (Propolis)	2.02±0.2^{a}	2.14±0.2^{a}	2.38±0.1^{b}	2.43±0.1^{b}	2.58±0.2^{b}
T3 (ZnONPs)	2.01±0.1^{a}	1.60±0.03^{b}	1.31±0.06^{c}	0.81±0.01^{c}	0.00±0.0^{c}
T4 (Propolis + ZnONPs)	2.00±0.2^{a}	1.10±0.02^{c}	0.00±0.0^{d}	0.42±0.01^{c}	0.00±0.0^{c}

Different letters within each column indicate significant differences ($p<0.05$).

Table 3. Microbial identification and antibiotic susceptibility of isolates recovered from cheese samples after 28 days of storage.

Isolate	Detected in treatment	Antibiotic susceptibility						
		DA	CIP	VA	AK	AZM	SXT	AX
S. aureus	T1, T2	**S**	**S**	**S**	**S**	**S**	I	R
E. coli	T1	**S**	**S**	**R**	**I**	**S**	I	R
Others	T1/T2							

ND, mean not detected. R, resistance; I, intermediate; S, sensitive; Clindamycin (DA-10), Ciprofloxacin (CIP-10), Vancomycin (VA-30), Amikacin (AK-10), Azithromycin (AZM-15), Trimethoprim sulphamethoxazole (SXT-25), Amoxicillin (AX-10).

Table 4. Antibacterial sensitivity of propolis and ZnO-NPs against bacterial isolates from Cheese samples.

Isolates species	Inhibition zone diameters (mm) of propolis and ZnONPs (mg/mL) against bacterial species		
	2.5	5.0	7.5
S. aureus	13±1.4^{c}	20±3.1^{b}	33±4.0^{a}
E. coli	10±2.2^{c}	19±2.7^{b}	28±3.8^{a}

The different letters mean significant differences in each raw at probability 0.05.