Research Article

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Efficiency of Using Local Propolis as A Preservative in Buffalo Milk Against *Escherichia coli* Contamination

Luma Awad Majeed Alzaidi, Basima Jasim Mohammed.

Department of Public Health, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah City, Iraq.
Corresponding Author Email Address: basima.jasim@qu.edu.iq

ORCID ID: https://orcid.org/0000-0003-1749-7941

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Abstract

The present study was designed to test the efficiency of local propolis as a natural preservative for buffalo milk against *E. coli* contamination. Here, 100 milk samples were collected from buffalo herds in different areas in Al-Diwaniyah City, Iraq, and transported in a cooled box to the Laboratory of Public Health, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah City, Iraq. Laboratory tests were conducted on the bacterial isolates of *Escherichia coli* using traditional and molecular methods, and virulence genes were investigated, in addition to conducting a sensitivity test for the bacterial isolates. In addition, the phylogenetic status of the bacterium was explored for understanding the genetic evolution. Local propolis extract in different concentrations (10 mg, 20 mg, and 40 mg) was added to tubes containing 1 ml of milk with a control group without addition. Al milk groups were stored at 5 ± 1°C for 10 days. Subsequently, bacterial counting was performed at time zero, 3 days, 7 days, and 10 days to determine bacterial contamination. *E. coli* was detected in the milk samples before propolis was added. The bacterium demonstrated high sensitivity to gentamycin (18.66±0.88mm) and amikacin (15.66±0.33mm), with very low sensitivity reaching 0 mm in amoxicillin and tetracycline. The bacterial counts revealed significant (p<0.05) protection against bacterial growth, especially at high concentrations. The sequencing revealed a distinct but close similarity with world E. coli isolates. The gene expression of the virulence genes was seen significantly (p<0.05) affected by propolis. The study demonstrates that the propolis protects against bacterial growth.

Keywords: Enterotoxigenic *E. coli*, coliform enteritis, food-borne diseases.

Introduction

Buffalo (*Bubalus bubalis*) milk has a higher nutritional value than cow milk as it contains high levels of fat, protein, minerals, and vitamins (1). This measure enhances human health and lifestyle, thereby significantly boosting the economic empowerment of smallholder producers (2,3). Unfortunately, the high nutritional value of buffalo milk results in a very short shelf-life using current conventional preservation methods. The mortality rate of *E. coli* infection is high, indicating a major threat to public health. *E. coli* is responsible for regular outbreaks due to the consumption of contaminated milk and dairy products worldwide (4, 5).

Several factors, such as poor farm management, have an impact on the quality of buffalo milk. Furthermore, the handling of buffalo milk during the collecting, processing, and storing times is important (6-9). The lack of recognizing the risk of *E. coli* contamination in milk procurement, processing, and everyday milk delivery activities which cause public health problems, also can affect the quality and integrity of buffalo milk (10-12).

Due to population growth, there is a demand for more animal-sourced foods such as meat, milk, and their products, which provides an attractive sector for the global economy. Buffalo milk is not only an alternative source of high-quality, highly nutritious milk, but it also has economic advantages. Compared to cow milk, buffalo milk contains more protein, fat, and milk solids and yields more dairy products such as cheese, yogurt, ice cream, and gelato (13). The protein in buffalo milk products has fewer allergenic properties and lower cholesterol levels. Due to its physiochemical and nutritional composition, dairy products made from buffalo milk have a longer shelf life, better taste, and even fewer grain formations. Buffalo milk contains a high concentration of unsaturated fatty acids, which improve human health by decreasing blood cholesterol levels (14,15).

E. coli is a representative member of the family of conditionally pathogenic microorganisms, which includes many strains of the species used in collaboration with humans in various contexts with potential risks. Practically, all contaminated milk is low in acidity and high in pH milk, and the percentage of its bacterial contaminant is very low. But due to the presence of a small number of contaminants, it can exhibit a strong shelf-life decrease in the form of a hygiene issue (microbial presence in spoiled products). Buffaloes have an immense impact on the developing world's economy and supply dairy products such as milk, meat, and cheese in Nigeria, among many other countries, with Bangladesh, India, Egypt, and Bangladesh supplying nearly 72% of world buffalo meat (16-19).

Research has demonstrated the synergistic activities of propolis with some antibiotics against pathogenic antibiotic-resistant strains, providing insights for medical and veterinary applications as an adjunct treatment for infectious diseases. Also, propolis showed selective cytotoxic activities according to its origin, which is advantageous for antimicrobial substance

candidates against specific pathogenic bacteria. The synergistic antimicrobial properties of propolis in combination with other natural products were also identified. All of these data support that the positive features make propolis a strong alternative to chemical preservatives, which have the potential to solve economic losses due to packed and canned food products (20-26).

The main antimicrobial activities of each component identified in the chemical composition of propolis, phenolic compounds, essential oils, and flavonoids have been investigated and demonstrated in many studies (27, 28). Compounds like galangin, caffeic acid, pinocembrin, kaempferol, chrysin, and quercetin are found in the chemical composition of propolis and have outstanding antibacterial activities. Furthermore, the appropriate conditions of propolis applications have been highlighted as 0.5% concentration, 24-hour activity, and acidic to neutral pH (29-34). The present study was designed to test the efficiency of local propolis as a natural preservative for buffalo milk against *E. coli* contamination.

Materials and Methods

Sample Collection and Bacterial Cultivation

One-hundred individual milk buffalo samples were aseptically collected and immediately transferred to a refrigerated environment. The animals were propagated at the same farm and within the same confinement and feeding regimen as that mentioned for both milk collection circuits. The bacteria were cultivated in Nutrient Agar, MacConkey Agar, and Eosin Methylene Blue Agar bases according to certain bacteriology methods (27).

Diagnostic Tests for *E. coli* Recovery

In addition, the screening test for the presence of *E. coli* was performed using EMB agar. An isolation streak was performed on the medium plate, and the development of the purple mucous colonies was watched after a 24-hour incubation period. *E. coli* species were known to have a manufactured metallic sheath mechanism on EMB agar, which distinguished them. These cells appeared on the liquid surrounding the colony and developed the aptitude to dissolve the metal, which shined in the reflected light. These diagnostic tests were performed to ensure that *E. coli* was recovered once treated, as stipulated in the experimental design of the current study. It was seen that viable *E. coli* bacteria were isolated in terms of the recovery contributions of treatments that far exceeded organisms treated with a lower concentration.

As a preliminary test, Gram staining was performed on the control and the propolis-treated group to verify cell morphology. A hanging drop microscopic examination was performed, and it was observed that the cells for the Gram staining appeared purple, and the shape was labeled as 'short rod'. The characteristics of E. coli growth in MCA, which is pink in color, and in EMB, which is green with a distinctive shine caused by the development of a thick cytoplasm, are described. According to the findings, the microorganisms that overcame the six concentration treatments were E. coli resistant.

Antibiotic susceptibility testing

The antibiotic resistance profile of *E. coli* was examined, and thenthe propolis extract was investigated for its antibacterial activity against this bacterium. Propolis has the ability to postpone food spoilage and prolong the shelf-life of various food items.

Determination of the antibiotic sensitivity was conducted by the standard Kirby-Bauer well-diffusion method using eight antibiotic agents to determine an antibiotic sensitivity pattern of *E. coli*. Each antibiotic was evaluated for each *E. coli* sample (35).

16S rRNA Gene-Based PCR

The study used the Geneaid Genomic DNA Purification Kit (Turkey) to extract bacterial DNA, estimating the amount using a NanoDrop. The PCR master mix was 25µl, the DNA was 5µl, the F or R primer was 10pmol 3µl, the PCR water was 14µl. The following table (1) represents the thermocycler conditions for each virulence gene.1.5% agarose was used in an 80-volt and 40-min electrophoresis. Then, the amplification bands were visualized using a UV-imager. The sequencing was done using the PCR purified bands.

Table 1: PCR thermocycler conditions plus primers for each virulence gene.

rssA Gene F TTGCTATTCGCGCATCATGC R AATATCAGCCCCCAATGCAC			
PCR steps	**Cycle**	**Temp. (°C)**	**Time (Sec)**
Initial denaturation	1	95	120
Denaturation		95	30
Annealing	35	57.3	30
Extension		72	60
Final extension	1	72	5X60
BssS gene F GTAGGGTGGGACATCAGCAC R CCAGAGCGTCTGACCAACTT			
Initial denaturation	1	95	120
Denaturation		95	30
Annealing	35	57.6	30
Extension	1	72	60
Final extension		72	5X60
BssR gene F ACCATGATTGCCCGACTGTT R TCAGGCCTTCTCAAGCATGG			
Initial denaturation	1	95	120
Denaturation		95	30
Annealing	35	*57.7, 56.6, 57.6	30
Extension		72	60
Final extension	1	72	5X60

Propolis extraction and GC-mass analysis

The propolis was let to freeze at -20°C for 24 hrs. for hardness. An electrical grinder then milled the propolis to obtain the powder. Later, 200 gm of the powdered propolis was soaked in 500 ml of distilled water in an amber glass container and left at room temperature 25±2 °C for 14 days. Throughout the soaking period, we shook the mixture twice daily for brief intervals. The mixture suspension of the extract was filtered by Whatman filter paper No. 4. To remove waxes and insoluble constituents, the suspension was subsequently frozen at -20°C for 24 hours, then re-filtered and repeated triplicate time. The remaining extract was incubated at 37°C for 3 days until the residual water was evaporated, and the resulting sticky-like substance was stored at -20°C until use

Preservation experiment setup

For the preservation experiment, finely-ground propolis was prepared with different concentrations (10mg, 20mg, and 40mg). Each concentration was added to 1 ml of milk with a control 1ml of milk with no propolis. After that, all milk groups were placed in 5±1 °C for 10 days. Later, a bacterial count was done at zero-time, 3 days, 7 days, and 10 days to identify the bacterial contamination utilizing the bacterial count.

RT-qPCR

The current test was performed to estimate the mRNA virulence gene level before and after the propolis extract was added, an additional indicator for the preservative effects against *E. coli.* The total RNA was obtained from RPE/choroid tissue after lysis by the TRIzol ® reagent kit according to manufacturer instructions. A concentration of 100 ng/µl RNA was utilized to synthesize cDNA by AccuPower ® RockScript RT PreMix kit (Bioneer company, South Korea). The total RT cDNA synthesis step was conducted at a temperature of 50 °C within 1 hour. The final RT-qPCR utilized a 5 µl concentration of cDNA while the quantity of 20 ng/µl was established for each marker through DNA analysis. For each primer-direction, a 1 µl fragment (10pmol) and 13 µl of water were occupied. The initialization process involved an initial denaturation step at 95 °C, followed by denaturation and annealing/extension detection (scan) steps at 95 °C and 60 °C, respectively. The operation times were set to 10 minutes, 20 seconds, and 30 seconds, respectively, for 1 cycle, 45 cycles, and 1 cycle.

Results

E. coli was detected in the milk samples before adding propolis as confirmed by the PCR with the presence of virulence genes (Figure 1).

The bacterium demonstrated high sensitivity to gentamycin (18.66±0.88mm) and amikacin (15.66±0.33mm) with very low sensitivity that reached 0 mm in amoxicillin ceftriaxone, and tetracycline (Table 2).

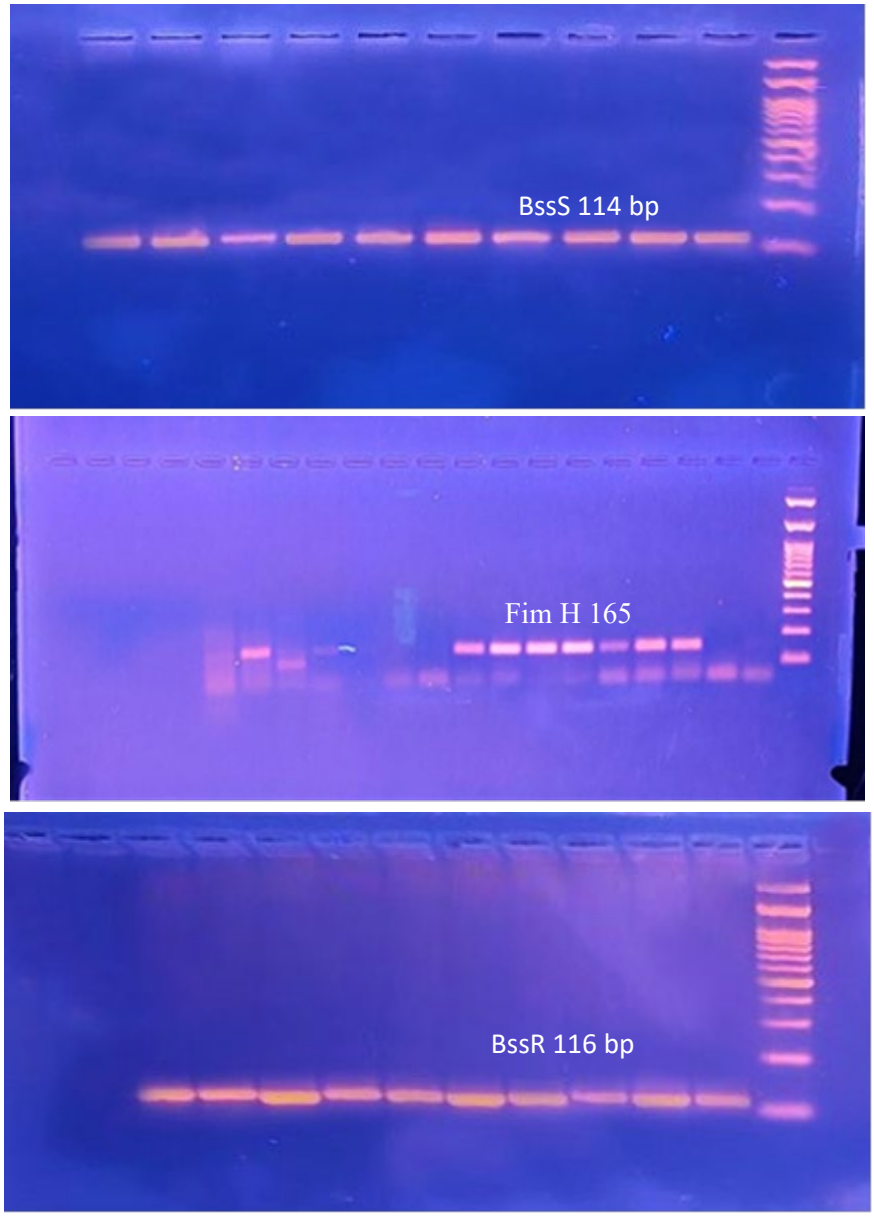

Figure 1: Image of agarose gel electrophoresis of virulence genes of *E. coli* isolated from buffalo milk. Vertical bands: ladder (3000-100). Horizontal bands: Positive PCR.

Table 2: Antibiotic sensitivity test

Antibiotic	***E. coli***
Amoxicillin	0±0 Aa
Gentamycin	18.66±0.88 Ba
Amikacin	15.66±0.33 BCa
Ceftriaxone	0±0 Aa
Trimethoprim	13.66±0.88 BCa
Tetracycline	0±0 Aa
Actinomycin	12.66±6.35 BCa
Vancomycin	11.66±5.85 Cb
LSD (P<0.05)	

The finding of the bacterial counts revealed significant (p<0.05) protection against bacterial growth, especially at high concentrations. No significant (p>0.05) differences were recorded at Zero time; however, it was with a significant (p<0.05) at 3, 7, 10 days of preservation (Table 3). The sequencing revealed distinct but close similarity with world isolates of *E. coli* (Figure 2). The propolis extraction procedure revealed different components as revealed by GC-mass findings (Table 4).

Table 3: Zone of inhibition (mm) of propolis against *E. coli* isolates

Concentration of propolis	**zero time**	**3 days**	**7 days**	**10 days**
10 µg /ml	14.06±0.37Aa	19.1±2.1Ab	22.43±0.33Ac	25.3±0.45Ac
20 µg /ml	14.28±0.37Aa	14.56±0.21Ba	22.76±3.07Ab	24±1.86Ab
40 µg /ml	14.14±0.37Aa	10.33±0.43Cb	18.43±0.31Bc	19.4±0.27Bc
Control	14.86±0.37Aa	19.26±0.37Ab	26.9±0.32Cac	28±0.17Ac
LSD (P<0.05)	3.11			

Different letters between any two means denote to the significant difference, Capital letters denote to the vertical comparison and small letters denote to the horizontal comparison

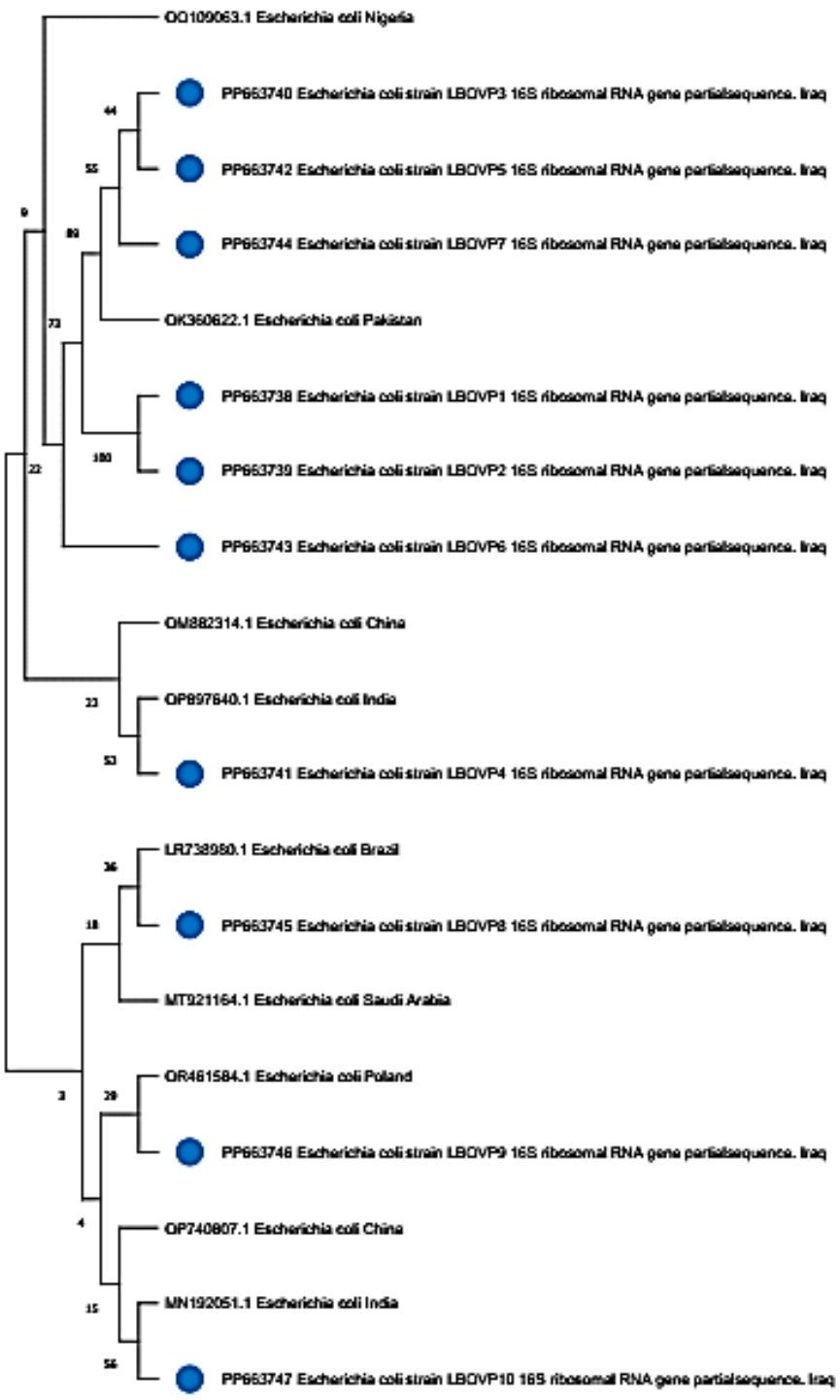

Figure 2: Phylogenetic tree of the *E. coli* isolated from buffalo milk and based on the *16S rRNA* gene.

Table 4: Results of GC-Mass analysis of propolis extract.

Peak	Retention time	Compound	Area %
1	2.17	Acetic acid	4.99
2	3.49	Methyl 2-hydroxy-2-methoxyacetate	4.86
3	4.04	2,3-Butanediol	66.64
4	4.95	2-Oxazolidinone, 3-amino-5-(4-morpholinylmethyl	1.30
5	5.05	Propanoic acid	0.90
6	5.57	3-Hexanone, 5-hydroxy-2-methyl-	0.95
7	8.95	Phenylethyl Alcohol	0.33
8	10.35	Catechol	0.65
9	10.57	4-Vinylphenol	0.39
10	11.81	Salicyl alcohol	0.80
11	11.97	2-Methoxy-4-vinylphenol	0.46
12	14.03	4-Vinylbenzene-1,2-diol	3.05
13	18.51	n-Hexadecanoic acid	2.90
14	20.47	Oleic Acid	6.82
15	23.99	Tetracontane	0.53
16	25.2	Benzoic acid, 2,4-dimethoxy-6-methyl-, 4-carboxy	0.47
17	25.95	4H-1-Benzopyran-4-one, 2,3-dihydro-5,7-dihydroxy	0.98
18	26.61	4H-1-Benzopyran-4-one, 5-hydroxy-7-methoxy	0.49
19	26.92	Heptacosyl heptafluorobutyrate	0.98
20	28.84	1-Hexacosanol	1.48

The gene expression of the virulence genes was seen significantly ($p<0.05$) affected by propolis. Figure 3 reveals these effects as expressed by mRNA fold change.

Discussion

The high level of public interest in milking products necessitates good-quality fresh produce.Pathogenic bacteria, especially *E. coli*, are important criteria used to evaluate the safety of milk. We propose that propolis, a bactericidal and anti-inflammatory compound, can eliminate E. coli. Compared with treatment of artificially antioxidant-treated buffalo milk, treatment of buffalo milk with propolis significantly reduces pathogenic *E. coli* levels. Furthermore, we discovered that adding propolis to buffalo milk can serve as a natural preservation technique, prolonging its shelf life without causing any harm (36).

Raw buffalo milk is contaminated with pathogenic bacteria, particularly from the E.a coli group. The results of our study prove that the treated buffalo milk met the required quality safety criteria, particularly for public consumption, as the milk treatment significantly reduced pathogenic *E. coli* levels. This finding supports the concept of utilizing natural preservatives, such as propolis, to extend the shelf life of buffalo milk (37, 38).

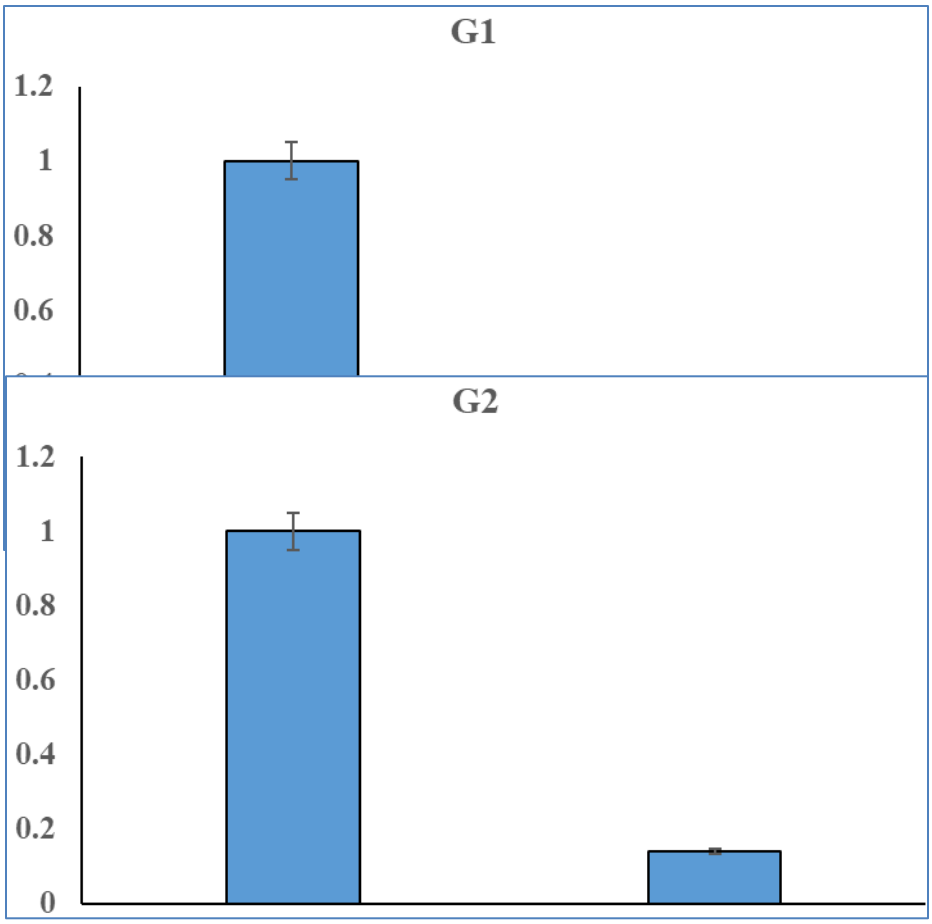

Figure 3: Fold change of mRNA expression of virulence genes after using propolis of the *E. coli* isolated from buffalo milk and based on the *16S rRNA* gene.

In previous studies, it was found that the addition of propolis extract inhibited *P. brevicorpium*, *P. freundenreich*, and *C. kutzner* during the storage period. Moreover, it deactivated enterotoxigenic and other enteropathogenic bacterial growth (39-46).

Furthermore, the antibacterial effects of propolis vary depending on its form, bacterial species, and assay conditions. In the same way, earlier studies have shown that propolis and essential oils together can kill more microbes and stop Listeria monocytogenes from growing in soy protein film used to cover meat products.Also, the combined preservative effects of propolis with cinnamon, allspice, and clove oil have been demonstrated on the shelf life of Atlantic chub mackerel fillets kept at 4 °C by delaying lipid oxidation and microbial growth. As a purifying agent encapsulated in gelatin, propolis is also recommended as an effective natural supplement to control Listeria spp. bacterial contamination during food maintenance. Moreover, propolis in

combination with Eucalyptus spp. preparation has great value as a biological preparation in preventing MYC toxin contamination in stored wheat grains. The report focuses on the potential for propolis application in stored products due to its natural origin, low cost, and no harmful residual side effects. The proficiency of propolis against food bacterial pathogens is probably due to its ability to damage the bacterial cell membranes as well as its antioxidant activity (47-49).

Conclusion

The present study demonstrated that the propolis was proven to be a natural effective preservative against *E. coli* contamination in buffalo milk. Predominantly, it reduced significantly bacterial growth, especially at higher additive concentrations, indicating its potent antimicrobial activity. The isolated *E. coli* strains are susceptible to the antibiotics; gentamycin and amikacin while resistant to antibiotics such as amoxicillin and tetracycline. These strains shared 88.6–95.9 percent nucleotide identity with global isolates of *E. coli*, indicating their similarities in strain characteristics within and across regions over the world. The results revealed that propolis could be used as an alternative to synthetic preservatives and antibiotics for the dairy industry. Furthermore, the findings could prompt further investigation into its potential antimicrobial activity and mechanisms of action.

Conflicts of interest

The authors declare that there is no conflict of interest.

Ethical Clearance

This work is approved by The Research Ethical Committee.

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كفاءة استخدام العكبر المحلي كمادة حافظة في حليب الجاموس ضد تلوث الإشريكية القولونية.

لمى عواد مجيد الزيدي، باسمة جاسم محمد.

فرع الصحة العامة، كلية الطب البيطري، جامعة القادسية، مدينة الديوانية، العراق

الخلاصة

تم تصميم الدراسة الحالية لاختبار كفاءة العكبر المحلي كمادة حافظة طبيعية لحليب الجاموس ضد تلوث الإشريكية القولونية. تم جمع 100 عينة حليب من قطعان الجاموس في مناطق مختلفة في مدينة الديوانية، العراق، ونقلت في صندوق مبرد إلى مختبر الصحة العامة، كلية الطب البيطري، جامعة القادسية، مدينة الديوانية، العراق. أُجريت اختبارات مخبرية على العزلات البكتيرية للإشريكية القولونية باستخدام الطرق التقليدية والجزيئية، وتم التحقيق في الجينات الضارة، بالإضافة إلى إجراء اختبار الحساسية للعزلات البكتيرية. بالإضافة إلى ذلك، تم استكشاف الحالة النسبية للبكتيريا لفهم التطور الجيني. تم إضافة مستخلص العكبر المحلي بتركيزات مختلفة (10 ملغ، 20 ملغ، و 40 ملغ) إلى أنابيب تحتوي على 1 مل من الحليب مع مجموعة ضابطة بدون إضافة. تم تخزين مجموعات الحليب في درجة حرارة 5 ± 1 درجة سيليزية لمدة 10 أيام. بعد ذلك، تم إجراء عد البكتيريا في الوقت صفر، 3 أيام، 7 أيام، و 10 أيام لتحديد التلوث البكتيري. تم الكشف عن الإشريكية القولونية في عينات الحليب قبل إضافة العكبر. أظهرت البكتيريا حساسية عالية للجنتاميسين (18.66±0.88ملم) وأميكاسين (15.66±0.33ملم) مع حساسية منخفضة جداً بلغت 0 ملم في الأموكسيسيلين والتتراسيكلين. كشفت نتائج عد البكتيريا عن حماية كبيرة ($p<0.05$) ضد نمو البكتيريا، خاصة في التركيزات العالية. أظهر تعاقب القواعد النتروجينية تشابهاً وثيقاً لكنه متميز مع عزلات الإشريكية القولونية العالمية. تأثر تعبير الجينات الضارة بشكل كبير ($p<0.05$) بالعكبر. تُظهر الدراسة أن العكبر يحمي ضد نمو البكتيريا.

الكلمات المفتاحية: الإشريكية القولونية المنتجة للسموم المعوية، التهاب الأمعاء القولوني، الأمراض المنقولة بالغذاء