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Role of lactoferrin in the treatment of *E. coli*-induced bovine mastitis

Mohamed Ibrahim Mostafa[1], Saber Ali Saad[2], Asmaa Elsayed Mohammed[3]*, Elsaid Mohamed Mohamed Saafan[4] and Amal Mohamed El-Sayed[5]

[1]*Department of Bacteriology, Animal Health Research Institute, Agriculture Research Center (ARC), Damanhur branch, Egypt*
[2]*Department of Food Hygiene, Animal Health Research Institute, Agriculture Research Center (ARC), Damanhur branch, Egypt*
[3]*Department of Bacteriology, Animal Health Research Institute, Agriculture Research Center (ARC), Sohag, Egypt*
[4]*Department of Food Hygiene, Animal Health Research Institute, Agriculture Research Center (ARC), Mansoura branch, Egypt*
[5]*Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Aswan University, Egypt*

ABSTRACT

Background: Bovine mastitis (BM) is a costly disease in the dairy industry. It is associated with reduced milk production and changes in milk composition and quality. *Escherichia coli* is the most important pathogen causing BM.
Aim: This study aimed to assess the efficacy of lactoferrin (LF) as an enhanced agent when combined with marbofloxacin for the treatment of *E. coli* BM.
Methods: Eighty lactating cattle and buffaloes, 40 each, aged 4–6 years suffering from BM underwent clinical examination. Milk samples were collected for bacteriological examination. The recovered *E. coli* isolates were subjected to serological, and polymerase chain reaction (PCR) analysis. BM cases were allocated into two groups: group (A) was treated with marbofloxacin combined with LF, and group (B) was treated with marbofloxacin alone.
Results: Fifty *E. coli* isolates were recovered. Six serogroup were identified: O55, O103, O26, O11, O44, and O124. All isolates were sensitive to marbofloxacin, while there was resistance to gentamycin and ampicillin (26% each), cefquinome (14%), cefixime (10%), and SuLFamethoxazole and trimethoprim (4%). The molecular identification of *E. coli* strains revealed that the *16S rRNA* gene was present in all identified *E. coli* strains (100%). All isolates resistant to antibiotics encoded BlaTEM, *aadB*, *and Sul1, whereas* the *qnrS* gene was not detected in any *E. coli* isolate. The broth dilution method did not detect growth when the marbofloxacin (6 µg/ml) was combined with LF (2 mg/ml). Group (A) showed significant improvements compared with group (B), with no recurrence rate.
Conclusion: LF can serve as a significant cotreatment agent for treating BM. As antibiotic resistance increases, LF-based drugs could play an important role in providing sustainable, effective alternatives for dairy animals.
Keywords: Bovine mastitis, Lactoferrin, *E. coli*, Marbofloxacin.

Introduction

Bovine mastitis (BM) is a mammary gland inflammation caused by physical trauma or bacterial invasion. It is the most prevalent and economically significant infectious disease in dairy farms worldwide (Sharun *et al*., 2021). *Escherichia coli* is the most prevalent Gram-negative pathogen causing clinical mastitis in dairy animals (Gao *et al*., 2017). Coliform mastitis causes high morbidity, and only 30%–50% of animals can regain their full production (Kutila *et al*., 2004) despite supportive and antibacterial therapy (Singh *et al*., 2024).

Management of BM is challenging because it is polygenic. Antibiotics are considered the cornerstone of treatment for BM. The efficiency of treating animals with clinical mastitis depends on the use of appropriate antimicrobials that eradicate pathogenic bacteria from udder tissue. Broad-spectrum antimicrobial drugs, namely, 3rd generation cephalosporins and fluoroquinolones, are prescribed for treating coliform BM. These drugs can be administered via intramammary, intramuscular, or direct intravenous injections (Abd-ELFatah *et al*., 2024). Although antimicrobial therapy is associated with rapid bacterial elimination and a high rate of survival in animals, it can be a significant cause for spreading antibiotic-resistant bacteria to humans (Fatemi *et al*., 2024).

The search for noninvasive therapeutic options that do not produce resistance is mandatory (Marques-Bastos

***Corresponding Author:** Asmaa Elsayed Mohammed. Department of Bacteriology, Animal Health Research Institute, Agriculture Research Center (ARC), Sohag, Egypt. Email: *dr_asmaa_lab@yahoo.com*

et al., 2023). Some alternatives, such as peptides, glycoproteins, and nanoparticles, have been used and proven effective (Saeed *et al*., 2023).

Lactoferrin (LF) is a glycoprotein naturally produced by polymorphonuclear neutrophils and epithelial cells. The protein is secreted by these cells at high levels in response to the inflammatory process. It has both bacteriostatic and bactericidal effects against a wide range of pathogens (Le *et al*., 2024).

The function of LF in mammary gland immunity is to modify and regulate macrophage, lymphocyte, and neutrophil function in response to nonspecific resistance to infection. LF increases the permeability of bacterial cell membranes and destroys the outer membrane of Gram-negative pathogens by binding to its lipopolysaccharide (LPS) and inhibiting its damaging effects. Low LF levels in clinical BM may be inadequate for preventing bacterial proliferation in the udder tissue (Yassin and Abd Elhady, 2023).

LF combined with antibiotics has a synergistic effect against BM. With an optimum dosage and duration of therapy, LF can help bacterial clearance from the animal mammary gland while simultaneously inhibiting the toxic properties of LPS (Diarra *et al*., 2002c).

The objective of the current study was to assess the efficacy of LF as an enhanced agent when combined with marbofloxacin for the reduction of bacterial resistance to this antibiotic in *E. coli* BM.

Material and Methods

Study sample

This study included 80 lactating cattle and buffaloes (40 each) from dairy farms aged 4–6 years. All animals had clinical mastitis. The study was conducted from January 2022 to October 2024 at the El-Beheira Governorate, Egypt.

Clinical examination of animals

Clinical examination of animals was conducted following Constable *et al*. (2016) to identify the clinical features of mastitis.

California mastitis test (CMT)

Equal volumes of CMT reagent and milk (2 ml from each quarter) were mixed thoroughly in a cup and then gently rotated for 10 seconds. The results were recorded and categorized into score 0 (negative), score 1 (trace), 2 (weakly positive), 3 (positive), and 4 (strongly positive) (Moroni *et al*., 2006).

Collection of milk samples

Milk samples were collected for bacteriological examination after cleaning and disinfecting the animal teats with soap, water, and alcohol (70%) (Kerro Dego and Tareke, 2003). The first jet of milk from each quarter of the udder was discarded. Then, 5–10 ml was taken in a sterile vial. The samples were labeled with the identification number of the affected animal and the date of collection. The samples were then brought to the Animal Health Research Institute Laboratory, Damanhur Laboratory, Egypt, for bacteriological examination.

Isolation, enumeration, and identification of E. coli

Milk samples from Clinical BM cases underwent centrifugation at 3,000 rpm for 20 minutes using a laboratory centrifuge (Sigma 1-7). After discarding the cream and supernatant, the resulting sediment was streaked onto MacConkey agar (Oxoid, UK) and EMB agar (Oxoid, UK), which were incubated at 37°C for 18–24 hours and subsequently examined for bacterial growth. *Escherichia coli* colonies on MacConkey's agar appeared as pink or red colonies lactose fermenter, while on EMB they appeared as bluish black colonies with a green metallic sheen. (Zinnah *et al*., 2007).

***E.coli* enumeration**

10-fold serial dilutions of milk samples were prepared using sterile peptone water. From the prepared dilutions 100 µl was plated on EMB agar. The inoculated plates were incubated at 37°C for 18–24 hours. The bacterial count was recorded from triple readings (Malt, 2013).

Microscopic examination

The direct film was prepared from pure culture and stained with gram stain to observe the morphological characteristics of *E. coli*, which is gram-negative short rod bacilli, that occur singly or in pairs (Cheesbrough, 2006).

Biochemical confirmation of *E. coli*

Pure cultures of a single colony from EMB agar were streaked onto nutrient agar (Oxoid, UK) for purification and then incubated at 37°C for 24 hours. Oxidase (Merk, Germany), triplicate sugar iron (Oxoid, UK), Indole, Methyl red (Oxoid, UK), Voges-Proskauer (Oxoid, UK), and Citrate tests (Oxoid, UK) were performed (De Boer and Heuvelink, 2000).

Serotyping of *E. coli*: A slide agglutination test was performed to identify *E. coli* using polyvalent and monovalent antisera was done (da Silva *et al*., 2001).

Antibiotic sensitivity testing of E. coli:

The 50 isolates of *E. coli* were examined for antibiograms using the disc diffusion method against six antibiotics (Oxoid). The antibiotic discs used were marbofloxacin (5 µg), cefixime (5 µg), suLFamethoxazole and trimethoprim (25 µg), gentamycin (10 µg), ampicillin (10 µg), and cefquinome (30 µg). The results were interpreted according to CLSI (He *et al*., 2024).

Molecular detection of antibiotic-resistance genes

DNA extraction and amplification

DNA extraction was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications based on the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 minutes. After incubation, 200 µl of 100% ethanol was added to the lysate. The samples were subsequently washed and centrifuged according to the manufacturer's instructions. The nucleic acid was then eluted with 100 µl of elution buffer. The oligonucleotide primers used were supplied by Metabion (Germany) (Table

Table 1. Primers sequences, target genes, amplicon sizes, and cycling conditions.

Genes	Primers (5'-3')		Condition of amplification					Reference
16S rRNA	GCTGACGAGTGGCGGACGGG TAGGAGTCTGGACCGTGTCT	253 bp	94°C 5 minutes.	94°C 30 s.	55°C 30 s.	72°C 30 s.	72°C 7 minutes.	Tivendale *et al*. (2004)
aadB	CTAGCTGCGGCAGATGAGC CTCAGCCGCCTCTGGGCA	219 bp	95°C 5 minutes.	94°C 1 minutes.	62°C 1 min	72°C 1 minutes.	72°C 5 minutes.	Doosti *et al*. (2016)
blaTEM	TCAACATTTTCGTGTCGCCC AACTACGATACGGGAGGGCT	445 bp	95°C 10 minutes	95°C 2 minutes.	57°C 35 s.	72°C 40 s.	72°C 7 minutes.	Jiang *et al*. (2022)
qrs	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	417 bp	94°C 5 minutes.	94°C 30 s.	48°C 45 s.	72°C 45 s.	72°C 5 minutes.	Robicsek *et al*. (2006)
Sul1	ATGGTGACGGTGTTCGGCATTCTGA CTAGGCATGATCTAACCCTCGGTCT	432 bp	95°C 5 minutes.	95°C 1 minutes.	55°C 1 min.	72°C 1 minutes.	72°C 5 minutes.	Toleman *et al*. (2006)

1). The 50 recovered *E. coli* isolates were confirmed by polymerase chain reaction (PCR) amplification of *the 16S rRNA* gene (Tivendale *et al*., 2004). Then, we screened for antibiotic resistance genes: *BlaTEM* gene for detection of β-lactam resistance (Jiang *et al*., 2022), *Sul1* encoding sulfonamide resistance (Toleman *et al*., 2006), *aadB* mediating gentamycin resistance (aminoglycosides) (Doosti *et al*., 2016), *and qnrs* encoding fluoroquinolone resistance (Robicsek *et al*., 2006). PCR amplification was conducted in an applied biosystem 2,720 thermal cycler (Life Technologies, Germany).

Analysis of PCR products

At room temperature, the products of PCR were separated by electrophoresis on 1.5% agarose gels (Applichem, Germany, GmbH) in 1× TBE buffer at 5 V/cm. For gel analysis, a gene ruler 100-bp ladder (Fermentas, Thermo, Germany), melilot 100-bp and 100-bp plus ladders (Qiagen, Gmbh, Germany), and Genedirex 50-bp DNA ladder RTU (Cat. No. DM012-R500) were used to determine the fragment sizes. The gel was photographed (Alpha Innotech, Biometra) and analyzed by computer software.

Detection of minimum inhibitory concentration (MIC) of marbofloxacin and LF using the Broth dilution method:

Preparation of bacterial inoculum

Escherichia coli strain O55 was sub-cultured onto EMB agar plates and incubated at 37°C for 24 hours. 3–4 colonies were selected, inoculated in tryptic soy broth, and then incubated at 37°C for 2–6 hours. Suspension turbidity was balanced to coordinate with 0.5 McFarland standards and then diluted to obtain a concentration of 10^5 CFU/ml approximately (Owuama, 2017).

LF preparation

Pure LF (Sigma Chemical Co.) was purchased. The following concentrations of LF solution were prepared, (5, 4, 3, 2, and 1 mg/ ml) in distilled water, sterilized by a 0.45-mm filter, and freshly used (Al Habty and Ali, 2023).

Preparation of marbofloxacin antibiotic

Marbofloxacin was diluted to different concentrations (5, 6, 7, 7.5, 9, 9.5, 9.8, 10, and 15 µg/ml) in distilled water in test tubes.

100 µl of bacterial concentration 10^5 CFU/ml were added to the prepared LF and marbofloxacin concentrations. The inoculated tubes and the positive control tube (tubes containing broth only without any treatment) were incubated at 37°C for 24 hours.

To determine the MIC, 100 µl of each clear tube was spread onto EMB agar and incubated at 37°C for 24 hours. The bacterial count was also recorded. The MIC is the lowest concentration of an antimicrobial agent that suppresses microbial growth (90%. The minimum bactericidal concentration (MBC) is the lowest antimicrobial concentration required to kill microorganisms. The mean MIC was calculated from triple readings for each test (Andrews, 2001).

The MICs of both LF and marbofloxacin were added to 100 µl of bacterial concentration 10^5 CFU/ml in a sterile tube to assess the synergistic effect of marbofloxacin and LF.

Treatment of infected patients

The 50 BM cases were allocated randomly in two groups (A) and (B), each consisting of 25 cases. Group (A) received marbofloxacin (marbocyl ®) (1 cm/50 kg bw) for 3 days, and LF (2 gm intramammary every 12 hours) for 3 days. Group (B) received marbofloxacin (marbocyl ®) 1 cm / 50-kg bw) for 3 days. The two

groups were injected with the nonsteroidall anti-inflammatory drug flunixin meglumine (finadyne®) at a dose of 1 cm/50 kg bw for three days.

Follow-up of cases to evaluate treatment outcome

The two groups were evaluated during the first week and two months after treatment to determine the efficacy of the treatment as follows:

Clinical examination: systemic clinical signs were monitored (Heart rate, rectal temperature, rumen motility, appetite, and general attitude). The udder was palpated for soreness, swelling, and hardness. The milk was inspected for clotting, color changes, and sourness at each milking. CMT was performed.

Bacteriological examination: *E. coli* enumeration and bacterial counts were recorded.

Statistical analysis

The obtained data were statistically analyzed to calculate the mean, SD, and *p* values using the independent sample *t* test and SPSS program according to (Snedecor and Cochran, 1980).

Ethical approval

This study was approved by the Institutional Ethics Committee and was conducted in accordance with local laws and regulations. The study was approved by the Medical Research Ethics Committee, Faculty of Medicine, Sohag University (OHRP #: IRB00013006) and was performed according to the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Testing, and Education by the New York Academy of Sciences, Ad Hoc Animal Research Committee.

Results

Of the 80 examined BM animals, 50 were positive for *E. coli* (62.5%). Thirty *East coli* strains were isolated from buffaloes and 20 *E. coli* strains were isolated from cattle. Six serogroups were identified: O55 and O103 (20% each), followed by O26 (18%) and O124 (12%) (Table 2).

Antibiotic sensitivity testing of 50 *E. coli* isolates revealed that all isolates were sensitive to marbofloxacin. The isolates showed resistance to gentamicin and ampicillin (26% each), cefquinome (14%), cefixime (10%), and SuLFamethoxazole and trimethoprim (4%) (Table 3).

Molecular identification by PCR confirmed that all 50 isolates of *E. coli* were positive for the *16S rRNA* gene. All beta-lactam-resistant isolates carried the *BlaTEM* gene. All suLFonamide-resistant isolates encode Sul1. All aminoglycoside-resistant isolates carried the *aadB* gene. The *qnrS* gene was not detected in any *E. coli* isolates (Fig. 1).

The broth dilution method revealed that the MIC of marbofloxacin was 6 µg/ml with a bacterial count of 4.16 log cfu/ml and MBC was 7 µg/ml, whereas the MIC of LF was 2 mg/ml with a bacterial count of 4.41 log cfu/ml and MBC of 3 mg/ml. No growth was detected when the marbofloxacin (6 µg/ml) was combined with LF (2 mg/ml) (Table 4).

Group (A), which received LF intramammary infusion with marbofloxacin, showed significant improvement in BM compared with group (B), which received marbofloxacin alone. Local soreness and edema in the udder improved within 3–5 days in group (A) and 5–7 days in group (B). Systemic signs improved within 18 to 24 hours in group (A), and within 24 to 36 hours in group (B). Milk properties returned to normal levels within 3–4 days in group (A), and 4–5 days in group (B). CMT returned to the normal level after 2–3 days in group (A), and 3–4 days in group (B) (Table 5).

There was a significant decrease in the *E. coli* count in group (A) compared with group (B). *The E. coli* count could not be detected after 4 days in group (A) and after 5 days in group (B) (Table 6). Re-evaluation

Table 2. Serotypes of *E. coli* strains isolated from BM animals.

Serogroup	Number (%)
O55	10 (20%)
O103	10 (20%)
O26	9 (18%)
O11	8 (16%)
O44	7 (14%)
O124	6 (12%)
Total	50

Table 3. Antimicrobial resistance profiles of *E. coli* isolated from BM animals.

Antibiotic	Sensitive	Intermediate	Resistant
Marbofloxacin (MAR 5)	35 (70%)	15 (30%)	--
Cefixime (CFM 5)	27 (54%)	18 (36%)	5 (10%)
Sulfamethoxazole and trimethoprim (SXT 25)	40 (80%)	8 (16%)	2 (4%)
Gentamicin (GM 10)	19 (38%)	18 (36%)	13(26%)
Ampicillin (AP 10)	25 (50%)	12 (24%)	13(26%)
Cefquinome (CEQ 30)	28 (56%)	15 (30%)	7(14%)

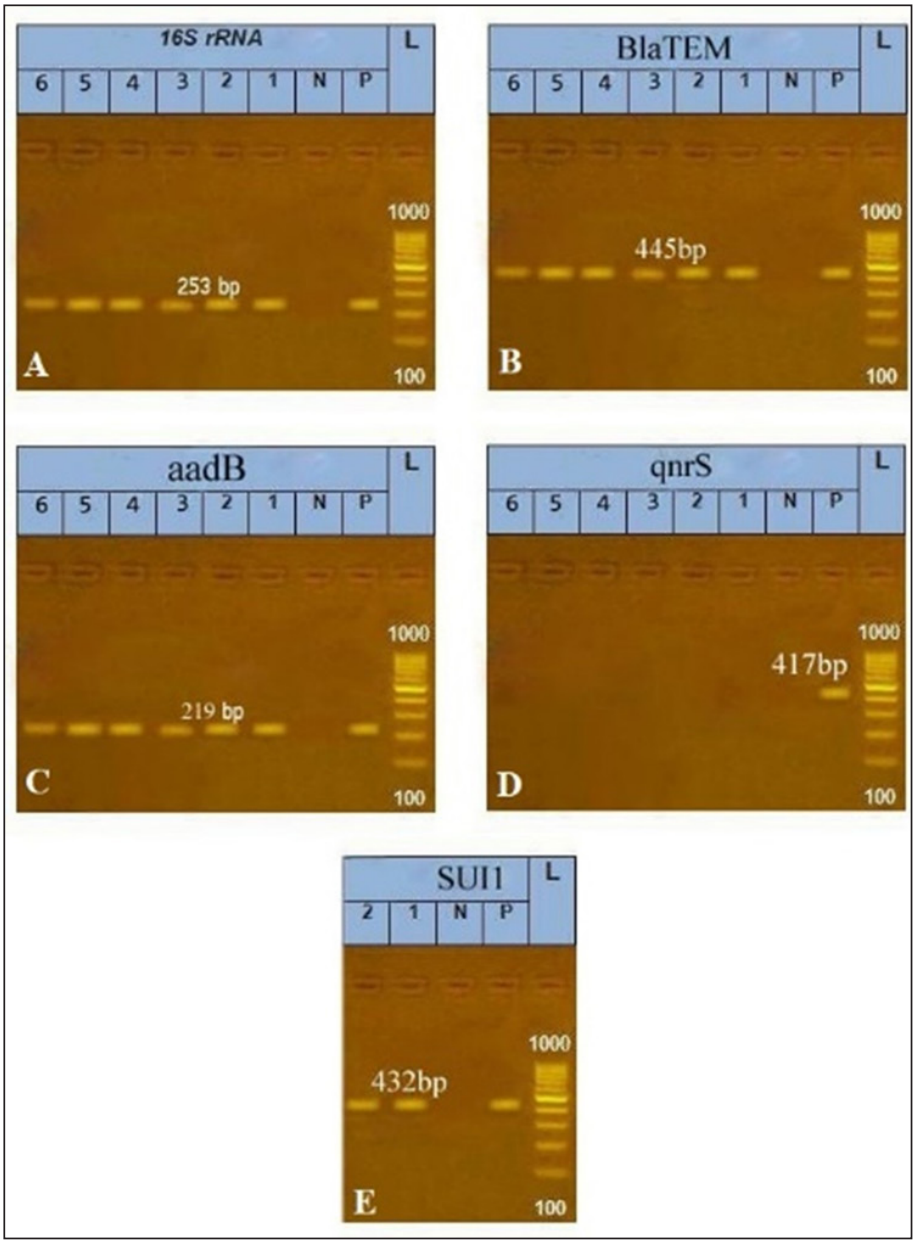

Fig. 1. Gel electrophoresis and PCR amplification for *E. coli*. Lane (L): DNA marker; lane (P): positive control; lane (N): negative control, (bp) base pair. (A): 1, 2, 3,4,5,6 lanes are positive for the *16S rRNA* gene (253 bp). (B): 1, 2, 3,4,5,6 lanes are positive for the *BlaTEM* gene (445 bp). (C): 1, 2, 3,4,5,6 lanes are positive for *aadB* gene (219 bp). (D): 1, 2, 3,4,5,6 lanes are negative for *qnrS* gene (417bp). (E): 1 and 2 lanes are positive for *Sul1* gene (432 bp).

of BM cases after 2 months revealed no recurrence in group (A), whereas recurrence occurred in three cases in group (B).

Discussion

BM is considered among the most common diseases in farm animals, resulting in a reduction in dairy milk consumption and high economic losses (Morales-Ubaldo *et al*., 2023). *Escherichia coli* is the major causative agent of clinical BM (Nery Garcia *et al*., 2024). *In the environment, E. coli can* invade the udders via the teat canal and cause intramammary infection (Burvenich *et al*., 2003).

In the present study, out of the 80 examined BM animals, there were 50 positive samples for *E. coli* (62.5%) lower results were obtained by Hameed *et al*. (2008), Ali *et al*. (2011), Bhanot *et al*. (2012), Lamey

Table 4. MIC of marbofloxacin and LF.

Positive control	Marbofloxacin conc.µg/ml		LF conc. mg/ml		Synergetic effects of 6 µg/ml marbofloxacin and 2 mg/ml LF.
	6 µg/ml	7 µg/ml	2 mg/ml	3 mg/ml	
6.23 log cfu/ml	4.16 log cfu/ml	ND	4.41 log cfu/ml	ND	ND

Table 5. Comparison of clinical outcomes between the treatment groups.

Clinical parameter		Group (A)	Group (B)	Significance (*p* value)
Improvement of local signs	Min.– max.	3–5 days	5–7 days	0.005
	Mean ± SD	3.8 ± 0.84*	5.8 ± 0.84	
Improvement of systemic signs	Min.– max.	18–24 hours	24–36 hours	0.003
	Mean ± SD	20.8 ± 2.3*	30 ± 4.5	
Improvement of milk properties	Min.– max.	3–4 days	4–5 days	0.05
	Mean ± SD	3.6 ± 0.5*	4.4 ± 0.5	
CMT	Min.– max.	2–3 days	3–4 days	0.02
	Mean ± SD	2.4 ± 0.55*	3.4 ± 0.55	

*Significant difference at *p* value ≤ 0.05 between groups for the same raw.

Table 6. Comparison of *E. coli* counts among the treatment groups.

Evaluation time	Parameter	Group (A)	Group (B)	Significance (*p* value)
Before treatment	Min. – max.	8.96–8.99	8.95–8.98	0.15
	Mean ± SD	8.98 ± 7.30	8.97 ± 7.30	
24 hours after treatment	Min. – max.	5.53–5.85	6.65–6.91	0.001
	Mean ± SD	5.71 ± 5.50**	6.80 ± 6.17	
48 hours after treatment	Min. – max.	2.27–2.96	3.53–3.89	0.001
	Mean ± SD	2.71 ± 2.50**	3.73 ± 3.27	
3 days after treatment	Min. – max.	1.36–1.77	2.38–2.78	0.001
	Mean ± SD	1.63 ± 1.18*	2.65 ± 2.17	
4 days after treatment	Min. –max.	-------	1.55–1.89	0.001
	Mean ± SD	ND**	1.75 ± 1.07	
5 days after treatment	Min. –max.	-------	-------	----
	Mean ± SD	ND	ND	

ND indicates that it was not detected.

*Significant difference at *p* value ≤ 0.05 between groups for the same raw.

**highly significant difference at *p* value ≤ 0.001between groups at the same raw.

et al. (2013), who observed *E. coli* at 15.38%, 16.18%, 16.3%, and 18.47%, respectively, in BM animals. The variations between the results of different reports may be due to the sample size, geographical distribution, management practice, and sanitary measures (Barua *et al*., 2014).

Buffaloes possess certain traits that can increase the risk of mastitis. Their udder is more pendulous than that of cattle (Moroni *et al*., 2006). In this study, 30 *E. coli* strains were isolated from buffaloes and 20 *E. coli* strains were isolated from cattle.

In the current study, six serogroups were identified: O55 and O103 (20% each), followed by O26 (18%), and the least was O124 (12%). These results are similar to those obtained by Mohamed *et al*. (2011) showed that the most prevalent *E. coli* serogroup from persistent BM cases were O55 (19.2%), O119 (12.3%), O114

(10.5%), O26 (7%), O111 (15.8%), O124 (12.3%), O157 (7%), and O44 (3.5%).

Antibiotics are considered the first-choice treatment for BM (El-Sayed and Kamel, 2021). The antibiotic sensitivity test is widely used in clinical investigations to determine the most effective antimicrobial drug for therapy (Singh *et al*., 2018). The antibiotic sensitivity testing of 50 *E. coli* isolates revealed that all isolates were sensitive to marbofloxacin. The isolates showed resistance to gentamicin and ampicillin (26% each), cefquinome (14%), cefixime (10%), and sulfamethoxazole and trimethoprim (4%). This finding agrees with Rahimiyan *et al*. (2021) w, who recommended the use of marbofloxacin as an effective drug in the treatment of *East coli* bone marrow infections. Fairbrother *et al*. (2015) and Martínez-Vázquez *et al*. (2018) concluded that 20% of the isolates were resistant to more than two groups of antibiotics and 20%–33% of *E. coli* isolates from BM cases were resistant to at least one drug. Yu *et al*. (2020) reported that all the isolated *E. coli* strains expressed multi-resistance to antimicrobial agents.

The abuse of antibiotics leads to antibiotic resistance in pathogenic strains. These resistances are coded by specific genes that may be located on the bacterial chromosome, plasmids, transposons, or gene cassettes that bind to integrons; thus, they are easily transmitted through isolates (Daka *et al*., 2012).

In this study, the molecular identification of antibiotic-resistance genes of *E. coli* strains revealed that all beta-lactam-resistant isolates possessed the *BlaTEM* gene. All sulfonamide-resistant isolates encode Sul1. All aminoglycoside-resistant isolates carried the *aadB* gene. The *qnrS* gene was not detected in any *E. coli* isolates. Similarly, Pishtiwan and Khadija (2019) detected *blaTEM* in 81% of *E. coli* isolates. Arabi *et al*. (2015) also detected Sul1 in 81% of *E. coli* isolates. In contrast, Yu *et al*. (2008) detected *the qnrS* gene in 14 (6%) *E. coli* isolates.

Previous *in vitro* mastitis studies revealed that LF had an antibacterial effect against some principal udder pathogens, especially *E. coli* (Kutila *et al*., 2003), and it can prevent sequelae related to the use of antimicrobial drugs (Jenssen and Hancock, 2009; Yen *et al*., 2011).

In this study, we detected the antibacterial activity of LF as a cotreatment with marbofloxacin using the broth dilution method, which revealed that the MIC of marbofloxacin was 6 µg/ml with a bacterial count of 4.16 log cfu/ml and MBC was 7 µg/ml, while the MIC of LF was 2 mg/ml with a bacterial count of 4.41 log cfu/ml and MBC was 3 mg/ml. No growth was detected when the marbofloxacin (6 µg/ml) was combined with LF (2 mg/ml). This finding agrees with that reported by Moravian *et al*. (2014), who found that all concentrations of bovine LF significantly inhibited Gram-negative bacteria such as *Salmonella, P. aeruginosa,* and *E. coli* growth. Diarra *et al*. (2002b) reported that combining LF with antimicrobial agents yielded better outcomes than combining LF with antimicrobials or LF alone.Kell *et al*. (2020) reported that LF can be used as an intramammary infusion to protect the udder with exogenous LF against new infections during the involution stage when the animal's own LF level is still low. LF has bacteriostatic or bactericidal effects and induces innate immunity in the host. Bukhari *et al*. (2016) advocated that LF in combination with antibiotics can improve udder defense mechanisms and inhibit bacterial proliferation.

In the present study, we assessed the synergistic effect of LF as a cotreatment agent with marbofloxacin in the treatment of BM animals. BM cases were divided randomly into two groups. Group (A) received 2 g of LF every 12 hours for 3 days, in combination with marbofloxacin (1 cm/50 kg bw) for 3 days. Group (B) was treated with marbofloxacin 1 cm / 50 kg bw) for 3 days. The two groups were injected with flunixin meglumine (finadyne®) at a dose of 1 cm/50 kg.

In this study, statistical analysis revealed a significant difference in posttreatment clinical parameters in group (A) compared to group (B). Group (A) showed rapid improvement in both local and systemic signs of BM. The time needed for the milk properties to return to normal levels in group (A) was less than that in group (B). CMT regained normal levels in group (A) earlier than in group (B). There was also a significant decrease in the *E. coli* count in group (A) compared with group (B). *The E. coli* count could not be detected after 4 days in group (A) and after 5 days in group (B). These results agreed with Kutila (2004) who used LF in treated BM cases and reported that systemic manifestations improved within 2–3 days, and local manifestations within 1 week. Bacteria were eliminated from enrofloxacin-treated animals within an average of 3.8 days and from LF-treated animals within 5.8 days. These results were in accordance with Petitclerc *et al*. (2007), who studied the effect of LF in combination with antibiotics in mastitis treatment, and Lacasse *et al*. (2008), who concluded that the combination of LF and antibiotics increases the cure rate as LF binds to the bacterial membrane through LPS, facilitating antibiotic penetration, and destroys the bacterial cell. Diarra *et al*. (2002a) demonstrated a synergistic effect of LF combined with antibiotics for BM treatment. Ongena *et al*. (2024) suggested that LF binding to lipid A in gram-negative bacteria causes discontinuity of the outer membrane and releases LPS, resulting in altered cell membrane permeability. Receptors for the N-terminal region of LF were noticed on the surface of some microorganisms. The binding of LF to these receptors causes cell death in Gram-negative bacteria due to disruption in its cell wall.

In this study, 2 g of LF was infused into the infected udder to increase LF levels approximately to those present in mastitic milk; these levels are within the effective range. Low levels do not play therapeutic roles. High levels can produce moderate-to-severe

inflammatory reactions in udder quarters and may even disturb or damage the normal function of the epithelial cells of the udder (Kell *et al*., 2020).

Intramammary LF infusion might cause local reactions in the udder quarters. This could be due to a foreign body reaction of the udder to the exogenous protein. This can be attributed to the contamination of LF with endotoxins, which induce an inflammatory reaction. The dose of LF (2 gm) used in this study contained insignificant amounts of endotoxins, which could cause mild local reactions (Izquierdo-Vega *et al*., 2023). Therefore, we decided to use LF at 12-h intervals for 3 days to prolong the period of therapeutic LF levels in milk so that LF would better eliminate bacteria and LPS.

The future perspectives of LF in treating BM are promising, with potential uses ranging from antimicrobial anti-inflammatory therapies to immune modulation and prevention. Future studies are needed to better understand the optimal use, dosing, and delivery mechanisms of LF in BM management. With antibiotic resistance becoming an increasing concern, LF-based drugs could play an important role in providing sustainable, effective alternatives for dairy animals.

Conclusion

BM is one of the most prevalent diseases in farm animals, leading to reduced dairy milk production and significant economic losses. *E. coli* is one of the primary causative agents of BM. Incorporating LF as a cotreatment with antibiotics, such as marbofloxacin, could limit the growth of *E. coli* bacteria better than marbofloxacin alone, thus enhancing cure rates.

Conflicts of interest

None.

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Authors' contributions

All authors made substantial contributions to all the following: substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data, acknowledgment of drafting the article or revising it critically for important intellectual content ; final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Mohamed Ibrahim Mostafa: Study sample collection, clinical examination of animals, collection of milk samples, isolation, and identification of *E. coli,* antibiotic sensitivity testing of *E. coli,* molecular detection of antibiotic-resistance genes, detection of MIC of marbofloxacin and LF by broth dilution method, treatment of the infected cases, follow-up of the cases for evaluation of the treatment outcome, and statistical analysis. Saber Ali Saad: Study sample collection, clinical examination of animals, collection of milk samples, treatment of infected cases, follow-up of the cases for evaluation of treatment outcome, and statistical analysis. Asmaa Elsayed Mohammed: Study sample collection, clinical examination of animals, collection of milk samples, isolation, and identification of *E. coli,* antibiotic sensitivity testing of *E. coli,* molecular detection of antibiotic-resistance genes, detection of MIC of marbofloxacin and LF, treatment of the infected cases, follow-up of the cases for evaluation of the outcome of treatment, statistical analysis, and journal correspondence. Elsaid Mohamed Mohamed Saafan: Study sample collection, clinical examination of animals, collection of milk samples, treatment of the infected cases, follow-up of the cases for evaluation of the treatment outcome, statistical analysis, and statistical analysis. Amal Mohamed El-Sayed: Study sample collection, clinical examination of animals, collection of milk samples, treatment of infected cases, follow-up of the cases for evaluation of treatment outcome, and statistical analysis.

Data availability

Data are available upon request.

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