Journal of Apiculture

2025.40(2) : 147-153
DOI: 10.17519/apiculture.2025.06.40.2.147

Original research article

Antibacterial Activity of Honey Bee Venom against Multidrug-resistant *S. aureus* and *E. coli* Isolated from Bovine Mastitis

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Abstract Bee venom (BV) consists of various bio-actives, including proteins, peptides, enzymes, and volatile metabolites. Over the last decade, the antimicrobial effect of BV against bacterial pathogens has been demonstrated through various *in vitro* and *in vivo* experiments. Because mastitis has a serious impact on the dairy industry, the present study aimed to evaluate the antibacterial activity of BV against multidrug-resistant *S. aureus* and *E. coli*, which are difficult to treat using antibiotics. In this study, 25 *E. coli* and 15 *S. aureus* strains from bovine mastitis were used for BV MIC testing. Based on the antibiotic resistance pattern, all isolates were categorized into three groups: susceptible, resistant, and multidrug-resistant. BV MICs for the *E. coli* and *S. aureus* isolates ranged from 16–128 μg/mL and 16–32 μg/mL, respectively. There were no significant differences between the antibiotic resistance and BV MICs of the isolates. However, two multidrug resistance (AMP-CEF-OXA-PEN) *S. aureus* isolates showed 2-fold higher BV MIC values than the other *S. aureus* isolates. Therefore, BV can be used as an adjunct treatment for mastitis caused by antibiotic-resistant bacteria. Further studies are needed to understand the resistance mechanisms of multidrug-resistant bacteria, and *in vivo* studies are required to evaluate the efficacy and safety of BV.

Keywords Bee venom, Mastitis, Multidrug-resistant bacteria

INTRODUCTION

Bovine mastitis (BM) is one of the most economically significant diseases that decreases milk production in dairy cattle. BM is an inflammatory disease of the mammary glands associated with the infection by several bacterial pathogens (Gomes *et al*., 2016). Many studies have analyzed to identify the primary cause and the major microbial pathogens causing mastitis (Ma *et al*., 2020; Sokolov *et al*., 2021). It was reported that mastitis is usually caused by *Escherichia coli*, *Streptococcus* spp., and *Staphylococcus aureus* (Dalanezi *et al*., 2020).

E. coli is the most common Gram-negative bacterium among the pathogens that cause acute mastitis during early lactation. *E. coli*-related mastitis, the common symptoms of the udder include redness, swelling, tenderness, and an abnormal texture of milk (Burvenich *et al*., 2003; Zhang *et al*., 2018).

S. aureus, one of the primary mastitis pathogens worldwide, causes chronic intramammary infections that respond poorly to antibiotics, disseminate within the herd, and lead to high economic losses. Classic mastitis control programs are based on hygiene and antibiotic therapy (Günther *et al*., 2017; Cheng and Han, 2020;

Received 31 May 2025; Revised 23 June 2025; Accepted 23 June 2025
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Duse *et al*., 2021).

Antibiotics are the most commonly used control measures owing to their remarkable effectiveness (Duse *et al*., 2021). Several antibiotics, including penicillin, ampicillin, tetracycline, and gentamicin, have been used to treat mastitis, and antibiotic treatment largely relies on the primary pathogen/s of mastitis (Gomes and Henriques, 2016). However, the misuse of antibiotics has led to the development of multidrug-resistant bacteria. In addition, the use of antibiotics in the treatment of mastitis directly affects consumers through the presence of antibiotic residues in milk (Gomes and Henriques, 2016; Cheng and Han, 2020). Antimicrobial drug resistance in pathogenic bacteria has become a present danger for many countries (WHO), and only a few alternatives are currently available (Cheng and Han, 2020). Therefore, the search for new candidates with novel modes of action is required.

Natural products, including plant- and animal-based products, are rich in bioactive compounds that exhibit diverse activities against various diseases (Cheng and Han, 2020). Among them, bee venom (BV) is a venomous cocktail of proteins, peptides, enzymes, and volatile metabolites secreted by the poison glands of honeybee as a protective mechanism. BV also contains many biochemically and pharmacologically active substances. Melittin is a compound that represents 40–60% of the dry BV weight (Abd El-Wahed *et al*., 2019). Owing to its constituents, BV has been proven to be an effective anti-inflammatory and antibacterial agent against several gram-positive (*Staphylococcus aureus* and *Streptococcus*) and gram-negative (*Escherichia coli*, *Klebsiella* and *Pseudomonas*) bacterial strains (Wehbe *et al*., 2019; Srichok *et al*., 2022). AL-Ani *et al*. (2015) reported that BV and its constituents, together with chemotherapy agents (vancomycin, oxacillin, and amikacin), have a synergistic effect against multidrug resistance bacteria.

Several studies have demonstrated the effectiveness of BV against mastitis pathogens, including *E. coli* and *S. aureus* (Han *et al*., 2007; Park *et al*., 2013). However, these studies have primarily focused on evaluating the antibacterial activity of BV. Therefore, this study aimed to compare the antibacterial activity of BV against antibiotic-susceptible, antibiotic-resistant, and multidrug-resistant *E. coli* and *S. aureus* isolated from cows with mastitis in Korea. In this study, we confirmed the applicability of bee venom to mastitis caused by multidrug-resistant bacteria for which antibiotics are difficult to use.

MATERIALS AND METHODS

1. Collection and preparation of bee venom (BV)

BV was supplied by the Department of Agricultural Biology of the National Institute of Agricultural Science, Rural Development Administration, Korea. BV was collected from honeybees, *Apis mellifera*, using electric BV-collection devices (Chungjin Biotech, Ansan, Korea) and purified under sterile laboratory conditions. Briefly, the devices were placed on the hive, and the honeybees were given weak electric stimulation to cause them to sting a glass plate. After drying the BV for sufficient time, it was collected by scraping. The collected BV was washed in sterile water and centrifuged at 10,000 × g at 4°C for 5 min to remove the residues. BV was lyophilized by freeze-drying and refrigerated at 4°C for later use. Purified BV was diluted in sterile distilled water and aseptically filtered through a 0.45 μm syringe filter (Advantec, Tokyo, Japan). BV was prepared in two-fold concentration series ranging from 0.5–512 μg/mL for the antibacterial experiment.

2. Bacteria and culture conditions

E. coli ($n=25$) and *S. aureus* ($n=15$) were isolated from milk samples of cows diagnosed with mastitis at dairy farms in Jeollabuk-do and Chungcheongbuk-do from 2019–2020. The strains were cultured on Blood agar (Kisan Biotech, Seoul, Korea) medium at 37°C, aerobic conditions for 24 h.

3. Minimum inhibitory concentration (MIC)

A broth microdilution assay was performed to evaluate the antibacterial effects of BV against the isolates. Two-dilution series of BV was prepared by adding 90 μL of Muller-Hinton (MH) broth and 10 μL of BV samples and then sequentially adding it to a 96-well plate to obtain final concentrations of 0, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 μg/mL. Each bacterial strain was cultured in MH broth, diluted, and adjusted to 0.5 McFarland standard by Sensititre reader (Thermo Fisher Scientific, US). Each 100 μL of bacterial suspension

was added to a 96-well plate containing the two-diluted BV and incubated at 37°C for 18 h. Bacterial growth was visually confirmed and the concentration at which proliferation did not occur was expressed as the minimum growth-inhibitory concentration. If there was a difference in the minimum growth inhibition concentration among strains of the same species, the minimum (Min) and maximum (Max) values were confirmed.

4. Antimicrobial susceptibility

MICs of *E. coli* were determined by the standard micro broth dilution method using the Sensititre system (TREK Diagnostic System, East Grinstead, UK) with antimicrobial testing plates containing the following 16 antimicrobials: Amoxicillin/Clavulanic Acid (AMC), Ampicillin (AMP), Cefepime (CEF), Cefoxitin (FOX), Ceftazidime (CAZ), Ceftiofur (CTF), Chloramphenicol (CHL), Ciprofloxacin (CIP), Colistin (CL), Gentamicin (GEN), Meropenem (MER), Nalidixic Acid (NAL), Streptomycin (STR), Sulfisoxazole (SXZ), Tetracycline (TET), and Trimethoprim/Sulphamethoxazole (TMP-SXT). Similarly, the MICs of *S. aureus* were determined using antimicrobial testing plates containing the following 10 antimicrobials: ampicillin (AMP), ceftiofur (CTF), cephalothin (CEP), erythromycin (EM), oxacillin (OXA), penicillin (PCN), penicillin/novobiocin (NB), pirlimycin (PIR), sulfadimethoxine (SDM), and tetracycline (TET). Resistance profiles (resistant, intermediate, or susceptible) were assigned according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2015).

RESULTS AND DISCUSSION

1. Antibiotic resistance and of isolates

All *E. coli* isolates were susceptible to ampicillin, cefoxitin, ceftazidime, ceftiofur, ciprofloxacin, meropenem, sulfisoxazole, whereas 40% and 36% of isolates were resistant to tetracycline and streptomycin, respectively. Moreover, below 12% of isolates were resistant to amoxicillin/clavulanic Acid, cefepime, chloramphenicol, colistin, gentamicin, nalidixic acid, and trimethoprim/sulfamethoxazole. All *S. aureus* isolates were susceptible to cephalothin, erythromycin, penicillin/novobiocin, pirlimycin, and tetracycline, whereas 47% and 33% of isolates were resistant to the penicillin and ampicillin, respectively. Additionally, 20% of the isolates were resistant to the ceftiofur and oxacillin, and sulfadimethoxine resistance was shown by 15% of *S. aureus* isolates (Fig. 1).

This study used 25 *E. coli* and 15 isolates of *S. aureus* obtained from dairy cows with mastitis (Jung *et al*., 2021). Both these pathogens are the most common causative agents of mastitis worldwide (Gilbert *et al*., 2013; Gomes *et al*., 2016; Rana *et al*., 2022). These two bacteria also show a high percentage of multi-resistance. Therefore, effective control measures against these pathogens are important.

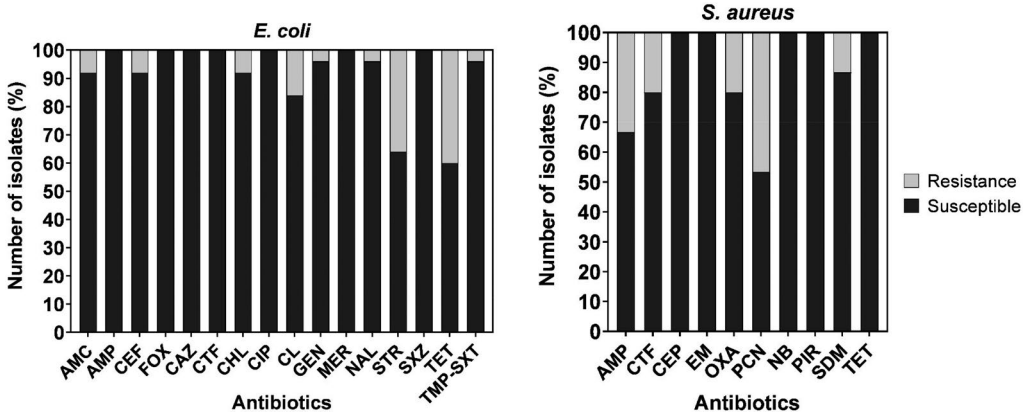

Fig. 1. Antibiotic susceptibility of the *E. coli* ($n=25$) and *S. aureus* ($n=15$) isolates. AMC, Amoxicillin/ Clavulanic Acid; AMP, Ampicillin; CEF, Cefepim; FOX, Cefoxitin; CAZ, Ceftazidime; CTF, Ceftiofur; CHL, Chloramphenicol; CIP, Ciprofloxacin; CL, Colistin; GEN, Gentamicin; MER, Meropenem; NAL, Nalidixic Acid; STR, Streptomycin; SXZ, Sulfisoxazole; TET, Tetracycline; TMP-SXT, Trimethoprim/ Sulphamethoxazole; CEP, Cephalothin; EM, Erythromycin; OXA, Oxacillin; PCN, Penicillin; NB, Penicillin/ Novobiocin; PIR, Pirlimycin; SDM, Sulphadimethoxime, and TET, Tetracycline.

2. Antibiotic resistance patterns of isolates

Based on the pattern of antibiotic resistance, all isolates were categorized into three groups: susceptible, resistant, and multidrug-resistant. Thirteen of the 25 *E. coli* isolates showed susceptibility to all tested antibiotics. The STR-TET resistance pattern was observed in five *E. coli* isolates, whereas the remaining resistance patterns were observed in each isolate. Five multidrug resistance patterns (CAZ-GEN-NAL/CAZ-STR-TET/ AMP-STR-TET/ CHL-STR-TET-TMP/SMX/ AMP-CHL-STR-TET) were observed for each isolate (Table 1). Seven of the 15 *S. aureus* isolates were susceptible to all the tested antibiotics. Among the five resistance patterns, three isolates exhibited the AMP-CEF-OXA-PEN multidrug-resistance pattern (Table 2).

The present study selected different sets of antibiotics to evaluate the antibiotic susceptibility of *E. coli* and *S. aureus* depending on the clinical use and CLSI guidelines. *E. coli* and *S. aureus* isolates showed different antibiotic resistance patterns. Most of the tested antibiotics were effective against most *E. coli* and *S. aureus* isolates. Over 30% of each *E. coli* and *S. aureus* isolates were resistant to the two antibiotics. In contrast, *E. coli* was resistant to tetracycline and streptomycin, whereas *S. aureus* was resistant to ampicillin and penicillin. Previous studies have reported different antibiotic resistance patterns in *E. coli* and *S. aureus* isolates (Oliver and Murinda, 2012; Yu *et al*., 2020; Mbindyo *et al*., 2021). Common antibiotics used for the treatment of mastitis include

Table 1. Antibiotic resistance patterns and the BV MICs of *E. coli* isolates

Resistance category	Antibiotic-resistant pattern	Number of isolates @ BV MIC (μg/mL)				Total
		16	32	64	128	
Susceptible	Susceptible	0	1	6	6	13
Resistance	CAZ	1	0	0	0	7
	CAZ-TET	0	0	0	1	
	STR-TET	0	0	5	0	
Multi-drug resistance	CAZ-GEN-NAL	0	1	0	0	5
	CAZ-STR-TET	0	0	1	0	
	AMP-STR-TET	0	0	0	1	
	CHL-STR-TET-TMP/SMX	0	0	0	1	
	AMP-CHL-STR-TET	1	0	0	0	

AMP, ampicillin; CHL, chloramphenicol; GEN, gentamicin; NAL, nalidixic acid; STR, streptomycin; TET, tetracycline; TMP-SXT, trimethoprim/ sulfamethoxazole; CAZ, ceftazidime

Table 2. Antibiotic resistance patterns and the BV MICs of *S. aureus* isolates

Resistance category	Antibiotic-resistant pattern	Number of isolates @ BV MIC (μg/mL)		Total
		16	32	
Susceptible	Susceptible	7	0	7
Resistance	SDM	1	0	5
	PEN	2	0	
	AMP-PEN	1	0	
	AMP-PEN-SDM	1	0	
Multi-drug resistance***	AMP-CEF-OXA-PEN	1	2	3

AMP, ampicillin; PEN, penicillin; CEF, cefepime SDM, sulfadimethoxime OXA, oxacillin. ***; $p \leq 0.001$, statistical differences referred to Multidrug resistance group to resistance and susceptible groups by ANOVA with Post Hoc Tukey HSD.

streptomycin, ampicillin, cloxacillin, penicillin, and tetracycline (Bhosale *et al*., 2014). However, different antibiotics are used for treatment depending on the primary cause of mastitis. In the present study, tetracycline, streptomycin, ampicillin, and penicillin selective pressures due to their overuse in the cattle industry might be the reason for the dissemination of resistant isolates among cows with mastitis.

Furthermore, three *S. aureus* isolates were resistant to penicillin, amoxicillin, oxacillin, and cefepime, which are potentially risky strains. Similarly, Park *et al*. (2012) reported that mastitis origin *Staphycocci* strains showed significantly less resistance to β-lactam antibiotics. Therefore, alternative treatments are required to control these multidrug-resistant strains.

3. Minimum Inhibitory Concentrations (MICs) of bee venom (BV) against *E. coli* and *S. aureus* isolates

The MIC values of bee venom (BV) against *E. coli* and *S. aureus* isolates showed different trends (Fig. 2). The mean MIC values were 81.3 ± 38.1 μg/mL for *E. coli* ($n=25$) and 18.1 ± 5.6 μg/mL for *S. aureus* ($n=15$). The MIC values ranged from 16–128 μg/mL for *E. coli* and 16–32 μg/mL for *S. aureus*, respectively. There was no significant relationship between BV MICs of *E. coli* isolates and antibiotic resistance patterns. However, BV MICs of the multidrug resistance isolates were shown to have significantly elevated compared to that in the resistant and susceptible groups.

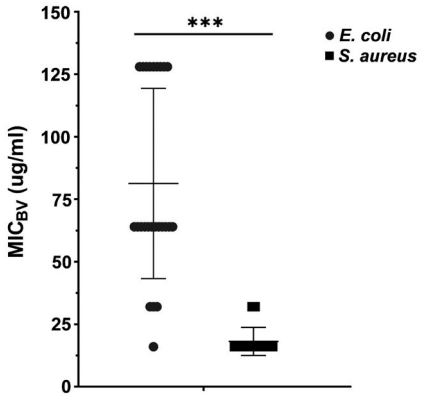

Fig. 2. Minimum inhibitory concentrations (MICs) of Bee venom (BV) against *E. coli* ($n=25$) and *S. aureus* ($n=15$) isolates. *p≤0.05; **p≤0.01; *** p≤0.001, statistical differences referred to BV MICs of *E. coli* and *S. aureus* isolates by t-test. Vertical bars show SD.

During the last decade, the antimicrobial action of BV against bacterial pathogens has been demonstrated through various *in vitro* and *in vivo* experiments. In the present study, there was a significant difference in the MIC values of BV between *E. coli* and *S. aureus* isolates. As opposed to the present study, Han *et al*. (2007) reported more or less similar BV MICs against mastitis origin *E. coli* (17.4 μg/mL) and *S. aureus* (21.4 μg/mL) isolates. Furthermore, another study reported comparatively high BV MICs against *E. coli* (62.5–250 μg/mL) and *S. aureus* (62.5–125 μg/mL) isolated from cattle with mastitis in Korea. Similar to the present study, several previous studies have reported a comparatively higher efficacy of BV against Gram-positive bacteria than against gram-negative bacteria, and major component of BV, mellittin, is more effective against Gram-positive than against Gram-negative bacteria (Čujová *et al*., 2014). Diverse mechanisms may be involved in disrupting the membrane structure, which has a lethal impact on bacterial pathogens.

In this study, isolates were categorized into three groups (susceptible, resistant, and multidrug resistance) depending on their antibiotic susceptibility results, and the MICs of BV against each isolate were compared with the antibiotic resistance patterns. There were no significant differences in the antibiotic resistance patterns and BV MICs of *E. coli* isolates. However, the two multidrug resistance *S. aureus* isolates showed significantly higher BV MIC values than the susceptible and resistant groups. Furthermore, both resistant and multidrug resistant isolates showed AMP and PEN resistance, and only CEF and OXA resistance were the excesses of the multidrug nature of the three *S. aureus* isolates. Common resistance mechanisms might be associated with the use of antibiotics (CEF and OXA) and BV. However, further studies with a higher number of CEF and OXA resistance isolates are needed to confirm this hypothesis.

Mastitis is a major challenge faced by dairy herds worldwide. Many bacterial species can cause infections in the udder, but most infections are caused by specific pathogens, including *S. aureus*, *E. coli*, *E. faecalis*, and *Streptococcus* spp. (Gomes *et al*., 2016).

In conclusion, the present *E. coli* and *S. aureus* isolates originated from cows with mastitis, and some isolates showed multidrug resistance. Therefore, there is a

potential risk of disseminating antibiotic-resistant strains among herds. There were no significant differences between the antibiotic resistance and BV MICs of the isolates. Thus, BV could be used as a substitute for antibiotics against antibiotic-resistant *E. coli*. However, two multidrug resistance (AMP-CEF-OXA-PEN) *S. aureus* isolates showed 2-fold high BV MIC values, indicating that common antibacterial mechanisms drive resistance against antibiotics and BV. Further studies are needed to gain deeper insight into these mechanisms. Furthermore, BV can be used effectively to control mastitis causing pathogens, including multidrug-resistant bacteria; however, *in vivo* studies are required to evaluate its efficacy and safety.

DISCLOSURE STATEMENT

The authors reported no potential conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by 2022 RDA (Rural Development Administration) fellowship program of NIAS (National Institute of Animal Science) and by the "Cooperative Research Program for Agriculture Science & Technology Development (Project title: Study of immunological responses by the application of bee venom and vaccines, Project No. PJ0142982021)" of RDA, Korea.

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