Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
31 (2): 237-245, 2025
DOI: 10.9775/kvfd.2024.33429

Research Article

Efficacy of Pyocyanin Isolated from *Pseudomonas aeruginosa* and *Lactobacillus plantarum* Against Methicillin Resistant *Staphylococcus* aureus Caused Bovine Mastitis

Demet CELEBİ [1,2 (*)] Ozgur CELEBİ [3] Sumeyye BASER [3] Betul ARI [4] Sila EKTAS KALAYCI [5] Adem KARA [5]

[1] Ataturk University, Faculty of Veterinary Medicine, Department of Microbiology, TR-25240 Erzurum - TÜRKİYE
[2] Ataturk University, Vaccine Application and Development Center, TR-25240 Erzurum - TÜRKİYE
[3] Ataturk University, Faculty of Medicine, Department of Medical Microbiology, TR-25240 Erzurum - TÜRKİYE
[4] Ataturk University, Faculty of Science, Department of Molecular Biology and Genetics, TR-25240 Erzurum - TÜRKİYE
[5] Erzurum Technical University, Faculty of Science, Department of Molecular Biology and Genetics, TR-25240 Erzurum - TÜRKİYE

(*) **Corresponding author:**
Demet ÇELEBİ
Phone: +90 537 666 6938
Cellular phone: +90 537 666 6938
Fax: +90 442 231 7244
E-mail: celebiidil@atauni.edu.tr

How to cite this article?

Celebi D, Celebi O, Baser S, Ari B, Ektas Kalayci S, Kara A: Efficacy of pyocyanin isolated from *Pseudomonas aeruginosa* and *Lactobacillus plantarum* against methicillin resistant *Staphylococcus* aureus caused bovine mastitis. *Kafkas Univ Vet Fak Derg*, 31 (2): 237-245, 2025.
DOI: 10.9775/kvfd.2024.33429

Article ID: KVFD-2024-33429
Received: 27.11.2024
Accepted: 25.02.2025
Published Online: 18.03.2025

Abstract

Staphylococcus aureus is an important pathogen causing mastitis in dairy cows and methicillin-resistant *Staphylococcus aureus* (MRSA) is found on farms globally. The risk of foodborne zoonotic infection from bovine MRSA poses significant challenges to veterinary medicine. These challenges are compounded by limited treatment options, particularly non-beta-lactam antibiotics such as tetracyclines, fluoroquinolones, etc. Innovative treatment strategies aiming to replace antibiotics in mastitis management are being investigated. The activity of pyocyanin and *Lactobacillus plantarum* was evaluated by Kirby-Bauer disc diffusion method, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), antibiofilm activity and fractional inhibitory concentration method. Cytotoxic effects were also analyzed by infecting a cell line with MRSA. The results indicated that pyocyanin produced a 15 mm inhibition zone, while *Lactobacillus plantarum* yielded a 10 mm zone; their combination resulted in an 18 mm zone, suggesting a synergistic effect. The MIC values were determined to be 64 µg/mL for pyocyanin and 128 µg/mL for *Lactobacillus plantarum,* both requiring an MBC of 512 µg/mL. Furthermore, pyocyanin exhibited a 58.6% antibiofilm effect, whereas *Lactobacillus plantarum* demonstrated a 50.3% effect. Gene expression analysis revealed that *Lactobacillus plantarum* was more effective in reducing MRSA DNA levels, underscoring its potential for use in combination therapy against MRSA infections.

Keywords: *Lactobacillus plantarum*, Mastitis, Pyocyanin, *Pseudomonas aeruginosa*

Introduction

Bovine mastitis is an inflammation of the mammary gland in dairy cattle. It mainly results in infections from the bacteria *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus* spp. [1]. These bacteria causing bovine mastitis primarily enter the mammary duct and reach the mammary gland. The invasion of these bacteria is facilitated by weakened defence mechanisms of the udder, inadequate hygiene conditions and inappropriate environmental factors (e.g. damp and dirty housing environment, inappropriate sanitation practices). This leads to rapid multiplication of bacteria and initiation of inflammatory processes, resulting in increased severity of mastitis. In addition, incorrect or incomplete veterinary interventions can also complicate the treatment of bacterial infection, leading to additional complications [2]. Mastitis is classified into clinical and subclinical forms. Clinical mastitis presents with visible symptoms such as swelling, redness and heat. The milk may appear abnormal, it may contain clots, flakes, or blood. The affected cows show systemic symptoms such as fever, appetite loss and drowsiness. Subclinical mastitis may not show significant symptoms, but is detected by high somatic cell count in milk [3]. Livestock-derived Methicillin Resistance *S. aureus* (MRSA) has become increasingly important in cases of bovine mastitis in recent years. MRSA, clonal complex 398 (CC398), is considered to be the main cause of bovine

mastitis. It is associated with farm animals and has been found in various dairy cows around the world, indicating that it is widespread in the dairy industry [4]. The presence of MRSA in dairy cows with mastitis leads to a decrease in the quality and quantity of milk. The infected cows are experiencing significant productivity losses, creating economic difficulties for dairy farmers. Livestock-derived MRSA can be transmitted between cows and humans, raising public health concerns. Bacteria can contaminate milk and dairy products and cause potential infections in people who consume these products or in contact with infected animals [5]. Milking hygiene is of great importance to prevent MRSA transmission in dairy cattle. One of the most common mistakes is not cleaning and disinfecting the milking equipment regularly. Milk residues left in the milking machines provide a favourable breeding environment for MRSA, facilitating animal-to-animal transmission. In addition, dirty or inadequately disinfected milking cloths and hands cause the bacteria to be carried directly to the teat. In addition, inappropriate use of bedding also increases the risk of infection. Bedding materials that are damp and contaminated with urine or faeces are a breeding ground for prolonged survival and spread of MRSA. Failure to change bedding regularly and ensure proper disinfection increases the risk of transmission from infected animals to healthy individuals. In addition, failure to detect and isolate infected cows early accelerates the spread of MRSA within the herd. Therefore, within the scope of effective hygiene practices, it is of great importance to clean teats with antiseptic solutions before and after milking, clean milking equipment with appropriate disinfectants after each use, change bedding materials regularly and keep infected animals separate. Maintaining cleanliness and appropriate maintenance techniques are essential to control cases of mastitis and prevent the spread of MRSA among dairy cattle [4]. MRSA strains are resistant to widely used antibiotics, which complicates treatment options for mastitis in cattle. This resistance leads to prolonged infections and increased costs of veterinary care, and further affects the dairy industry. Parallel to antibiotic resistance, MRSA is a critical factor in the pathogenicity and continuity of infections [5]. The ica genes (icaA, icaB, IcaC, and icaD) are part of the intercellular adhesion (ica) locus responsible for the synthesis of polysaccharide adhesive (PIA), which is necessary for biofilm formation in *S. aureus*. Biofilms provide a protective environment for bacteria, making them more resistant to antibiotics and host immune response [6]. Studies have shown a high prevalence of icaABCD genes in MRSA isolates. For example, one study that all MRSA strains tested were positive for these genes, which suggests biofilm formation is a common feature among these resistant strains. This suggests that MRSA's biofilming ability contributes significantly to virulence and persistence in clinical settings [7]. Relationships also extend to other genes that play a role in adhesion and biofilm development, such as clfA (adhesion factor A) and clfB (adhesion factor B), which are surface proteins that facilitate adherence to host tissues. The presence of these genes in conjunction with the root genes complicates treatment options by increasing the ability of MRSA to create infection and biofilm [8,9]. That's why researchers have turned to searching for alternative treatments depending on the growing antibiotic resistance. Pyocyanin is a blue-green pigment produced by the gram-negative bacterium *Pseudomonas aeruginosa*. It is a toxic secondary metabolite with a defined chemical structure, which exhibits antibiotic activity against Gram-positive bacteria in particular [10]. It causes oxidative stress and cell damage in MRSA cells by producing reactive oxygen species (ROS). Furthermore, it inhibits ATP production by disrupting the energy metabolism of the bacterium and suppresses biofilm formation, making it difficult to adhere to surfaces. Due to these properties, pyocyanin is being investigated as a potential antimicrobial agent against antibiotic-resistant bacteria, but its clinical use is limited due to toxicity. Studies have that psoriasis is effective against MRSA, with minimum inhibitor concentration (MIC) values ranging from 8 to 400 µg/mL [11]. *Lactobacillus plantarum* is a genus of probiotic bacteria known for its beneficial effects on human health. *L. plantarum* develops in the human gastrointestinal system. It can effectively resist low pH levels and high bile concentrations, which allows it to colonize and show its beneficial effects. Research has shown that *L. plantarum* has antibacterial activity against MRSA [12]. Studies have shown that the cell-less superfluid may inhibit the growth of MRSA and have dose-dependent effects on bacterial vitality and biofilm formation. This suggests that *L. plantarum* can effectively reduce MRSA populations in various environments, including wound infections [13]. In the light of this information, in this study, we evaluated the antibacterial and antibiotic activity of pyocyanin isolated from *P. aeruginosa* and *L. plantarum* against MRSA, which is a major factor in mastitis, and its effect on cell vitality and cytotoxicity, and on virulence genes.

Material and Methods

Ethical Approval

Since the *S. aureus* ATTC 23235 bacterial strain and *L. plantarum* ATCC 8014 bacterial strain used in our study were the reference strains, ethical approval is not required.

Isolation of Methicillin Resistant *S. aureus* and *L. plantarum*

Commercially purchased *S. aureus* ATTC 23235 and *L. plantarum* ATCC 8014 were grown on blood agar, nutrient

agar and mannitol saline agar plates (Oxoid, Hampshire, UK). Bacteria inoculated by line inoculation method were then incubated at 37°C for 24-48 h. For further analyses, they were stored at -80°C in medium containing 10% glycerol (v/v).

Production of Pyocyanin

Pyocyanin isolated from *P. aeruginosa* (CAS No. 85-66-5, Sigma, Aldrich) was purchased commercially. Confirmed by High Performance Liquid Chromatography (HPLC) with a rate of ≥98%. It was prepared as a stock solution of 1024 µg/mL of pyocyanin dissolved in double distilled water in 1:1 ratio.

Antimicrobial Susceptibility Testing of Methicillin Resistant *S. aureus*

Antimicrobial susceptibility test was performed using disc diffusion technique. The isolated strains were tested for susceptibility to cefoxitin (Cef; MRSA indicator), penicillin (Pen), ampicillin-sulbactam (Amp-Sul), amoxicillin-clavulanic acid (AmoCla), tetracycline (Tet), cefotaxime (Ceft) and erythromycin (Ery) (Oxoid). In addition, inhibition zone diameters were evaluated after 20 µL impregnation of pyocyanin and *L. plantarum* on 6 mm discs. The selected antimicrobials represent drugs used in human and animal industry and were selected according to the National Antimicrobial Resistance Monitoring System (NARMS) registry. The test was performed on Mueller Hinton agar medium (MH, Oxoid) and incubated at 37°C for 24 h. The inhibition zones obtained were determined with digital callipers and compared with the specific zone diameters for resistance or susceptibility based on European Committee on Antimicrobial Susceptibility Testing (EUCAST)/Clinical Laboratory Standards Institute (CLSI) standards.

Determination of Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Fractional Inhibitory Concentration (FIC)

Minimum Inhibitor Concentration was determined by liquid microdilution test on Mueller Hinton Agar liquid medium. Briefly, 100 µL Mueller Hinton Broth (MHB) (MilliporeSigma) medium and 50 µL pyocyanin at concentrations ranging from 1024-16 µg/mL and *L. plantarum* were added to the wells by dilution technique. Then, the prepared bacterial colonies were inoculated 50 µL into all wells according to McFarland 0.5 scale (10^8CFU/mL) and incubated at 37°C for 24 h. After incubation, the wells in which bacterial growth stopped were determined. Then, the minimum bactericidal concentration was determined in the medium in which up to 5 colonies were observed to grow by inoculation from the wells with higher concentrations than the well in which bacterial growth stopped. Fractional inhibitor concentration was determined at pyocyanin 1024, 512, 256, 128, 16 µg/mL and *L. plantarum* 1024, 512, 256, 128, 16 µg/mL concentrations and synergistic, additive and antagonistic activity in the wells were evaluated using the Fractional Inhibitory Concentration (FIC) index. FIC index ≤0.5 was considered as synergistic, 0.5-1 as additive, 1-4 as indifferent and ≥4 as antagonistic. The dilution steps for *L. plantarum* were performed using the double dilution method, and two-fold dilutions were performed consecutively from the initial concentration. Thus, the antibacterial effects of L. plantarum in combination with pyocyanin at different concentrations were analysed [14].

Determination of Antibiofilm Activity

After determining the MIC, a 0.1% solution was used for 15 min to determine the presence of biofilm in the wells. The wells were stained with crystal violet and then washed with phosphate-buffered saline (PBS). Remaining biofilm layer 33% (volume/volume) after dissolution with glacial acetic acid, microplate, spectrophotometric measurements at 595 nm using a microplate reader (Sunrise™, TECAN, Switzerland). Eradication biofilm inhibition rate with the formula [(OD (control)-OD (test)/OD (control))x100] to obtain the percentage evaluated [15].

Cell Culture

Cytotoxicity Study: The cytotoxic effect of the given substances in healthy cells was investigated in the healthy cell line dermal fibroblast. Cells were grown and maintained in DMEM medium containing 10% fetal bovine serum (FBS). The grown cells were seeded in 96 well plates and incubated at 37°C for 24 h at 5%CO_2. At the end of the incubation, the substances given at a concentration of 200-6.25 µM were prepared from the master stock and given to the cells and incubated at 37°C for 24 h at 5%CO_2. At the end of incubation, WST-8 (Water-Soluble Tetrazolium-8) assay was performed according to the manufacturer's kit protocol to evaluate cell viability and metabolic activity. The WST-8 assay contains a tetrazolium salt that is converted into water-soluble orange coloured formazan by mitochondrial dehydrogenase enzymes. WST-8 reagent was added to the cell culture at the end of the specified incubation period and incubated at 37°C for a specified time (usually 1-4 h). Cell viability was then determined by measuring the colour change spectrophotometrically at a wavelength of 450 nm. The colour intensity is directly proportional to the number of metabolically active cells and the result of the test was evaluated according to this principle [16].

Determination of Resistance Gene Expression

Extraction and Homogenisation of Pellets: MRSA, MRSA+ Pyocyanin, MRSA+ *L. plantarum* and MRSA+ Pyocyanin + *L. plantarum* in polystrene tubes (Eppendorf,

USA) were centrifuged at 500 xg x10 m at 4ºC to obtain pellets. The supernatant was collected in another tube and 20 micro glass balls were placed in these tubes on the pellet and these tubes were placed in the Tissue lyser LT system (Qiagen, USA) device whose header was previously cooled in -80°C deep freezer and lysed for 1 min. The lysed samples were spun in a centrifuge to collect the lysate on the edge of the tube to the bottom and these samples were kept in -80°C deep freezer for 5 min. Then they were put back into the Tissue lyser LT and lysed for 1 min. This digestion process was repeated 3 times until the samples were completely homogenised. When the pellet was completely homogenised, the samples were removed and the RNA isolation stage was started [17].

RNA Isolation and cDNA Synthesis: RNA isolation from the samples was performed according to the kit protocol using the PureLink RNA mini kit (Thermo Fisher Scientific, USA) "Protocol for purification of RNA from bacterial cells" protocol. In this context, fresh lysozyme solution was prepared first. For this solution, 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, and 10 mg/mL lysozyme were mixed in ultrapure water. This solution was used as 100 µL for each 1x10^9 bacterial cell population. After this step, Lysis Buffer was prepared with 10 µL 2-mercaptoethanol in 1 mL with the materials available in the kit. 10% SDS solution was also prepared in ultrapure water. After these solutions were prepared, 100 µL of lysozyme solution and 500 µL of 10% SDS solution were added into the tubes homogenised above and these tubes were incubated at room temperature for 5 min using heating block. Then 350 µL Lysis Buffer was added to this mixture. The lysate in the tubes was inverted and vortexed at intervals until completely homogenised. The lysate was then homogenised using a DAIHAN homogeniser with a fine blade at 26000 rpm. The homogenate was centrifuged at 2600xg for 5 min at room conditions and the particles were allowed to settle to the bottom. The supernatant was taken into clean tubes and 1.5 vol of 100% ethanol (Merck, USA) was added to these tubes. Then vortexed until completely dissolved. This mixture was loaded onto the columns in the Purelink RNA mini kit (Thermo Fisher Scientific, USA) at a rate of 700 µL each time. The columns were centrifuged sequentially at 12.000 g for 30 sec and the RNAs were loaded onto the columns and then these columns were washed first with washing solution 1 and then twice with washing solution 2 by centrifugation at 12.000 g for 30 sec at each stage. After washing, the columns were centrifuged 1 time at 12.000 g for 3 min to dry the columns, then the columns were placed in sterile new 1.5 mL polystrene tubes (Eppendorf, USA), 60 µL of the solution given in the kit was pipetted into the centre of the membrane in the column, and these columns were centrifuged at 12.000 g for 1 min and pure RNAs were collected in the polystrene tube. The purity of the collected RNAs was determined by Optizen NanoQ Lite micro-volume spectrophotometer (Mecasys, South Korea) and the RNA quantity obtained are given in *Table 1*.

Table 1. *RNA quantity and purity values obtained from the samples*

Samples	Quantity (ng/ul)	Purity
MRSA	3.70	1.87
MRSA+ Pyocyanin	5.48	1.89
MRSA+ *L. plantarum*	2.65	1.96
MRSA+ Pyocyanin + *L. plantarum*	7.22	2.16

The obtained RNAs were equilibrated with DNAse and RNAse free ultrapure water (Sigma, USA) to 25 ng/10 µL for the next step. After the equilibration process, complementary DNA was synthesised to ensure that the obtained RNAs could be amplified by PCR. At this stage, Pathwayscanner cDNA Reverse Transcription Kit (Micromolecules, Türkiye) was used, and according to the kit protocol, the enzyme, dNTP mix and random primers in the kit were mixed and pipetted into PCR tubes as 10 µL. Afterwards, total RNA, which was equalised to 25 ng/10 µL in the previous section, was put into the same tubes. These tubes were synthesised cDNA in Applied Biosystems® ProFlex™ PCR System thermal cycler using step 1, 25°C, 10 min; step 2 37°C, 120 min; step 3 85°C, 5 min cycles. The cDNAs obtained were stored at -20°C.

Quantitative Real-time PCR Study, Determination of Gene Expression: In the study, the expression levels of resistance genes in control and treatment groups of examples were analysed by qRT-PCR method. The primers used to investigate the changes in the expression of these genes are given below in 5'-3' order. The genes and primers were given in *Table 2*. In gene expression studies, cDNAs were used. These cDNAs were analysed in qRT-PCR according to the Pathwayscanner cyber green qPCR MasterMix (Micromolecules, Türkiye) protocol. The reaction mix contained Hot Start Taq DNA polymerase (enzyme), SYBR Green I dye, dNTPs (0.2 mM each), $MgCl_2$ (3 mM), and stabilizers in a proprietary buffer. In the study, cDNAs were amplified using Applied Biosystems QuantStudio 5 Real-Time PCR device. Step 1 for qRT-PCR reaction to take place: Enzyme activation: 95ºC-3 min; 2nd step: Denaturation: 95ºC-15 sec; Primer binding-chain elongation: 60ºC-1 min; Step 3: Melting curve: 95ºC-15 sec, 60ºC-1 min, 95ºC-15 sec. Ct values of the peaks obtained during the replication process were used to determine gene expression and gene expression was calculated by 2-ΔΔΔCt method. Endogenous control 16 S rRNA expression was used as calibration and correction factor.

Table 2. Genes and primers				
Gene		**Primer (5'-3')**	**Size bp**	**Reference**
icaA (intercellular adhesion)	F	GAGGTAAAGCCAACGCACTC	151	[18]
	R	CCTGTAACCGCACCAAGTTT		
icaB (intercellular adhesion)	F	ATACCGGCGACTGGGTTTAT	140	
	R	TTGCAAATCGTGGGTATGTGT		
icaC (intercellular adhesion)	F	CTTGGGTATTTGCACGCATT	209	
	R	GCAATATCATGCCGACACCT		
icaD (intercellular adhesion)	F	ACCCAACGCTAAAATCATCG	211	
	R	GCGAAAATGCCCATAGTTTC		
nbA (fibronectin binding proteinA)	F	AAATTGGGAGCAGCATCAGT	121	
	R	GCAGCTGAATTCCCATTTTC		
nbB (fibronectin binding proteinB)	F	ACGCTCAAGGCGACGGCAAAG	197	
	R	ACCTTCTGCATGACCTTCTGCACCT		
clfA (clumping factorA)	F	ACCCAGGTTCAGATTCTGGCAGCG	165	
	R	TCGCTGAGTCGGAATCGCTTGCT		
clfB (clumping factorB)	F	AACTCCAGGGCCGCCGGTTG	159	
	R	CCTGAGTCGCTGTCTGAGCCTGAG		
16S rRNA	F	GGGACCCGCACAAGCGGTGG	191	
	R			

Table 3. Percent inhibition (%) of biofilm formation of MRSA at different concentrations						
Agents	**Concentrations (µg/mL)**					
	1024	**512**	**128**	**64**	**32**	**16**
Pyocyanin	58.6	48.6	40.6	31.1	17.3	15.1
L. plantarum	50.3	50	40.1	21.9	12.7	0
Pyocyanin+ *L. plantarum*	17.2	8.6	3.9	2.7	0.5	0
MRSA: *Methicillin Resistance Staphylococcus aureus*						

Results

Antimicrobial Susceptibility Testing of MRSA Results

Disc diffusion zone diameters of Pyocyanin, *L. plantarum* and Pyocyanin+*L. plantarum* against MRSA. The MRSA suspension has an inhibition area of 15 mm in the test with pyocyanin. The inhibition area in the test with *L. plantarum* was 10 mm. In a combination of pyocyanin and *L. plantarum*, the inhibition area of MRSA was measured at 18 mm. This suggests that when the two agents were used together, they create a synergistic effect, inhibiting the growth of MRSA more effectively.

Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC) and Fractional Inhibitory Concentration (FIC) Results

The MIC and MBC values of pyocyanin and *L. plantarum* versus MRSA and the FIC index for pyocyanin+*L. plantarum*. MIC, Pyocyanin: 64 µg/mL *L. plantarum*: 128 µg/mL. These results indicate that Pyocyanin has a lower MIC value over MRSA. Pyocyanin needs less concentration to inhibit the growth of MRSA. *L. plantarum* shows a higher MIC, which indicates that its effect is weaker. MBC, Pyocyanin: 512 µg/mL, *L. plantarum*: 512 µg/mL. MBC values indicate that both agents need a similar concentration to kill MRSA. FIC showed synergistic effect with the FIC index of 0.36 at pyocyanin+*L. plantarum* 128 µg/mL.

Antibiofilm Activity Results

Antibiofilm activity of pyocyanin, *L. plantarum* and pyocyanin+*L. plantarum* on MRSA was given as % in *Table 3*. At the highest concentration (1024 µg/mL), pyocyanin provides a biofilm inhibition of 58.6%. As the concentration decreases, the inhibition percentage was also decreasing, with values of 48.6% at 512 µg/mL and 40.6% at 128 µg / mL. This indicates that pyocyanin effectively inhibits biofilm formation. *L. plantarum* produces an inhibition of 50.3% at 1024 µg/mL, while

at lower concentrations its effect is reduced. It drops to 12.7% at 32 µg/mL and 0% at 16 µg/mL. This suggests that *L. plantarum* was effective at higher concentrations, but its effect at lower concentrations remains limited. In a combination of pyocyanin and *L. plantarum*, the inhibition percentage was relatively low at all concentrations. Values such as 8.6% were obtained at the highest concentration (1024 µg/mL) at 17.2% and 512 µg/mL.

Cell Culture Results

Cell culture cytotoxicity results were given in *Fig. 1*. In the control group, proliferation was accepted as 100%. In 200 µM pyocyanin application, proliferation was around 140%. In 100 µM pyocyanin application, proliferation reached 160%. When *L. plantarum* was applied alone, proliferation decreased to around 80% at 50 µM. In the combination of pyocyanin and *L. plantarum*, proliferation decreased to 60% at 50 µM. The lowest proliferation, around 40%, was observed in the combination of 25 µM pyocyanin + *L. plantarum*.

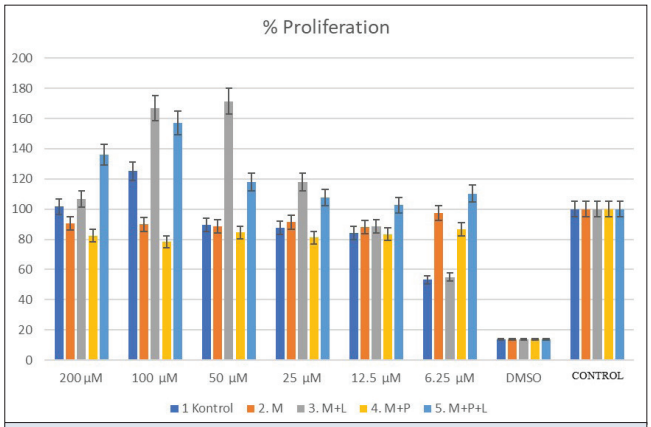

Fig 1. Cell culture results. M: MRSA, L: *L. plantarum,* P: Pyocyanin

Genes Expression Results

icaA and icaB Expression: The icaA gene expression of pyocyanin and *L. plantarum* on MRSA was shown in *Fig. 2*. Target, repeat number and Cq (Cycle quantification) values were determined for each sample. MRSA and MRSA+ Pyocyanin Cq values, i.e. DNA quantity, are very close to each other (21.193 and 21.22). MRSA+ *L. plantarum* Cq value (27.812) was higher than the other samples. This means that the DNA amount of this sample was less. MRSA+ Pyocyanin + *L. plantarum* Cq value (21.351) was slightly higher than MRSA and MRSA+ Pyocyanin but still lower than MRSA+ *L. plantarum*. MRSA+*L. plantarum* gave the most significant result by reducing the quantity of DNA compared to the other sample groups *(Fig. 2)*. The icaB gene expression of pyocyanin and *L. plantarum* on MRSA and MRSA was shown in *Fig. 2*. MRSA and MRSA+ Pyocyanin, Cq values (19.796 and 19.821) were very close to each other, indicating that the DNA quantity of these samples was similar. MRSA+ *L. plantarum* Cq value (16.591) was significantly lower than the other samples. This indicates that MRSA+ *L. plantarum* has a higher amount of DNA. MRSA+ Pyocyanin + *L. plantarum* Cq value (19.626) was slightly higher compared to MRSA and MRSA+ Pyocyanin, but still lower than MRSA+ *L. plantarum*. icaB expression was most suppressed in MRSA+ Pyocyanin *(Fig. 2)*.

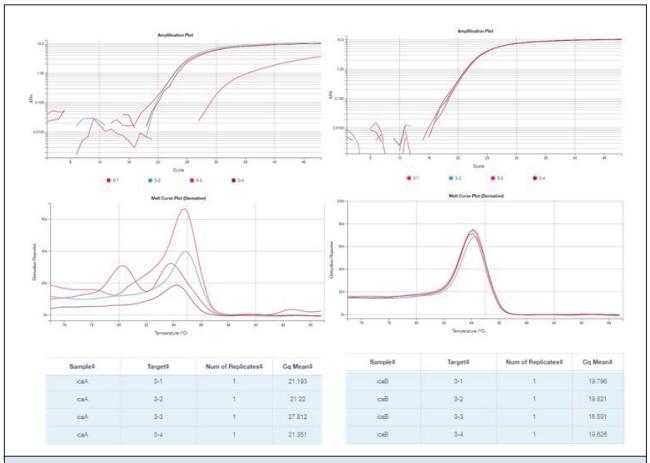

Fig 2. icaA and icaB expression levels. 3-1: MRSA, 3-2: MRSA+ Pyocyanin, 3-3: MRSA+ *L. plantarum,* 3-4: MRSA+ Pyocyanin + *L. plantarum*

icaC and icaD Expression: The icaC gene expression of pyocyanin and *L. plantarum* on MRSA and MRSA was shown in *Fig. 3*. MRSA and MRSA+ Pyocyanin Cq values (29.454 and 29.908) were close to each other, indicating that the DNA quantity of these samples was similar. MRSA+ *L. plantarum*, Cq value (27.511) was lower than the other samples. This indicates that the quantity of DNA of MRSA+ *L. plantarum* was higher. MRSA+ Pyocyanin + *L. plantarum* Cq value (29.919) was slightly higher compared to MRSA and MRSA+ Pyocyanin but still lower than MRSA+ *L. plantarum (Fig. 3)*. The icaD gene expression

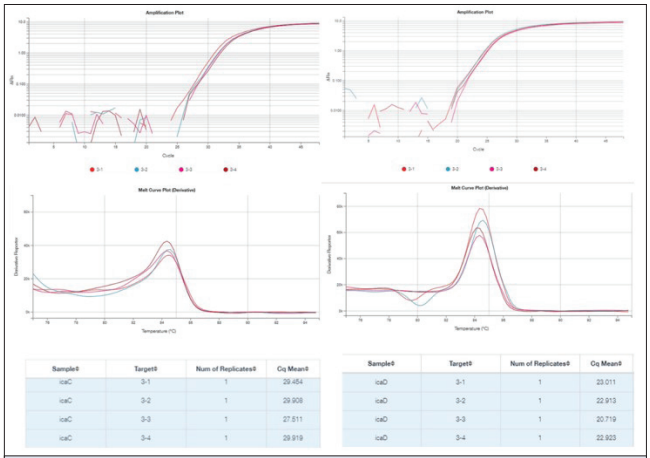

Fig 3. icaC and icaD expression levels. 3-1: MRSA,3-2: MRSA+ Pyocyanin, 3-3: MRSA+ *L. plantarum,* 3-4: MRSA+ Pyocyanin + *L. plantarum*

of pyocyanin and *L. plantarum* on MRSA was shown in *Fig. 3*. MRSA and MRSA+ Pyocyanin Cq values (23.011 and 22.913) were very close to each other, indicating that the DNA quantity of these samples was similar. MRSA+ *L. plantarum*, Cq value (20.719) was significantly lower than the other samples. This indicates that the amount of DNA was higher in MRSA+ *L. plantarum*. MRSA+ Pyocyanin + *L. plantarum* Cq value (22.923) was slightly higher compared to MRSA and MRSA+ Pyocyanin but still lower than MRSA+ *L. plantarum (Fig. 3)*.

nbA and nbB Expression: The nbA gene expression of pyocyanin and *L. plantarum* on MRSA was shown in *Fig. 4*. MRSA and MRSA+ Pyocyanin Cq values (20.591 and 20.266) were close to each other, indicating that the DNA quantity of these samples was similar. MRSA+ *L. plantarum*, Cq value (17.59) was lower than the other samples. This indicates that the quantity of MRSA+ *Lactobacillus plantarum* DNA was higher. MRSA+ Pyocyanin + *L. plantarum* Cq value (19.953) was higher compared to MRSA and MRSA+ Pyocyanin but still lower than MRSA+ *L. plantarum (Fig. 4)*. The nbB gene expression of pyocyanin and *L. plantarum* on MRSA was shown in *Fig.4*. MRSA+ *L. plantarum* has the lowest Cq value (25.784), indicating that MRSA+ *L. plantarum* has the highest quantity of DNA/RNA. The Cq values of MRSA, MRSA+ Pyocyanin and MRSA+ Pyocyanin + *L. plantarum* were close to each other (28.134, 28.553 and 28.463), but MRSA+ Pyocyanin has the highest Cq value, indicating the lowest quantity of DNA/RNA *(Fig. 4)*.

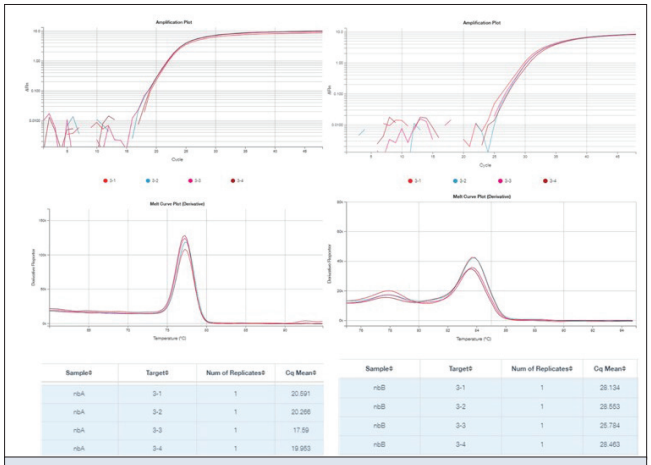

Fig 4. nbA and nbB expression levels. 3-1: MRSA, 3-2: MRSA+ Pyocyanin, 3-3: MRSA+ *L. plantarum,* 3-4: MRSA+ Pyocyanin + *L. plantarum*

ClfA and ClfB Expression: The ClfA gene expression of pyocyanin and *L. plantarum* on MRSA was shown in *Fig. 5*. MRSA+ *L. plantarum* has the lowest Cq value (25.577), indicating the highest amount of DNA/RNA in this sample. MRSA+ Pyocyanin has the second lowest Cq value (28.314). MRSA+ Pyocyanin + *L. plantarum*

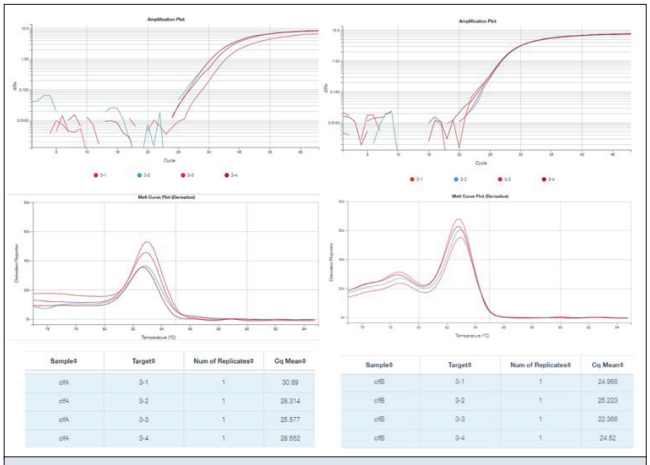

Fig 5. ClfA and ClfB expression levels. 3-1: MRSA,3-2: MRSA+ Pyocyanin, 3-3: MRSA+ *L. plantarum,* 3-4: MRSA+ Pyocyanin + *L. plantarum*

Cq value 28.682 was slightly higher than MRSA+ *L. plantarum*. MRSA has the highest Cq value (30.89), indicating the lowest concentration of genetic material *(Fig. 5)*. The ClfB gene expression of pyocyanin and *L. plantarum* on MRSA and MRSA was shown in *Fig.5*. MRSA+ *L. plantarum* has the lowest Cq value (22.366), indicating the highest amount of DNA/RNA in MRSA+ *L. plantarum*. MRSA has the second lowest Cq value (24.968). MRSA+ Pyocyanin + *L. plantarum*, Cq value 24.52, was slightly lower than MRSA. MRSA+ Pyocyanin has the highest Cq value (25.223), indicating the lowest concentration of genetic material *(Fig. 5)*.

Relative Quantification Results

RQ (Relative Quantification) shows the gene expression level of each sample *(Fig. 6)*. clFA; MRSA+ Pyocyanin, highest RQ value, *L. plantarum* and MRSA+ Pyocyanin + *L. plantarum* lower RQ values, MRSA+ Pyocyanin + *L. plantarum* RQ value was close but lower than MRSA+ *L. plantarum*. clFB; MRSA+ Pyocyanin, higher RQ value, MRSA+ *L. plantarum* lower RQ value, MRSA+ Pyocyanin + *L. plantarum* lowest RQ value, showing a significant decrease compared to MRSA+ Pyocyanin and MRSA+ *L. plantarum*. icaA, icaC, icaD, nbA, nbB; In general, RQ values are higher in MRSA+ Pyocyanin. Between MRSA+ *L. plantarum* and MRSA+ Pyocyanin + *L. plantarum*, they remain at similar levels for some genes, but MRSA+ *L. plantarum* is generally higher *(Fig. 6)*.

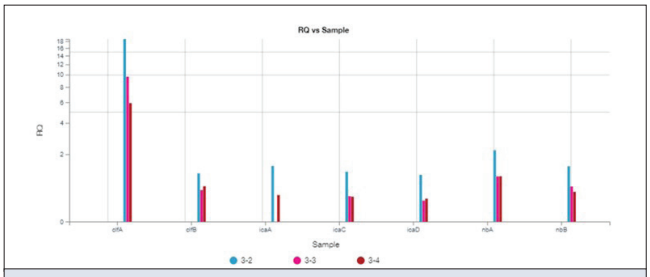

Fig 6. Relative quantification results. 3-2: MRSA+ Pyocyanin, 3-3: MRSA+ *L. plantarum,* 3-4: MRSA+ Pyocyanin + *L. plantarum*

Discussion

The relationship between bovine mastitis and MRSA is significant, particularly concerning the implications for animal health, milk quality, and public health. Antibiotic resistance developed by MRSA and biofilm therapy make it more difficult to prevent infection. In this percentage, we evaluated the therapeutic activity of pyocyanin and *L. plantarum in vitro* as alternative therapies in this study. Lactic acid bacteria inhibit the growth of pathogens through the secretion of bacteriocins, H_2O_2 or other antimicrobial compounds, as well as the adhesion properties of epithelial cells that inhibit pathogen adhesion through specific competition or steric hindrance [19]. The ability of *L. casei* to prevent bMEC invasion against two bovine strains of *S. aureus* RF122 and Newbould305 causing acute and moderate mastitis was evaluated. *Lactobacillus casei* strains affected the adhesion and/or internalisation of *S. aureus* in a strain-dependent manner. *L. casei* CIRM-BIA 667 reduced *S. aureus* Newbould305 and RF122 internalisation by 60% to 80% [20]. Several *Lactobacillus* strains, such as *L. plantarum* and *Lactobacillus brevis*, have demonstrated the ability to inhibit common mastitis-causing pathogens such as *S. aureus* and *E. coli*. For example, *L. plantarum* KLDS 1.0344 reduced bacterial growth and the expression of proinflammatory cytokines such as IL-6 and TNF-α in response to lipopolysaccharide (LPS) stimulation in both *in vitro* and *in vivo* studies [21]. In another study, the potential of *L. lactis* LMG 7930 strain to be used as an antibiotic alternative in the treatment of mastitis in dairy ruminants was investigated. In particular, the probiotic properties of this strain against pathogens causing mastitis were investigated. *L. lactis* LMG 7930, a nisin-producing strain, is widely used in the food industry and is considered safe for human consumption. *L. lactis* showed antagonistic properties against various pathogens in *in vitro* tests. In particular, it has a broad spectrum of inhibitory activity against Gram-positive bacteria. However, it could not inhibit the growth of *E. coli*. The strain was able to ferment various carbohydrates such as galactose, glucose and fructose. It also fermented eight of the 14 carbohydrates metabolised by some mastitis isolates. Surface hydrophobicity of *L. lactis* was found to be moderate and electron donor and acceptor properties were found to be low. Auto-aggregation and co-aggregation abilities were found to be low. However, the ability of *L. lactis* to adhere to bovine mammary epithelial cells was found at a certain level. *L. lactis* tended to reduce the internalisation of some pathogens, but this effect was not statistically significant [22]. Forty lactobacilli, including *L. plantarum*, were isolated from healthy bovine milk samples and selected isolates were evaluated for their probiotic properties on microorganisms [23]. However, no gene expression was evaluated in this study. Among the lactobacilli isolates, varying levels of activity (9 to 19 mm) against the bovine mastitogens *S. aureus, E. coli* and *Streptococcus dysgalactiae* were observed in a well diffusion assay. These isolates showed auto-aggregation (ranging from 14.29±0.96% to 62.11±1.09%) and co-aggregation (ranging from 9.21±0.14% to 55.74±0.74%) with mastitogens after 2 h. *L. plantarum* CM49 showed sensitivity to most antibiotics tested and showed strong inhibitory effects in co-culture experiments with an average $\log_{10}$ reduction of 3.46 for *S. aureus*, 2.82 for *E. coli* and 1.45 for *S. dysgalactiae*. Moreover, *L. plantarum* CM49 significantly reduced the adhesion rate of mastitogens in bovine mammary cell line and mouse model, demonstrating its potential efficacy in preventing mastitis [23]. Pyocyanin is a pigment produced by *P. aeruginosa* that exhibits notable antibacterial properties against a wide range of pathogens, including *S. aureus*. The mechanism of action of pyocyanin is primarily attributed to its ability to generate reactive oxygen species (ROS), such as superoxide radicals and hydrogen peroxide, through redox cycling. These ROS cause oxidative stress within the bacterial cells, leading to damage to cellular components, including proteins, lipids, and DNA. Pyocyanin also disrupts bacterial energy metabolism by interfering with mitochondrial function and ATP production. Furthermore, it has been shown to inhibit biofilm formation by *S. aureus*, which is a critical factor in its pathogenicity and resistance to antibiotics. Overall, the bactericidal effect of pyocyanin is due to its ability to induce cellular damage through oxidative stress, energy depletion, and biofilm disruption, making it a promising candidate for combating antibiotic-resistant infections [24]. Pyocyanin shows strong antibacterial activity primarily against Gram-positive bacteria. Studies have shown that it is effective against organisms such as *S. aureus* and *Bacillus* species, but its activity against Gram-negative bacteria such as *E. coli* is relatively lower. One study reported that pyocyanin produced inhibition zones of 24 mm against *S. aureus* and 26 mm against *S. typhi*, indicating strong antibacterial effects against these pathogens. However, no studies have been performed against *S. aureus*, the causative agent of mastitis [25]. In the disc diffusion test, pyocyanin 15 mm, *L. plantarum* 10 mm and their combinations 18 mm zone diameter were determined. Minimum inhibitory concentration was determined as 64 µg/mL for pyocyanin and 128 µg/mL for *L. plantarum*. Both agents require a minimum bactericidal concentration of 512 µg/mL. In the antibiofilm activity test, pyocyanin showed 58.6% inhibitory effect and *L. plantarum* showed 50.3% inhibitory effect. In gene expression analyses, *L. plantarum* was more effective in reducing the amount of DNA of MRSA.

Declarations

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author (D. Çelebi) on reasonable request.

Funding Support

The authors declared that this study has received no financial support.

Ethical Approval

Ethical approval is not required for this study.

Competing Interests

The authors have no conflicts of interest to declare.

Author Contributions

D.C., O.C., S.B., B.A., S.E.K., A.K.: Concept, Design, Supervision, Resources, Materials Data, Collection and/or Processing, Analysis and/or Interpretation, Literature Search, Writing and Critical Reviews.

References

1. Campos B, Pickering AC, Rocha LS, Aguilar AP, Fabres-Klein MH, de Oliveira Mendes TA, Fitzgerald JR, de Oliveira Barros Ribon A: Diversity and pathogenesis of *Staphylococcus aureus* from bovine mastitis: Current understanding and future perspectives. *BMC Vet Res,* 18 (1):115, 2022. DOI: 10.1186/s12917-022-03197-5

2. Shahzad MA, Yousaf A, Ahsan A, Irshad H, Riaz A, Khan A, Ullah I, Sattar S, Bostan N, Javed S: Virulence and resistance profiling of *Staphylococcus aureus* isolated from subclinical bovine mastitis in the Pakistani Pothohar region. *Sci Rep,* 14 (1):14569, 2024. DOI: 10.1038/s41598-024-65448-9

3. Fidelis CE, Orsi AM, Freu G, Goncalves JL, Santos MVD: Biofilm formation and Antimicrobial resistance of *Staphylococcus aureus* and *Streptococcus uberis* isolates from bovine mastitis. *Vet Sci,* 11 (4):170, 2024. DOI: 10.3390/vetsci11040170

4. Kadlec K, Entorf M, Peters T: Occurrence and characteristics of livestock-associated methicillin-resistant *Staphylococcus aureus* in quarter milk samples from dairy cows in Germany. *Front Microbiol,* 10:1295, 2019. DOI: 10.3389/fmicb.2019.01295

5. Khazaie F, Ahmadi E: Bovine subclinical mastitis-associated methicillin-resistant *Staphylococcus aureus*, selective genotyping and antimicrobial susceptibility profile of the isolates in Kurdistan province of Iran. *Iran J Microbiol,* 13 (1): 65-73, 2021. DOI: 10.18502/ijm.v13i1.5494

6. González-Vázquez R, Córdova-Espinoza MG, Escamilla-Gutiérrez A, Herrera-Cuevas MdR, González-Vázquez R, Esquivel-Campos AL, López-Pelcastre L, Torres-Cubillas W, Mayorga-Reyes L, Mendoza-Pérez F: Detection of *mecA* genes in hospital-acquired MRSA and SOSA strains associated with biofilm formation. *Pathogens,* 13 (3):212, 2024. DOI: 10.3390/pathogens13030212

7. Ghasemian A, Najar-Peerayeh S, Bakhshi B, Mirzaee M: High prevalence of icaABCD genes responsible for biofilm formation in clinical isolates of *Staphylococcus aureus* from hospitalized children. *Arch Pediatr Infect Dis,* 3 (3):e20703, 2015. DOI: 10.5812/pedinfect.20703v2

8. Ghasemian A, Peerayeh SN, Bakhshi B, Mirzaee M: Comparison of biofilm formation between methicillin-resistant and methicillin-susceptible isolates of *Staphylococcus aureus*. *Iran Biomed J,* 20 (3):175, 2016. DOI: 10.7508/ibj.2016.03.007

9. Tahmasebi H, Dehbashi S, Jahantigh M, Arabestani MR: Relationship between biofilm gene expression with antimicrobial resistance pattern and clinical specimen type based on sequence types (STs) of methicillin-resistant *S. aureus*. *Mol Biol Rep,* 47, 1309-1320, 2020. DOI: 10.1007/s11033-019-05233-4

10. Leanse LG, Zeng X, Dai T: Potentiated antimicrobial blue light killing of methicillin resistant *Staphylococcus aureus* by pyocyanin. *J Photochem Photobiol B,* 215: 112109, 2021. DOI: 10.1016/j.jphotobiol.2020.112109

11. Marey MA, Abozahra R, El-Nikhely NA, Kamal MF, Abdelhamid SM, El-Kholy MA: Transforming microbial pigment into therapeutic revelation: Extraction and characterization of pyocyanin from *Pseudomonas aeruginosa* and its therapeutic potential as an antibacterial and anticancer agent. *Microb Cell Fact,* 23 (1):174, 2024. DOI: 10.1186/s12934-024-02438-6

12. Sürmeli M, Maçin S, Akyön Y, Kayikçioğlu AU: The protective effect of *Lactobacillus plantarum* against meticillin-resistant *Staphylococcus aureus* infections: An experimental animal model. *J. Wound Care,* 28 (3): 29-34, 2019. DOI: 10.12968/jowc.2019.28.Sup3b.S29

13. Dubey AK, Sharma M, Parul, Raut S, Gupta P, Khatri N: Healing wounds, defeating biofilms: *Lactiplantibacillus plantarum* in tackling MRSA infections. *Front Microbiol,* 14:1284195, 2023. DOI: 10.3389/fmicb.2023.1284195

14. Çelebi D, Çelebi Ö, Taghizadehghalehjoughi A, Başer S, Parlak E: HepG2 karaciğer hücre hattı üzerinde bakterilerle oluşturulan infeksiyon modelinde nanopartikül çinko+ çinko borat bileşiklerinin sinerjik etkisinin belirlenmesi. *Flora J Infect Dis Clin Microbiol,* 29 (1): 52-61, 2024. DOI: 10.5578/flora.202401755

15. Celebi D, Taghizadehghalehjoughi A, Baser S, Genc S, Yilmaz A, Yeni Y, Yesilyurt F, Yildirim S, Bolat I, Kordali S: Effects of boric acid and potassium metaborate on cytokine levels and redox stress parameters in a wound model infected with methicillin-resistant *Staphylococcus aureus*. *Mol Med Rep,* 26 (3):294, 2022. DOI: 10.3892/mmr.2022.12809

16. Seifabadi ZS, Rezaei-Tazangi F, Azarbarz N, Darioush BN, Mohammadiasl J, Darabi H, Pezhmanlarki-Tork S: Assessment of viability of wharton's jelly mesenchymal stem cells encapsulated in alginate scaffold by WST-8 assay kit. *Med J Cell Biol,* 9 (1): 42-47, 2021. DOI: 10.2478/acb-2021-0007

17. Kim J, Lee S, Darlington AP, Kim J: Impact of fleQ deficiency on resource allocation and heterologous gene expression in *Pseudomonas putida* across various growth media. *Microb Biotechnol,* 17 (11):e70054, 2024. DOI: 10.1111/1751-7915.70054

18. Atshan SS, Shamsudin MN, Karunanidhi A, van Belkum A, Lung LTT, Sekawi Z, Nathan JJ, Ling KH, Seng JSC, Ali AM: Quantitative PCR analysis of genes expressed during biofilm development of methicillin resistant *Staphylococcus aureus* (MRSA). *Infect Genet Evol,* 18, 106-112, 2013. DOI: 10.1016/j.meegid.2013.05.002

19. Reid G, Burton J: Use of *Lactobacillus* to prevent infection by pathogenic bacteria. *Microbes Infect,* 4 (3): 319-324, 2002. DOI: 10.1016/s1286-4579(02)01544-7

20. Bouchard DS, Rault L, Berkova N, Le Loir Y, Even S: Inhibition of *Staphylococcus aureus* invasion into bovine mammary epithelial cells by contact with live *Lactobacillus casei*. *Appl Environ Microbiol,* 79 (3): 877-885, 2013. DOI: 10.1128/AEM.03323-12

21. Chen Q, Wang S, Guo J, Xie Q, Evivie SE, Song Y, Li B, Huo G: The protective effects of *Lactobacillus plantarum* KLDS 1.0344 on LPS-induced mastitis *in vitro* and in *vivo*. *Front Immunol,* 12:770822, 2021. DOI: 10.3389/fimmu.2021.770822

22. Armas F, Camperio C, Marianelli C: *In vitro* assessment of the probiotic potential of *Lactococcus lactis* LMG 7930 against ruminant mastitis-causing pathogens. *PLoS One,* 12 (1):e0169543, 2017. DOI: 10.1371/journal.pone.0169543

23. Izhar MZ, Nawaz M, Yaqub T, Avais M, Anjum AA: *In vitro* characterization of probiotic potential of *Lactobacillus plantarum* CM49 against selected cattle mastitogens. *BMC Microbiol,* 24 (1):310, 2024. DOI: 10.1186/s12866-024-03464-5

24. Baron SS, Rowe JJ: Antibiotic action of pyocyanin. *Antimicrob Agents Chemother,* 20 (6): 814-820, 1981. DOI: 10.1128/AAC.20.6.814

25. Özyürek SB, Gür SD, Bilkay IS: Investigation of antimicrobial activity of pyocyanin produced by *Pseudomonas aeruginosa* strains isolated from different clinical specimens. *Hacettepe J Biol Chem,* 44 (1): 1-6, 2016. DOI: 10.15671/HJBC.20164417526