Applied Microbiology: Theory & Technology
https://ojs.wiserpub.com/index.php/AMTT/

UNIVERSAL WISER PUBLISHER

Research Article

A Quorum-Sensing-Based Genetic Circuit for Bioengineered Bacterial Treatment of Bovine Mastitis

Adrija Adhikary[1], Abeg Dutta[1*], Udit Ghosh[1], Sourin Chatterjee[2], Shreyash Borkar[1], Shubhamay Das[1]

[1]Department of Biological Sciences, Indian Institute of Science Education and Research, Kolkata, India
[2]Department of Mathematics and Statistics, Indian Institute of Science Education and Research, Kolkata, India
E-mail: abegdutta99@gmail.com

Received: 24 October 2024; **Revised:** 17 February 2025; **Accepted:** 6 March 2025

Abstract: Bovine mastitis is a significant challenge for the dairy industry, affecting milk production, quality, and cow health. Traditional antibiotic-based treatments raise concerns about resistance and residual antibiotics in dairy products, creating an urgent need for sustainable alternatives. This study explores a genetically modified organism (GMO)-based, antibiotic-free treatment targeting *Staphylococcus aureus*, a primary mastitis-causing pathogen. This study proposes using the bioengineered defensin Nisin PV, which has enhanced stability and reduced susceptibility to proteolytic degradation compared to Nisin A. This makes it a more effective, pathogen-specific alternative to broad-spectrum antibiotics, reducing off-target effects and supporting responsible antimicrobial stewardship. To disrupt biofilm formation, this study utilizes deoxyribonuclease I (DNase I), an enzyme that degrades extracellular deoxyribonucleic acid (DNA), a key structural biofilm component. This genetically modified bacteria detects *S. aureus* through its agr quorum sensing system and responds by producing Nisin PV and DNase I. Experimental results confirm DNase I's efficacy asgainst biofilm formation and validate this quorum sensing-based pathogen detection mechanism. Molecular dynamics simulations further suggest that Nisin PV resists cleavage by bacterial strains resistant to Nisin A. A regulated lysis mechanism is proposed to ensure controlled therapeutic release. This study highlights the potential of bioengineered defensins as an effective, sustainable alternative to antibiotics, offering a promising strategy to reduce mastitis incidence and economic losses in the dairy industry.

***Keywords*:** autoinducing peptide I (AIP-I), genetically modified organism (GMO), bovine mastitis, Nisin, DNase I, quorum sensing, biofilm

1. Introduction

Bovine mastitis, an inflammatory disease of the mammary glands, remains one of the most prevalent and costly challenges in the dairy industry. It significantly impacts milk production and quality, leading to economic losses worldwide [1]. A study in India estimated that subclinical mastitis caused economic losses of 48.53% due to reduced milk yield and 36.57% due to veterinary treatment costs, with projected annual losses reaching thousands of dollars in Maharashtra and Pune alone [2]. Mastitis remains a significant concern in U.S. dairy herds, leading to substantial

DOI: https://doi.org/10.37256/amtt.6120255872

economic losses, averaging $444 per cow per year, considering factors such as reduced milk yield, treatment costs, and increased culling rates [3], while climate change is projected to increase raw milk losses due to mastitis across Europe, with the highest losses in the Mediterranean region [4].

Mastitis results from bacterial infections that invade udder tissue, triggering an inflammatory immune response [5]. It progresses through four stages: infection, subclinical mastitis, clinical mastitis, and recurrent clinical mastitis, with treatment becoming increasingly difficult as the disease advances [6]. Early intervention is crucial, yet subclinical mastitis remains asymptomatic, complicating detection while significantly affecting milk production and cow health [7]. *Staphylococcus aureus* and *Streptococcus agalactiae* cause persistent subclinical infections, while *Streptococcus uberis* and *Escherichia coli* contribute as environmental pathogens [8]. A study in Ethiopia identified *Staphylococcus aureus* as the most common pathogen (44.9%), followed by *Streptococcus* spp. (25.3%) and *Escherichia coli* (8.8%) [9].

Antibiotic treatments face increasing limitations due to rising antibiotic resistance and residual contamination in dairy products. *Staphylococcus aureus*, in particular, exhibits high resistance due to horizontal gene transfer and biofilm formation, which shields bacteria from antibiotics [10, 11]. Prolonged antibiotic use further accelerates resistance by selectively eliminating susceptible bacteria while promoting the survival of resistant strains [12]. This highlights the urgent need for alternative therapeutic strategies. Recent advances in bioengineering have led to innovative solutions, with defensins emerging as promising antimicrobial agents. These small cationic peptides exhibit broad-spectrum activity against bacteria, fungi, and viruses, while also modulating immune responses [13-15].

This study proposes a novel bioengineered approach leveraging the quorum-sensing system of *Staphylococcus aureus* for early pathogen detection by introducing the two-component agrC-agrA signal transduction system into a proposed chassis, *Lactococcus lactis* LMG 7930. This would enable it to sense the AIP-I molecules produced by the pathogenic *Staphylococcus aureus*. This strain was previously used as a potential probiotic and has been shown to reduce inflammation due to mastitis when injected into the udder [16]. Upon detection, the engineered bacteria would produce DNase I to disrupt biofilms and Nisin PV, a bacteriocin resistant to nisin resistance protein (NSR) from *Streptococcus uberis* [17]. Expression of these proteins is regulated by the P2 promoter which is activated in the presence of AIP-I by phosphorylated agrA. The incorporation of a Lysis E7-based kill switch ensures the rupturing of the chassis organism and allows the involved gene products to be released out of the cell. The Lysis E7 gene would be placed under a phosphorylated agrA sensitive weak promoter P3, such that the rate of production of Lysis E7 is lesser compared to DNase I and Nisin PV, allowing their accumulation prior to release by lysis. The agrC-agrA component is designed to be oriented opposite to the P2 and P3 promoters to prevent leaky gene expression.

Figure 1 illustrates the gene circuit. The proposed strategy aims to provide a sustainable alternative to antibiotics, reducing mastitis incidence and economic losses while promoting responsible antimicrobial stewardship.

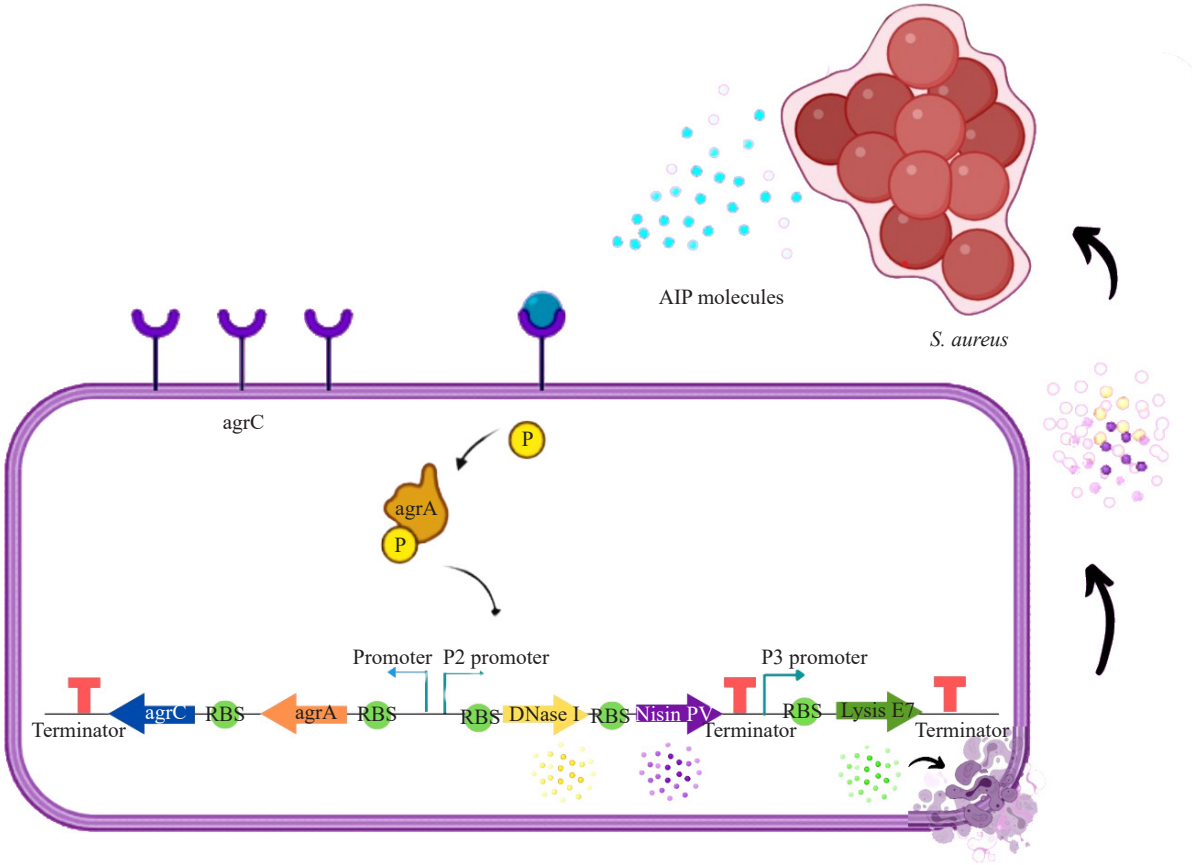


Figure 1. The working principle of the GMO. When the concentration of AIP-I exceeds a specific threshold as a result of the presence of *Staphylococcus aureus* cells, AIP-I molecules attach to agrC. Subsequently, the agrC-AIP-I complex functions as a kinase, leading to the phosphorylation of agrA. The phosphorylated agrA then binds to both P2 and P3, triggering the activation of Nisin PV and DNase I production downstream of P2, while Lysis E7 production occurs downstream of P3. However, the production rate of Nisin PV and DNase I surpasses that of Lysis E7 due to the heightened affinity of phosphorylated agrA for the P2 promoter compared to the P3 promoter. Therefore, our genetically modified organism will only undergo lysis after a substantial accumulation of Nisin PV and DNase I. If the AIP-I concentration is not above a certain threshold, then the GMO will not be switched on. RBS: ribosome binding site

2. Materials and methods

2.1 *Materials*

All resource materials can be accessed from the Supplementary Material.

2.2 *Construction of the gene circuit*

Cloning of all biological parts was performed using a standardized cloning protocol, with various steps elaborated as follows.

2.2.1 *Preparation of competent cells*

A single colony was picked and inoculated in 10 mL of lysogeny broth (LB) and grown overnight at 37 °C on a shaker. The overnight culture (1 mL) was added to 100 mL of prewarmed LB in a 500 mL conical flask and grown at 37 °C until the optical density (OD) reached 0.5. The culture was cooled on ice for 5 minutes and transferred to clean falcon tubes. The cells were collected by centrifugation, and the supernatant was discarded. The pellet was resuspended in TFB-I buffer (composition available in Supplementary Material) and incubated on ice for 90 minutes. The cells were then collected by centrifugation (5 minutes, 4,000 g, 4 °C), resuspended in ice-cold TFB-2 buffer (composition available in Supplementary Material), and stored as 100 µL aliquots at -80 °C.

2.2.2 *Plasmid purification*

Plasmids were extracted using the Wizard® Plus SV Minipreps DNA Purification System Kit (Promega) according to the manufacturer's instructions.

2.2.3 *Polymerase chain reaction (PCR) amplification*

PCR reactions were prepared on ice. Prior to starting the PCR program, tubes were flicked and spun in a minispin centrifuge. The PCR program followed is described in Figure 1 (Supplementary Material). The composition of the PCR mix is detailed in Table 1.

Table 1. PCR mix for amplification

Component	Amount
Forward primer (10 µM)	2.5 µl
Reverse primer (10 µM)	2.5 µl
Template DNA (10 ng/µl)	3 µl
Nuclease-free water (NFW)	17 µl
New england biolabs (NEB) Q5® High-Fidelity 2X Master Mix	25 µl

2.2.4 *PCR cleanup*

PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System Kit (Promega) according to the manufacturer's instructions.

2.2.5 *Gel extraction*

DNA was purified from agarose gels using the Wizard® SV Gel and PCR Clean-Up System Kit (Promega) according to the manufacturer's instructions.

2.2.6 *Restriction digestion*

Digestion mixtures were prepared according to Table 2, mixed gently by flicking, and briefly spun. The mixtures were incubated at 37 °C for 5.5 hours in a thermocycler using restriction enzymes from Promega.

Table 2. Mixture for restriction digestion

Component	Amount
Restriction enzyme	1 µl each
Buffer (B)	2 µl
Bovine serum albumin (BSA)	0.2 µl
MilliQ water	up to 20 µl
Template DNA	1 µg

2.2.7 *Gel electrophoresis*

A 1% agarose gel was prepared by dissolving 1 g of agarose in 100 mL of 1X TAE buffer (composition available in Supplementary Material). The solution was microwaved for 2-3 minutes with occasional swirling and cooled to 50 °C. Ethidium bromide (EtBr) was added to a final concentration of 0.2-0.5 µg/mL. The gel was cast and allowed to solidify for 20-25 minutes at room temperature. Samples were mixed with loading dye, and electrophoresis was run at 80-120 V until the dye front reached approximately 75-80% of the gel length (~1-1.5 hours). The gel was visualized under UV light.

2.2.8 *Ligation*

Reaction components (Table 3) were assembled in a microcentrifuge tube on ice, with T4 DNA Ligase added last. The molar ratio of insert to plasmid was maintained at 3 : 1 using the NEBio Calculator. After gentle mixing and a brief spin, the reaction was incubated overnight at 16 °C. Heat inactivation was performed at 65 °C for 10 minutes, and the mixture was chilled on ice before transformation.

Table 3. Mixture for ligation

Component	Amount
T4 DNA Ligase Buffer (10 X) (should be thawed and resuspended in room temperature)	2 µl
Vector DNA 4 kb	50 ng (0.020 pmol)
Insert DNA (1 kb)	37.5 ng (0.060 pmol)
Nuclease free water	up to 20 µl
T4 DNA ligase	1 ul

2.2.9 *Transformation*

Competent cells (100 µL) were taken from -80 °C and placed on ice. The ligation mixture (10 µL) or miniprep plasmid (1-2 µL) was added carefully. The mixture was incubated on ice for 30 minutes, followed by a 45-second heat shock at 42 °C. Cells were then incubated in 1 mL of super optimal broth with catabolic repressor (SOC) media at 37 °C for 1.5 hours with shaking. After incubation, 100 µL of the culture was spread on an agar plate, and the remaining culture was centrifuged, resuspended in a small volume of SOC, and spread onto a second plate. Plates were incubated at 37 °C for 12-16 hours.

2.2.10 *Colony polymerase chain reaction (PCR)*

PCR tubes were prepared with 10 µL of NFW. Colonies were picked, streaked onto a new plate, and transferred to PCR tubes containing NFW. A reaction mixture (Table 4) was added to the tubes. After spinning, PCR was performed following the program outlined in Figure 1 (Supplementary Material).

Table 4. Mixture for colony PCR

Component	Amount
Go Taq Green DNA Master Mix	12.5 µl
Template DNA	5 µl
VF2	1 µl
VR	1 µl
Nuclease free water	5.5 µg

2.3 *DNase I biofilm assay*

2.3.1 *Protocol for studying biofilm formation*

A microtiter plate assay was used to assess bacterial adherence. Bacteria were grown in 96-well plates, rinsed to remove planktonic cells, and stained with crystal violet dye. Cultures were inoculated into a growth medium and incubated overnight at 37 °C. Pseudomonas cultures were diluted to 0.1 OD and added to 96-well plates. After a 72-hour incubation at 37 °C, non-adherent cells were removed by washing with phosphate buffer saline (PBS). Wells were stained with 0.1% crystal violet for 15 minutes, washed with PBS, and dried. Stained biofilms were solubilized in 33% acetic acid and incubated for 15 minutes. The absorbance at 595 nm was measured using a plate reader, with 33% acetic acid as the blank.

2.3.2 *Standardization of biofilm assay*

A standard Biofilm test in a 96-well plate was used to characterize DNase I. Pseudomonas aeruginosa was chosen because it is gram-negative and easily available. Before beginning the DNase I treatment, the technique had to be standardised by changing the experimental conditions to find the parameters that would produce the most biofilm. In our trials, the media type was altered, during dilution, and incubation days. Following a review of the literature, luria broth (LB), nutrient broth (NB), and tryptone soytone broth (TSB) were identified to be suitable for the formation of biofilms. To find the optimal incubation period for biofilm growth in 96 well plates at 37 °C, bacterial biofilm was cultivated without DNase I in various media for various duration of incubation (24 hours and 72 hours). How the yield of biofilm varied for two distinct secondary cultures prepared in 96-well plates was also checked. Before adding the primary culture to the 96-well plate, the optical density was diluted to between 0.1 and 0.05 OD with fresh medium. This was then incubated for the optimized 72 hours, according to the results of the previous experiment for various duration of incubation. Here, a variety of media were utilized (LB, NB, and TSB) to see whether the choice of media and dilution had any combined effects on biofilm yield. Biofilm quantification was carried out by measuring the absorbance at 595 nm. In conclusion, the 96-well plate biofilm assay was standardised by selecting NB media for bacterial growth, 0.1 OD, and 72-hour incubation for maximum biofilm output.

2.3.3 *DNase I-biofilm assay*

To optimize the concentration of DNase I required for treatment, the effect of a gradient of DNase I in the range of 0-50 µg/mL was studied. As a negative control for the experiment, uninoculated wells containing media and diluent were treated similarly and the readings obtained were subtracted from the test readings. Positive control wells containing inoculated media without DNase I were considered as “Control A595 nm”. The biofilm percentage reduction was then calculated as:

$$\frac{\text{Control A}^{595}\text{ nm} - \text{Test A}^{595}\text{ nm}}{\text{Control A}^{595}\text{ nm}} \times 100$$

In order to determine the optimum concentration and time for DNase I treatment, an assay was done at two levels:

1. **Pretreatment:** It was done to determine the optimum concentration of DNase I which gives maximum degradation. A serial dilution of DNase I (0-25 µg/ml) was prepared in NB media. Primary cultures were directly diluted to this media and added to 96 well plates as per the procedure mentioned in the above protocol. The layout used can be seen in Figure 2 available in supplementary material. Biofilm was allowed to develop in the presence of DNase I for 72 hours. Plates were washed and processed as per the procedure mentioned in the above protocol. Absorbance was measured at 595 nm to quantify biofilm. Biofilm percentage reduction at different concentrations was compared and analysed.

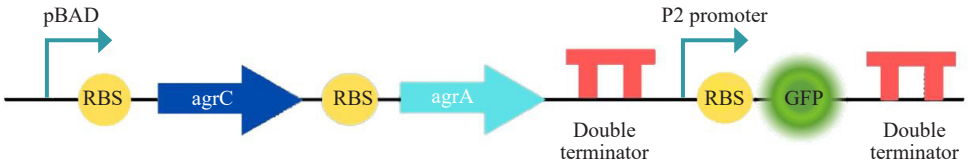


Figure 2. The regulatory circuit comprises agrC and agrA genes, which are constitutively expressed via the pBAD promoter featuring a strong ribosomal binding site and a double terminator. P2 promoter downstream is activated in the presence of AgrA. Later to test if the AIP I sensing module was working properly or not, a GFP reporter gene was incorporated downstream of the P2 promotor. The reasoning was that in the presence of AIP-I molecules, the P2 promoter could sense and activate the GFP gene which would then give an effective readout, hence confirming that the model senses AIP-I in its presence. RBS: ribosome binding site

2. **Posttreatment:** This was done to determine the optimum time required for maximum biofilm degradation for the optimal concentration determined by pretreatment. Pseudomonas biofilm was allowed to develop in 96 well plates for 72 hours as per the protocol mentioned before. Well constituents, particularly bacteria, were then washed off in dH20. Media containing optimum concentration determined by pretreatment was then added into each well and incubated for different times ranging from 0-50 minutes. Plates were further processed by adding Crystal violet followed by Acetic acid. Biofilm quantification was carried out at 595 nm. Biofilm percentage reduction was calculated and further analysed.

3. **Cloning and expression of DNase I:** Initially, the gene fragment of the DNase I coding sequence with biobrick prefix and suffix which was synthetically obtained from Twist bioscience was amplified using Routine PCR and biobrick-specific primers. This was then ligated into the linearized plasmid backbone of PSB1C3 after restriction digestion by EcoRI and PstI following normal bio brick assembly. *E. Coli* DH5α was transformed and further verified using colony PCR using sequencing primers. The size of the gene fragment after amplifying with sequencing primer was simulated as 1,161 bp using Snapgene in-silico PCR simulator. For expressing the protein in *E. Coli* BL21, a composite part of DNase I was cloned, which consists of Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible Plac promoter (R0010) along with strong Ribosome Binding Site (B0034) and double terminator (B0015). This part was obtained synthetically from Twist Bioscience. The gene fragment was amplified by Routine PCR using biobrick-specific primers and then ligated to the linearized plasmid backbone of PSB1C3 following normal biobrick assembly. Double digestion by EcoRI and PstI was done using optimized protocols for double digestion. *E. Coli* DH5α was initially transformed and transformants were screened and verified using colony PCR using sequencing primers. Snapgene in silico PCR simulator was used to determine the size of gene fragments after colony PCR. Further, the plasmids were extracted from transformed colonies and transformed *E. Coli* BL21 for expressing the protein. There was not enough time to achieve protein purification due to COVID-19 restrictions and hence industrially prepared bovine pancreatic DNase I.

2.4 *Fluorescence assay to assess the improved AIP-I sensor*

The assembly of 4 fragments (including the plasmid backbone) was performed using the NEB HiFi DNA Assembly kit. An LB agar plate containing bacteria with a unidirectional circuit and another LB agar plate with a bidirectional circuit containing bacteria were obtained. Separate primary cultures of both bacteria were provided in 10 ml of LB agar supplemented with Chloramphenicol. The cultures were incubated overnight at 37 °C. Autoclaved conical flasks (250 ml) were filled with 25 ml of fresh LB media (Containing chloramphenicol). Half of the flasks were inoculated with 1 ml

of the primary culture of unidirectional circuit bacteria, while the other half were inoculated with 1 ml of the primary culture of bidirectional circuit bacteria. The flasks were incubated at 37 °C until reaching an OD of 0.6 at 600 nm. Different concentrations of AIP-I molecules were added to induce the flasks. To assess fluorescence, a Polystyrene Nunc black 96-well fluorescence plate was acquired. Triplicates of 100 µl samples from each flask were added to the 96-well plate. LB media (100 µl) was added to each well for sample dilution. As a negative control, 200 µl of LB media was added to three wells. Fluorescence measurements were taken using a plate reader with an excitation wavelength of 395 nm and a fluorescence wavelength measured of 509 nm. The flasks were incubated at 30 °C to allow for protein expression, and fluorescence was measured at various time points. Fluorescence from negative control (LB medium) was subtracted from the readings to obtain net fluorescence.

2.5 *Molecular modeling*

2.5.1 *Molecular docking and molecular mechanics poisson-boltzmann surface area (MM-PBSA)/molecular mechanics generalized born surface area (GBSA) based free energy calculations*

In order to characterize the efficacy of engineered nisin (Nisin PV) molecular docking of Nisin A and Nisin PV was performed respectively with the NSR. Nisin A structure was mutated using PyMol and a PRO-VAL mutation was added to the 29th and 30th residues respectively to produce Nisin PV. These studies were inspired by the bioengineering nisin study [18]. Residues 22 to 34 from the N-terminus of the protein data bank (PDB) structure with ID 1WCO were used as a representative of the segment of Nisin that enters the tunnel region of NSR for cleavage.

For the NSR molecule, the PDB ID 4Y68 was used. Waters associated with proteins were removed and polar hydrogens were added during pre-processing. Kollman charges were added to the protein. To determine a starting configuration for the NSR-nisin complex, a docking program Autodock for ligand and protein binding was used where the Nisin (A/PV) was set as ligand and NSR was set as the receptor. The docking of Nisin A with the tunnel region of NSR was performed and the lowest energy stage was the starting configuration. The docking location was found to be consistent with the ligand-binding site of Nisin as discussed in the literature [18]. The ligand-binding site for Nisin A was noted and Nisin PV was docked using the same coordinates.

The ensemble of poses generated from protein-protein molecular docking was fed into a MM/PBSA and MM/GBSA implementation to perform binding free energy estimations and rescore the binding poses. Residue-wise calculations for each Nisin subtype were also performed. The HawkDock server [19] was used for this purpose.

3. Results

3.1 *Detecting the presence of staphylococcus aureus through AIP-I-sensor*

In *Staphylococcus aureus*, the causative pathogen of Bovine Mastitis, agrA, and agrC are essential components of the agr quorum-sensing system, which enables bacterial communication based on population density [20]. agrC functions as a sensor histidine kinase that detects and binds to AIP-I produced by neighboring bacteria. Upon AIP-I binding, agrC undergoes autophosphorylation, activating agrA, which then regulates the expression of quorum-sensing pathway genes, including those controlling virulence factors, RNAIII and Rot [21, 22].

To explore the potential of using this quorum-sensing mechanism for pathogen detection, the agrC-agrA twocomponent signal transduction system was introduced into *Lactococcus lactis* LMG 7930. The engineered strain was designed to sense AIP-I molecules produced by *S. aureus*, providing a foundation for quorum-sensing-based genetically modified organisms (GMOs) for targeted Bovine Mastitis treatment.

A genetic circuit incorporating agrC and agrA under constitutive expression (pBAD promoter, iGEM registry: B0034) was constructed to facilitate AIP-I detection. The regulatory module included a double terminator (iGEM registry: B0015) to ensure stable expression. In the presence of AIP-I, activated agrA binds to the P2 promoter, increasing downstream gene expression. To assess the functionality of this sensing module, green fluorescent protein (GFP) was placed under the P2 promoter, allowing fluorescence detection upon AIP-I exposure (Figure 2). Exogenous AIP-I supplementation was used to evaluate the module's responsiveness, demonstrating that the system successfully detected and activated downstream gene expression in response to AIP-I.

3.1.1 *AIP-I production*

AIP-I molecules were produced in *E. coli* BL21 carrying the agrB-agrD construct under the IPTG-inducible pLac promoter. Following IPTG induction at 30 °C for 10 hours, the culture supernatant was collected, filtersterilized, and lyophilized.

3.1.2 *Verification of AIP-I presence and activity*

The presence of AIP-I was assessed using a GFP-based reporter system. *E. coli* BL21 containing the agrA-agrC system and the P2-GFP construct was incubated with or without AIP-I. Fluorescence intensity was measured at the initial time point and subsequently at hourly intervals from 5 to 10 hours. A significant increase in fluorescence was observed in the AIP-I-treated samples compared to the untreated controls (Figure 3), confirming the activation of the reporter system.

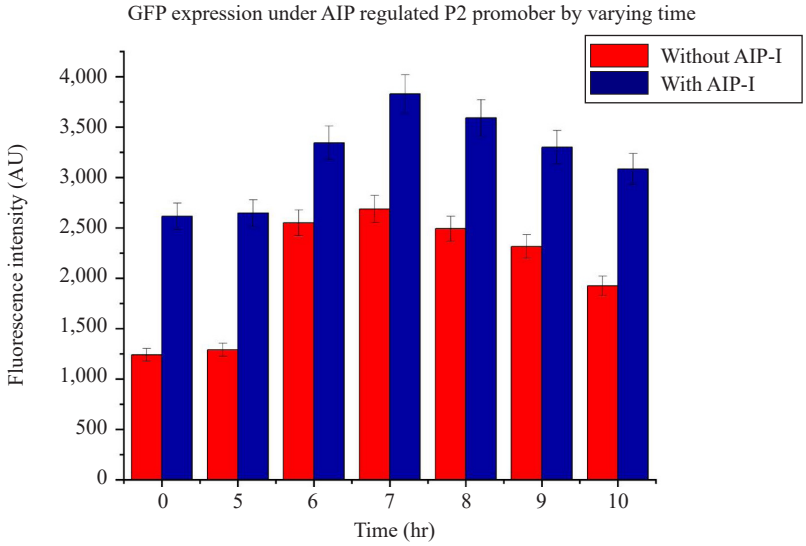


Figure 3. GFP expression with AIP-I alongside without AIP-I across varying time. The error bar represents the standard deviation of the results

3.1.3 *Assessing efficacy of AIP-I-sensor by concentration-dependent activity*

The relationship between AIP-I concentration and GFP expression was evaluated (Figure 4). *E. coli* BL21 cells expressing the AIP-I sensor and GFP reporter construct were exposed to varying concentrations of AIP-I. Fluorescence measurements indicated a positive correlation between AIP-I concentration and GFP expression. These results confirm the dose-dependent activation of the sensing and regulatory module (agrC, agrA, P2, and GFP).

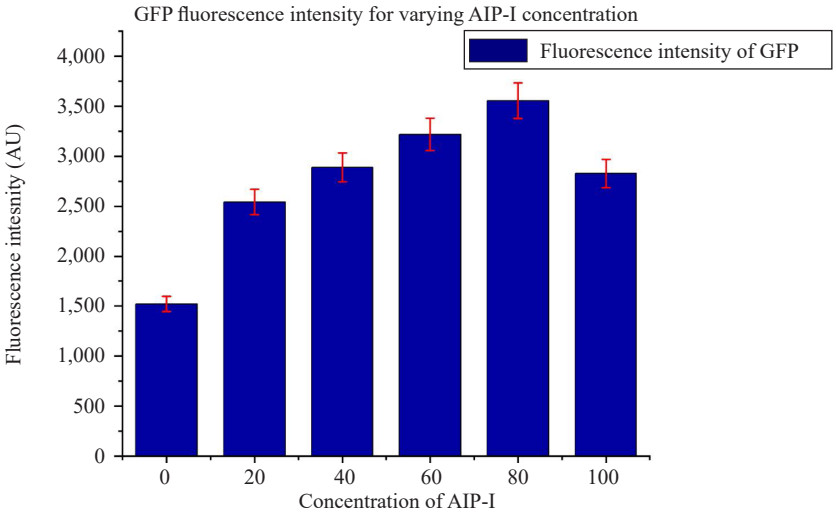


Figure 4. GFP expression with varying concentration of AIP-I. The error bar represents the standard deviation of the results

It is important to note that at very high concentrations of AIP-I, the fluorescence intensity decreased, suggesting the occurrence of toxicity resulting from the elevated concentration of AIP-I.

3.2 *DNase I inhibits biofilm formation*

The ability of DNase I, regulated by the P2 promoter, to degrade biofilms was assessed. To establish the optimal conditions for biofilm degradation, biofilm formation by *Pseudomonas aeruginosa* was evaluated under different growth conditions.

3.2.1 *Concentration-dependent DNase I activity*

Biofilm formation was quantified under varying DNase I concentrations and incubation times. As shown in Figure 5, biofilm formation was highest in NB after 72 hours of incubation. Additionally, cultures with a starting OD of 0.1 consistently produced more biofilm than those with an OD of 0.05 (Figure 6). These findings establish the optimal conditions for assessing DNase I activity.

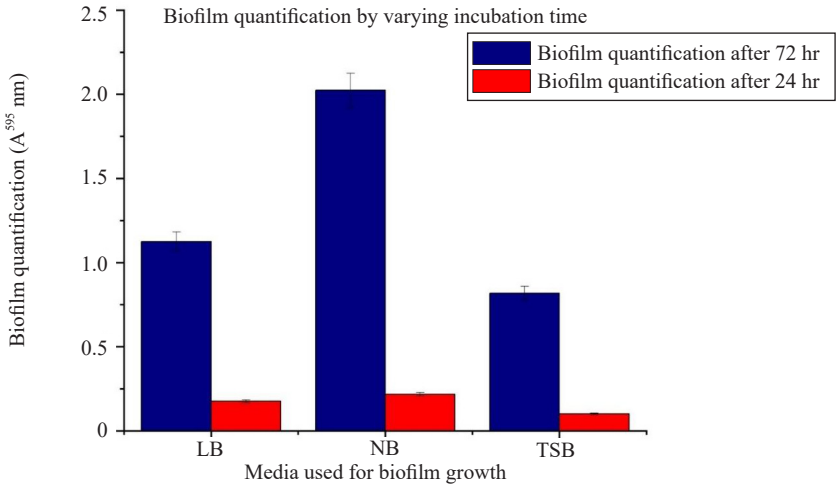


Figure 5. Biofilm yield in 96-well plate comparison in different growth media broths with incubation at 37 °C for 72 and 24 hours. The error bar represents the standard deviation of the results

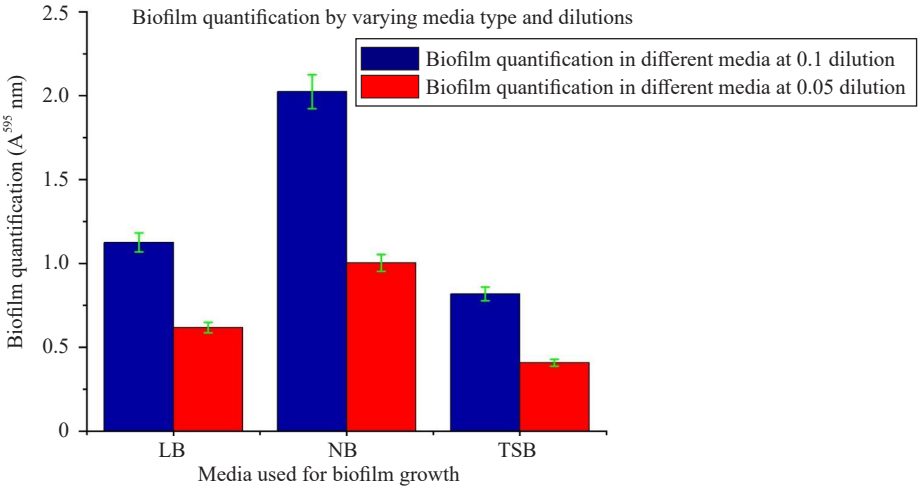


Figure 6. Biofilm yield comparison in different growth media with starting OD of 0.05 and 0.1 on incubation at 37 °C for 72 hours. The error bar represents the standard deviation of the results

The activity of DNase I on biofilm was quantified in terms of biofilm percentage reduction (BPR) calculated by the equation in section 2.3.3.

Initially, the effect of DNase I on biofilm formation was assayed by supplying different concentrations of DNase I (0, 5, 10, 15, 20, 25 µg/ml) in NB, as seen in Figure 7. The biofilm was quantified after incubation at 37 °C for 72 hours. 10 µg/ml was able to reduce biofilm formation by 43%; subsequent increase in DNase I concentration did not reduce biofilm formation. Subsequently, the ability of 10 µg/ml DNase I to degrade pre-formed biofilm was tested with varying treatment duration. The biofilm was allowed to form in NB for 72 hours at 37 °C. The preformed biofilm was treated with 10 µg/ml DNase I and incubated at 37 °C for a different duration before quantifying the residual biofilm. Incubation for 40 minutes degraded 82% of the biofilm. These can be seen in Figure 8. Hence, this experiment proves that DNase I can degrade biofilm and supports the working hypothesis of DNase I's ability to degrade biofilm produced by *S. aureus*.

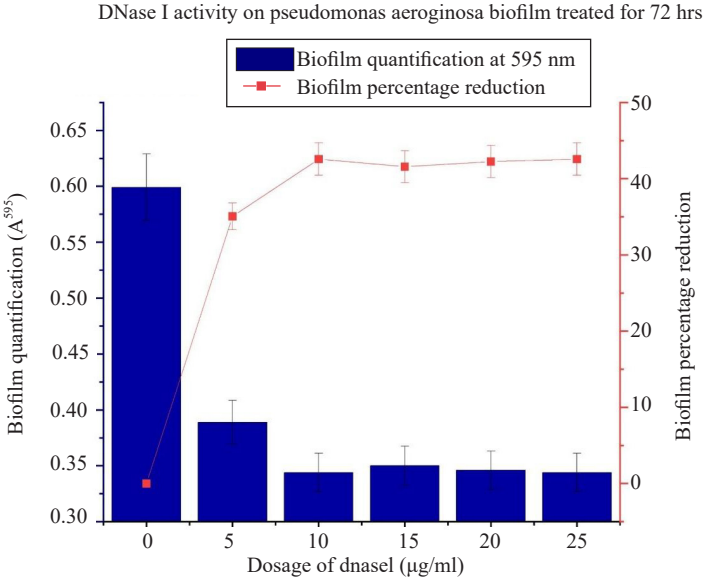


Figure 7. Biofilm formation in NB in the presence of different DNase I concentrations after 72 hours incubation at 37 °C. The error bar represents the standard deviation of the results

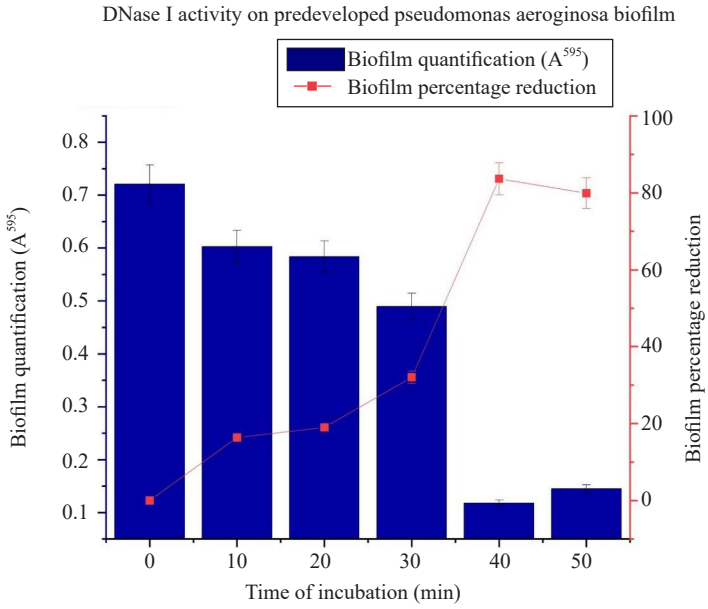


Figure 8. Quantification of biofilm degradation with time by treating pre-formed biofilm with 10 µg/ml DNase I and incubating at 37 °C for different durations. The error bar represents the standard deviation of the results

3.3 *Molecular modelling*

3.3.1 *Molecular docking of nisin with NSR*

The docking results are summarized in Table 5. The highest magnitude pose corresponds to the binding of Nisin A/PV to the tunnel region of NSR, as shown in Figure 9.

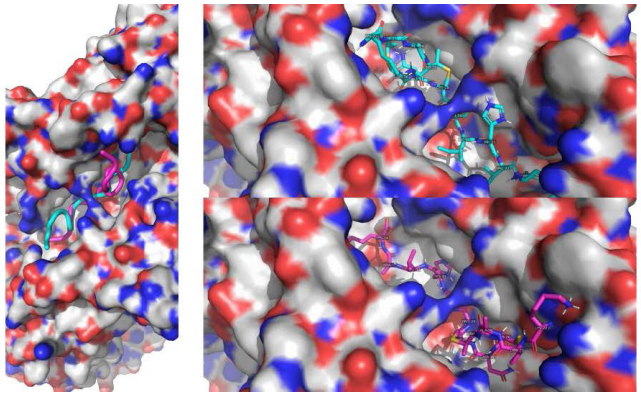


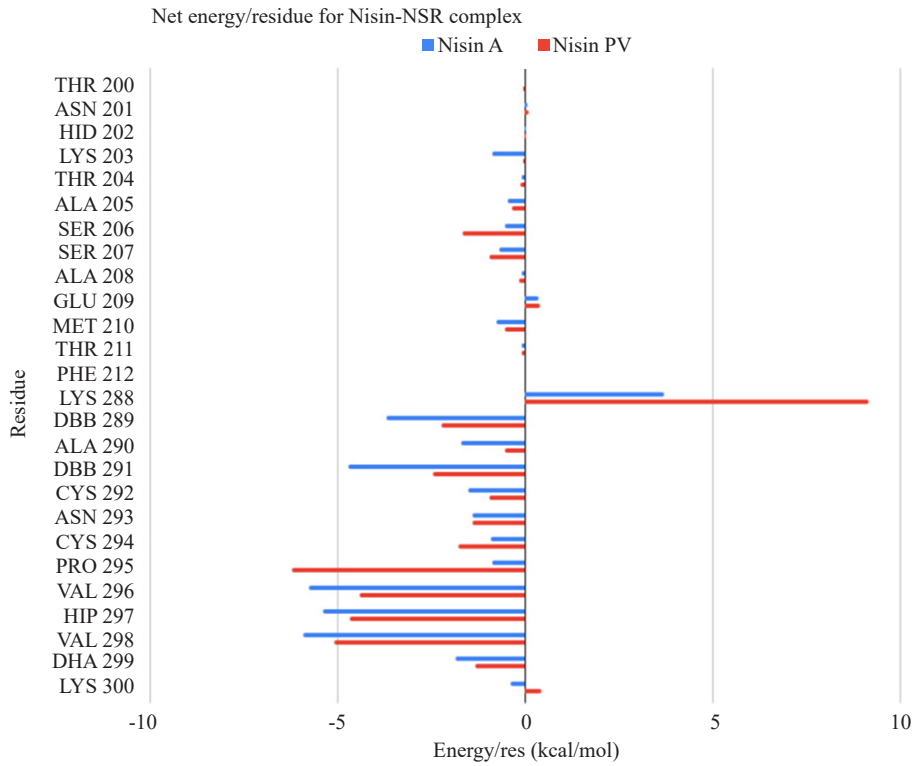
Figure 9. Docking of residues 22-34 N-terminus end of NisinA (cyan) and Nisin PV (magenta) to the tunnel region of NSR. Only the lowest free energy poses are shown

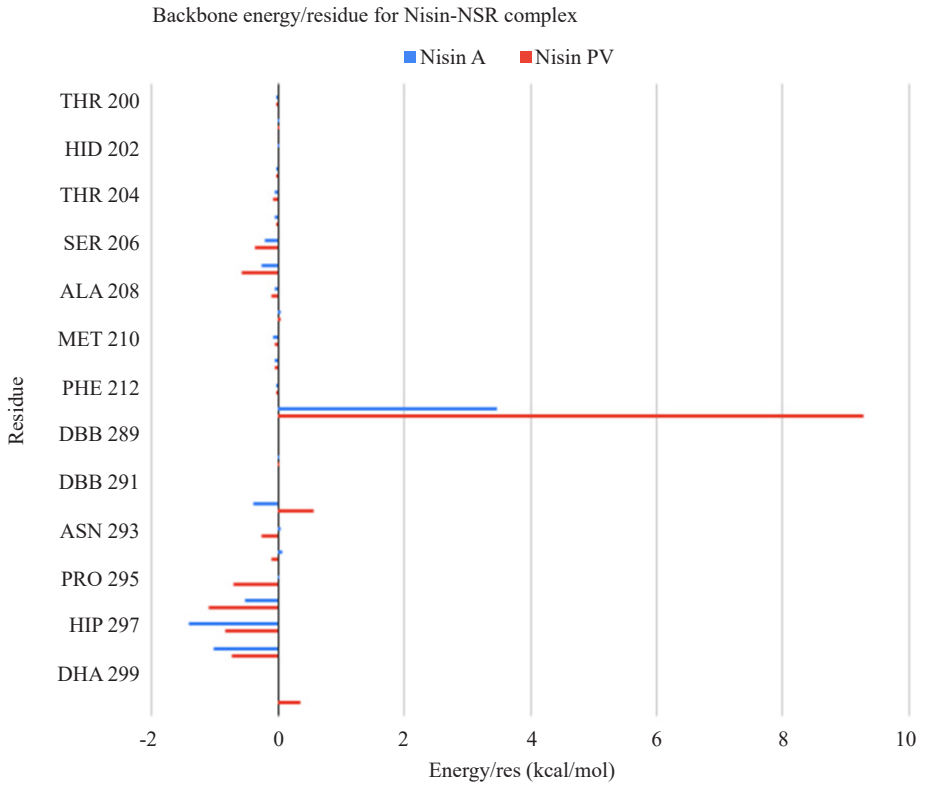
Table 5. Affinity (Autodock score) of Nisin A vs Nisin PV for top 9 docking poses. The lowest free energy pose corresponds to the docking of Nisin in the tunnel region of NSR

Pose	Affinity (kcal/mol)	
	Nisin A	Nisin PV
1	-9.3	-9.5
2	-8.9	-9.1
3	-8.7	-9
4	-8.7	-8.9
5	-8.7	-8.8
6	-8.6	-8.8
7	-8.6	-8.7
8	-8.5	-8.4
9	-8.5	-8.4

3.3.2 *MM-PB/GB-SA based free energy calculations*

MMPBSA calculations (Figure 10) reveal that at residue 29, the residue at which NSR is supposed to cleave Nisin, Nisin PV shows significantly higher total free energy than the native Nisin, indicating that cleavage of Nisin PV is potentially more energetically expensive than cleavage of Nisin A.





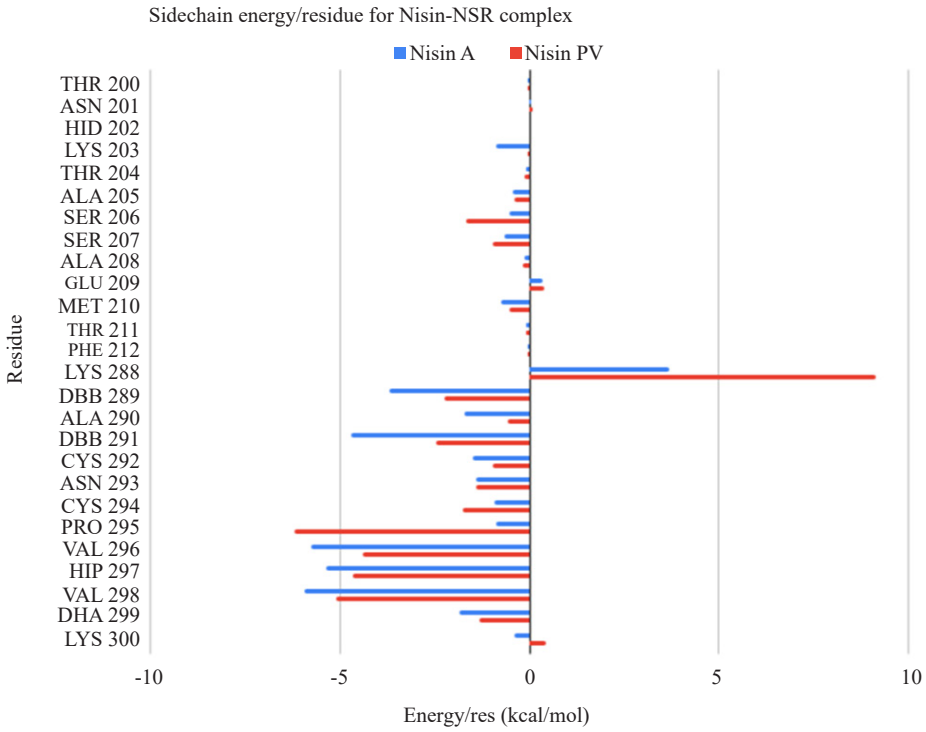


Figure 10. Residue-wise free energy for Nisin-NSR interaction

4. Discussion

A GMO-based therapy has been proposed to target the quorum-sensing system of *Staphylococcus aureus*, a common causative agent of bovine mastitis. This approach leverages the quorum-sensing pathway of *S. aureus* to induce DNase I production in response to bacterial presence. The proposed strategy ensures that the genetic circuit is activated only when *S. aureus* cells are present, and the AIP-I concentration exceeds a specific threshold. Future refinements of this study could involve quantifying the precise AIP-I concentration required for activation to standardize the system's functionality. The selected promoter ensures that lysis does not occur until a sufficient accumulation of DNase I and Nisin PV has been achieved, facilitating biofilm degradation and increasing bacterial susceptibility to antimicrobial treatments. Due to the higher affinity of phosphorylated AgrA for the P2 promoter compared to P3, the production rate of Nisin PV and DNase I is greater than that of Lysis E7.

Unlike broad-spectrum antibiotics, the bacteriocin Nisin PV exhibits pathogen-specific activity. The genetic circuit incorporates Lysis E7 to lyse the GMO, ensuring the effective release of Nisin PV to target *S. aureus*. This mechanism facilitates both bacteriocin delivery and the elimination of the GMO. Notably, Nisin plays a crucial role in the quorum-sensing system of *Lactococcus lactis*, where extracellular Nisin-A induces the transcription of the *nisin* gene cluster. This cluster encodes proteins responsible for biosynthesis, post-translational modification, immunity, and signaling functions associated with Nisin-A. Previous studies have reported that Nisin-A mutants can still participate in quorum sensing, albeit to varying degrees [23]. Given this background, it is plausible that Nisin PV may interact with the quorum-sensing system of *L. lactis*, potentially leading to the upregulation of the *nisin* gene cluster and enhancing the production of Nisin-A and related peptides. This interaction could further improve the antimicrobial properties of the proposed therapeutic system. However, the present study did not investigate the specific interaction between Nisin PV and *L. lactis* quorum sensing, leaving this as an avenue for future research.

To prevent unintended gene transfer, a kill switch can be introduced to induce lysis of the GMO in the absence of the target pathogen. The *thyA* gene in the chassis organism could be modified using CRISPR-Cas9 [24], rendering the mutant unviable in environments with low thymidine or thymine concentrations. The *thyA* mutation results in thymidine auxotrophy, preventing the bacteria from synthesizing thymidine and making them reliant on an external supply [25]. During cultivation in a bioreactor, thymidine could be supplemented to sustain the bacteria. The final formulation could be stored at 4 °C with thymidine supplementation to maintain minimal bacterial growth. Upon introduction into the udder, the bacteria would proliferate in the presence of thymidine. In the event of pathogen detection, Lysis E7 would lyse the GMO, facilitating bacteriocin release. If no pathogen is encountered, the absence of thymidine in the udder environment would cause the chassis to perish, preventing horizontal gene transfer and minimizing unintended microbiome disruption.

Compared to conventional antibiotic treatments for bovine mastitis, this approach offers several advantages. First, it specifically targets *S. aureus*, minimizing collateral damage to the microbiota. However, experimental validation of Nisin PV's impact on the udder microbiome remains necessary [26, 27]. *S. aureus* is a major pathogen associated with bovine mastitis [28], and reducing its prevalence could significantly improve treatment outcomes. Second, the strategy reduces antibiotic overuse, thereby mitigating the risk of antibiotic resistance. Third, the biofilm-a major barrier to antibiotic penetration-may be more effectively disrupted by DNase I secretion than by antibiotics alone. Moreover, since quorum sensing is a common mechanism employed by bacterial pathogens to regulate virulence and biofilm formation, this approach may have broader applications beyond bovine mastitis.

The chassis organism selected for this therapeutic system is the probiotic strain *L. lactis* LMG7930. The treatment could be administered as a single-dose intramammary injection via the teat canal [29]. To ensure the safety and efficacy of this GMO-based therapy, further in vivo studies are required to assess interactions with the native microbiome, evaluate potential environmental risks, and optimize delivery mechanisms. This study provides a proof-of-concept for the development of GMO-based antibacterial treatments, which could have significant implications for the future of antimicrobial therapy.

While the designed kill switch minimizes the risk of unintended gene transfer, horizontal gene transfer (HGT) remains a consideration in environmental applications. Integrating the kill switch into the bacterial chromosome reduces plasmid loss and limits gene mobility, but rare events such as natural transformation or transduction cannot be entirely ruled out. Additionally, the potential impact of the GMO on the bovine udder microbiome is unknown and would

require further investigation to assess microbiome resilience and potential off-target effects. Any future application of this approach would need to adhere to strict biosafety and regulatory guidelines to ensure containment and minimize ecological risks. Moreover, strategies such as environmental DNA monitoring or quantitative real-time PCR (qPCR)-based detection could be employed to track the persistence of genetic material, reinforcing containment measures.

In this approach, *Staphylococcus aureus* is targeted, because it is responsible for almost half of the cases of bovine mastitis [9]. Mycobacteria as the primary causes of bovine mastitis are considered of low occurrence [30]. Commonly, it is the lipophilic species of the *Corynebacterium* genus that cause mastitis and they usually have prolonged incubation time and more meticulous or finicky growth requirements [31].

The authors of this study acknowledge that all concentration measurements were conducted in *Escherichia coli*, and while the experiments specifically focused on the impact of DNase I on biofilm formation in *Pseudomonas aeruginosa*, the study was not extended to or involved experiments with *Staphylococcus aureus* biofilm. It is well-documented that extracellular DNA (eDNA) is a significant component of biofilms formed by both *Pseudomonas aeruginosa* [32] and *Staphylococcus aureus* [33]. Given that DNase I is an enzyme known to degrade eDNA, the study is based on the working hypothesis of expecting similar outcomes with *Staphylococcus aureus* biofilm degradation as observed in the experiments with *Pseudomonas aeruginosa*. This working hypothesis is supported by the shared reliance of these bacterial species on eDNA as a structural component of their biofilms.

The proposed solution is sustainable as it reduces dependence on traditional antibiotics, thereby lowering the risk of antimicrobial resistance and minimizing antibiotic residues in dairy products. By leveraging a targeted bacteriocin (Nisin PV) and a biofilm-disrupting enzyme (DNase I), this approach is more effective against *Staphylococcus aureus* than conventional treatments, which often struggle with resistant strains and biofilm-associated infections. Furthermore, the genetically engineered system ensures precise release of therapeutic agents, minimizing environmental contamination. However, further in vivo validation is necessary to assess long-term efficacy, regulatory considerations, and large-scale implementation feasibility.

In conclusion, this study provides a preliminary exploration of a quorum-sensing-based approach to combat bovine mastitis using bioengineered defensins. While our findings highlight the potential of this strategy, further research is necessary to assess its feasibility, efficacy, and safety in real-world applications. This work lays the groundwork for future investigations aimed at developing sustainable alternatives to antibiotic-based treatments in the dairy industry.

Resource availability

All materials were sourced from organizations mentioned in the Funding section of the manuscript. Data generated from the work is available on the iGEM website. Data from Mathematical Modelling has been uploaded to GitHub.

Author contributions

All the authors have contributed equally to this work.

Funding

The project is supported by IISER Kolkata, Promega Corporation, NEB, Genscript, and the iGEM Impact Grant supported by the Fredrick Gardner Cottrell Foundation.

Acknowledgements

The authors would like to thank Mr. Anshuman Jaysingh, Ms. Lakshmi Prakash, Ms. Soumi Bhattacharyya, Ms. Debshruti Biswas, and Mr. Debdeep Chatterjee for their continuous support, guidance, and help during the work. Additionally, we would like to thank Prof. Supratim Dutta for his valuable insights and guidance. Finally, we would

like to thank the Department of Biological Sciences, IISER Kolkata, for helping us with lab equipment to carry out this project.

Conflict of interest

All authors declare no other competing interests.

References

[1] Kebede N, Tilahun A. Review on dairy cow mastitis and its economic impact. *International Journal of Advanced Research in Biological Sciences*. 2023; 10(2): 109-125.
[2] Sinha MK, Thombare NN, Mondal B. Subclinical mastitis in dairy animals: incidence, economics, and predisposing factors. *The Scientific World Journal*. 2014; 2014(1): 523984.
[3] Puerto MA, Shepley E, Cue RI, Warner D, Dubuc J, Vasseur E. The hidden cost of disease: I. Impact of the first incidence of mastitis on production and economic indicators of primiparous dairy cows. *Journal of Dairy Science*. 2021; 104(7): 7932-7943.
[4] Guzmán-Luna P, Nag R, Martínez I, Mauricio-Iglesias M, Hospido A, Cummins E. Quantifying current and future raw milk losses due to bovine mastitis on European dairy farms under climate change scenarios. *Science of the Total Environment*. 2022; 833: 155149.
[5] Thompson-Crispi K, Atalla H, Miglior F, Mallard BA. Bovine mastitis: Frontiers in immunogenetics. *Frontiers in Immunology*. 2014; 5: 493.
[6] Girma A, Tamir D. Prevalence of bovine mastitis and its associated risk factors among dairy cows in Ethiopia during 2005-2022: a systematic review and meta-analysis. *Veterinary Medicine International*. 2022; 2022(1): 7775197.
[7] Paramasivam R, Gopal DR, Dhandapani R, Subbarayalu R, Elangovan MP, Prabhu B, et al. Is AMR in dairy products a threat to human health? An updated review on the origin, prevention, treatment, and economic impacts of subclinical mastitis. *Infection and Drug Resistance*. 2023; 16: 155-178.
[8] Kibebew K. Bovine mastitis: a review of causes and epidemiological point of view. *Journal of Biology, Agriculture and Healthcare*. 2017; 7(2): 1-14.
[9] Birhanu M, Leta S, Mamo G, Tesfaye S. Prevalence of bovine subclinical mastitis and isolation of its major causes in Bishoftu Town, Ethiopia. *BMC Research Notes*. 2017; 10: 1-6.
[10] Guo Y, Song G, Sun M, Wang J, Wang Y. Prevalence and therapies of antibiotic-resistance in *Staphylococcus aureus*. *Frontiers in Cellular and Infection Microbiology*. 2020; 10: 107.
[11] Lister JL, Horswill AR. *Staphylococcus aureus* biofilms: recent developments in biofilm dispersal. *Frontiers in Cellular and Infection Microbiology*. 2014; 4: 178.
[12] Águila-Arcos S, Álvarez-Rodriguez I, Garaiyurrebaso O, Garbisu C, Grohmann E, Alkorta I. Biofilm-forming clinical Staphylococcus isolates harbor horizontal transfer and antibiotic resistance genes. *Frontiers in Microbiology*. 2017; 8: 2018.
[13] Field D, Cotter PD, Hill C, Ross RP. Bioengineering lantibiotics for therapeutic success. *Frontiers in Microbiology*. 2015; 6: 1363.
[14] Cruz KCP, Enekegho LO, Stuart DT. Bioengineered probiotics: synthetic biology can provide live cell therapeutics for the treatment of foodborne diseases. *Frontiers in Bioengineering and Biotechnology*. 2022; 10: 890479.
[15] Benitez-Chao DF, León-Buitimea A, Lerma-Escalera JA, Morones-Ramirez JR. Bacteriocins: an overview of antimicrobial, toxicity, and biosafety assessment by in vivo models. *Frontiers in Microbiology*. 2021; 12: 630695.
[16] 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*. 2017; 12(1): e0169543.
[17] Perez-Ibarreche M, Field D, Ross RP, Hill C. A bioengineered nisin derivative to control *Streptococcus uberis* biofilms. *Applied and Environmental Microbiology*. 2021; 87(16): e0039121.
[18] Field D, Blake T, Mathur H, O'Connor PM, Cotter PD, Paul Ross R, et al. Bioengineering nisin to overcome the nisin resistance protein. *Molecular Microbiology*. 2019; 111(3): 717-731.
[19] Wang E, Sun H, Wang J, Wang Z, Liu H, Zhang JZH, et al. End-point binding free energy calculation with MM/PBSA and MM/GBSA: strategies and applications in drug design. *Chemical Reviews*. 2019; 119(16): 9478-9508.

[20] Thoendel M, Horswill AR. Random mutagenesis and topology analysis of the autoinducing peptide biosynthesis proteins in *Staphylococcus aureus*. *Molecular Microbiology*. 2013; 87(2): 318-337.
[21] Geisinger E, George EA, Muir TW, Novick RP. Identification of ligand specificity determinants in AgrC, the *Staphylococcus aureus* quorum-sensing receptor. *Journal of Biological Chemistry*. 2008; 283(14): 8930-8938.
[22] Boisset S, Geissmann T, Huntzinger E, Fechter P, Bendridi N, Possedko M, et al. *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. *Genes & Development*. 2007; 21(11): 1353-1366.
[23] Kuipers OP, Beerthuyzen MM, de Ruyter PG, Luesink EJ, de Vos WM. Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. *Journal of Biological Chemistry*. 1995; 270(45): 27299-27304.
[24] Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*. 2013; 8(11): 2281-2308.
[25] Hayashi N, Lai Y, Fuerte-Stone J, Mimee M, Lu TK. Cas9-assisted biological containment of a genetically engineered human commensal bacterium and genetic elements. *Nature Communications*. 2024; 15(1): 2096.
[26] Derakhshani H, Fehr KB, Sepehri S, Francoz D, De Buck J, Barkema HW, et al. Invited review: Microbiota of the bovine udder: contributing factors and potential implications for udder health and mastitis susceptibility. *Journal of Dairy Science*. 2018; 101(12): 10605-10625.
[27] Dean C, Slizovskiy I, Crone K, Pfennig A, Heins B, Caixeta L, et al. Investigating the cow skin and teat canal microbiomes of the bovine udder using different sampling and sequencing approaches. *Journal of Dairy Science*. 2021; 104(1): 644-661.
[28] Porcellato D, Meisal R, Bombelli A, Narvhus JA. A core microbiota dominates a rich microbial diversity in the bovine udder and may indicate presence of dysbiosis. *Scientific Reports*. 2020; 10(1): 21608.
[29] Merriman KE, Poindexter MB, Kweh MF, Santos JE, Nelson CD. Intramammary 1, 25-dihydroxyvitamin D3 treatment increases expression of host-defense genes in mammary immune cells of lactating dairy cattle. *The Journal of Steroid Biochemistry and Molecular Biology*. 2017; 173: 33-41.
[30] Schiller I, RayWaters W, Vordermeier HM, Jemmi T, Welsh M, Keck N, et al. Bovine tuberculosis in Europe from the perspective of an officially tuberculosis free country: trade, surveillance and diagnostics. *Veterinary Microbiology*. 2011; 151(1-2): 153-159.
[31] Dobinson HC, Anderson TP, Chambers ST, Doogue MP, Seaward L, Werno AM. Antimicrobial treatment options for granulomatous mastitis caused by Corynebacterium species. *Journal of Clinical Microbiology*. 2015; 53(9): 2895-2899.