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In vitro antibiofilm and quorum sensing inhibition activities of selected South African plants with efficacy against bovine mastitis pathogens

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ABSTRACT

Bovine mastitis is a critical pathology in global dairy herds, causing economic losses due to decreased production and increased culling. The continuous use of conventional antibiotics has led to antimicrobial resistance and treatment failures. The ability of mastitis bacteria to form biofilms is associated with resistance, while quorum sensing plays a vital role in biofilm formation. This complex interplay between quorum sensing (QS) and biofilm formation challenges mastitis management, necessitating the development of alternative therapeutics to combat this microbial threat effectively. This study aimed to investigate the biofilm forming ability (BFA) of 29 bacterial strains isolated from milk from cattle diagnosed with clinical cases of mastitis in Brain Heart Infusion broth (BHI) and Tryptic Soy broth (TSB). Of these strains, 93.10 % (27) and 68.97 % (20) demonstrated BFA in TSB and BHI, respectively. Compared to BHI, TSB appeared to enhance BFA of the bacteria except for *Streptococcus* (*Str.*) *uberis* strains. Four selected South African plants with known antibacterial activities were tested for their antibiofilm and antiquorum sensing activities against the biofilm-forming mastitis isolates. *Searsia lancea* demonstrated antibiofilm forming activity against all organisms tested. All the plants demonstrated good biofilm disruption ability (BDA) against 24 h preformed biofilms of the isolates except for *Erythrina caffra*, while *S. lancea* displayed good BDA against all the 48 hour preformed biofilms of the bacteria. Generally, the plants' antibiofilm activities appeared to improve as the biofilm matured, with few exceptions. The ethanol extracts of *S. lancea* demonstrated MQSIC activity at 2.50 mg/mL, and the lowest $MQSIC_{50}$ value (< 0.08 mg/mL), demonstrating its quorum quenching ability. The ability of these plants, especially *S. lancea*, to inhibit QS and biofilms at various developmental stages may play a pivotal role in managing mastitis infections and curbing the emergence of antimicrobial resistance. Nonetheless, further research is needed to elucidate the precise mechanisms underlying the inhibition of quorum signaling and biofilms, and to identify the specific compounds responsible for the observed activities.

1. Introduction

Bovine mastitis is a critical and multifactorial disease with severe consequences for dairy cow welfare and the industry's economy, causing decreased milk production and milk quality, heightened healthcare expenses, and even animal mortality (Melchior et al., 2006; Viguier et al., 2009; Maity and Ambatipudi, 2021). The disease is characterized as inflammation of the mammary gland, and can have infectious or non-infectious causes (Bradley, 2002). It is an important pathology in dairy herds, with reduced production and increased culling rates contributing to substantial economic losses (Azooz et al., 2020; Sharun et al., 2021). Mastitis is typically classified based on clinical features and etiology, with infectious causes, primarily bacterial infections, being the most common. These bacterial pathogens, including *Staphylococcus (S.) aureus, Streptococcus (Str.) agalactiae, Streptococcus (Str.) dysgalactiae, Streptococcus (Str.) uberis, Escherichia* (*E.*) *coli*, and *Klebsiella pneumoniae*, have been frequently identified in mastitis cases (Klaas and Zadoks, 2018; Ashraf and Imran, 2020; Cadona et al., 2021).

Bovine mastitis can present as subclinical or clinical, ranging from mild to severe cases (Klaas and Zadoks, 2018). Subclinical mastitis, detectable through tests like microbiology (for identification of intramammary infection) and somatic cell counts in milk, lacks apparent clinical signs. In contrast, clinical mastitis exhibits visible milk changes, such as clots or flakes, along with infection and inflammation signs like fever, udder and lymph node swelling, pain, and redness (Ashraf and Imran, 2020; Azooz et al., 2020). While many cases

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of mastitis resolve with standard antibiotic treatment, some can progress to the point of culling, and in severe instances, spontaneous death may occur (Cadona et al., 2021; Sharun et al., 2021).

Antibiotics are the standard treatment for bovine mastitis, but in some cases, antibiotics fail to resolve the infection, leading to chronic mastitis. Incorrect and prolonged antibiotic use in such cases poses a substantial risk by promoting antibiotic resistance, a significant global threat to both human and animal health. It is worth noting that mastitis is the leading cause of antimicrobial administration in farming, particularly in lactating cows, which is why the prudent use of antibiotics in udder health needs to be promoted (Higham et al., 2018). Unfortunately, traditional antibiotic treatments have been associated with the development of antibiotic-resistant strains, extending to commonly used antibiotics like penicillin, amoxicillin, tetracycline, amikacin, gentamicin, and erythromycin. Recent studies have raised concerns about the rising resistance to newer antimicrobials, including piperacillin, ceftazidime, cefquinome, tigecycline, colistin, and vancomycin. Additionally, the issue of drug residues has been on the rise (Carvalho-Castro et al., 2017; Bonardi et al., 2023). This underscores the critical need for effective strategies to manage and prevent bovine mastitis while mitigating the risks associated with antibiotic use in this context.

The ability of bacteria to produce biofilms is one of the various factors associated with antibacterial resistance (Du Preez, 2000; Sordillo, 2005). This highlights the importance of understanding biofilm formation (BF) and its role in chronic infections, emphasizing the challenges it presents for effective antibiotic treatment in both veterinary and human medicine. Biofilms are intricate organisations of microorganisms to form structured communities surrounded by a biopolymer matrix, which is capable of adhering to biological or non-biological surfaces, and it confers increased resistance to antimicrobial agents and the host's immune system, when compared to planktonic cells (Burmølle et al., 2010). Biofilm formation has been suggested to be the default mode of bacterial growth (Flemming et al., 2009). Biofilm-associated infections are often resistant to antibiotic treatment, making them challenging to eradicate (Bjarnsholt et al., 2013).

Strains of the major mastitis pathogens such as *E. coli, S. aureus, Str. uberis, Str. dysgalactiae*, and *Str. agalactiae* have been documented to possess biofilm forming capabilities *in vitro,* and this plays an important role in the virulence of these bacteria, contributing to an important mechanism of protection and resistance to antimicrobials (Melchior et al., 2006; Høiby et al., 2010). The development of biofilms in response to external stimuli has been linked with a variety of genes that encode distinct components, including proteins and extracellular polymeric substances (EPS), which play crucial roles in the formation and establishment of biofilms by these bacteria (Domka et al., 2007; Landini, 2009). Previous research endeavours have reported on genetic elements that undergo distinct patterns of expression in the context of biofilm development by *E. coli*. Employing the technology of DNA microarrays, several studies have contributed to elucidating this intricate process. Among the genes identified as being differentially expressed during biofilm formation are the luxS gene, responsible for the synthesis of AI-2 (autoinducer-2) synthase; the fliC gene encoding flagellin; the fimA gene governing the synthesis of type I fimbriae, and the csgA gene responsible for the production of curli fimbriae (Pratt and Kolter, 1998; De Kievit and Iglewski, 2000). Other studies have revealed specific genetic determinants associated with BF in *Str. agalactiae, Str. uberis* and *Str. dysgalactiae* isolated from milk samples. In *Str. agalactiae*, the set of genes identified as pivotal contributors to BF are rib, cylE, bca, cfb, pavA, scpB, bac, lmb, fbsA, fbsB, PI1, PI2a, and PI2b (Kaczorek et al., 2017; Bonsaglia et al., 2020). Kaczorek et al. (2017) associated sua, pauA/skc, gapC, cfu, lbp, hasA, hasB, and hasC genes with BF in *Str. uberis*, while in *Str. dysgalactiae* they found eno and napr genes. These investigations have substantially advanced the understanding of the genetic determinants and molecular mechanisms underlying BF within streptococcal species and *E. coli* of mastitis origin.

Quorum sensing (QS) represents a pivotal virulence factor intimately associated with and acknowledged to play a fundamental role in the intricate mechanism governing BF (dos Reis et al., 2011). This communication system is a distinctive attribute of bacterial populations, distinguished by the secretion and subsequent detection of signal molecules, termed autoinducers. Once a critical threshold concentration of these autoinducers is achieved within the bacterial community, it triggers a series of orchestrated behaviors, thereby conferring a quorum and leading to a collective response (dos Reis et al., 2011). Notably, in the case of *E. coli*, a noteworthy molecule akin to autoinducer 2 (AI-2) originally identified in *Vibrio harveyi* (where the luxS gene encodes AI-2 synthase) has been ascertained to exert a profound influence on the BF process. Specifically, when AI-2 or its homologue is present in the extracellular milieu at a concentration that falls below a critical threshold, it serves as the instigating factor for a cascade of signal transduction events, ultimately culminating in a substantial alteration of the behavioral traits exhibited by the entire bacterial population (De Kievit and Iglewski, 2000; Prüß et al., 2006). This intricate interplay between QS and BF underscores the sophisticated regulatory mechanisms that bacteria employ to adapt and thrive in environment. This resilience poses a significant challenge in the management and treatment of infectious diseases, necessitating ongoing research and the development of alternative therapeutics to combat microbial threats effectively.

Several plant products have been investigated with the aim of developing alternative products useful in inhibiting BF and QS mechanism in microorganisms (Bouyahya et al., 2017; Vinothkannan et al., 2018; Meng et al., 2022). Hence, the objective of this study was to screen the selected South African plant extracts against biofilm-forming bacteria isolated from clinical cases of bovine mastitis for antibiofilm and quorum sensing inhibition activities. The selected plants in this study have demonstrated good bacterial growth inhibitory activity against major mastitis pathogens (Akinboye et al., 2023), but little to nothing is known about their antibiofilm activity against these mastitis-causing bacteria and their QS inhibitory activity (QSIA). Several similar studies have shown that plants with antibacterial activities have antibiofilm activity and QSIA (Adeyemo et al., 2022; Ogbuadike et al., 2023; Erhabor et al., 2024), and therefore this study hypothesized that the selected plants demonstrate antibiofilm and quorum sensing inhibition activities against biofilm-forming bacteria isolated from clinical cases of bovine mastitis. This study is therefore aimed at determining the antibiofilm activity and quorum sensing inhibition (QSI) of the selected plant extracts.

2. Materials and methods

2.1. Collection of plant material and preparation of the extracts

The plant leaves were collected from two locations: the Manie van der Schijff Botanical Garden at the University of Pretoria and the Onderstepoort campus (Faculty of Veterinary Science) of the University of Pretoria. Herbarium voucher specimens were prepared and stored at the H.G.W.J. Schweickerdt Herbarium, University of Pretoria, South Africa (Table 1). Leaves were placed in open mesh bags and air-dried indoors at room temperature under well-ventilated

Table 1
Selected plants and their herbarium accession number.

Family	Plant species	Accession numbers
Anacardiaceae	*Searsia lancea* (L.f.) F.A.Barkley	PRU 128113
Fabaceae	*Erythrina caffra* Thunb.	PRU 128360
Leguminosae	*Indigofera frutescens* L.f.	PRU 128111
Phyllanthaceae	*Antidesma venosum* E.Mey. ex Tul.	PRU 128361

conditions. The dried leaves were finely ground using a Janke and Künkel model A10 mill. These powdered samples were stored in air-tight glass containers in a dark room at room temperature.

For plant extraction, acetone and ethanol solvents were used. Each solvent involved soaking 20 g of powdered leaves in 200 ml of the respective solvent, with periodic manual agitation. After 24 h, the supernatant liquid was carefully collected and filtered through Whatman No. 1 filter paper into pre-weighed glass vials. The collected extracts were concentrated by drying them under a stream of cold air. This extraction process was repeated three times to ensure thorough extraction. The resulting dried extracts were weighed, and their yields were determined by dividing the mass by the initial quantity of plant material used.

2.2. Test bacterial strains

Bacterial strains (Table 2) were derived from the biobank collection of the Milk Laboratory, Department of Animal Production Studies, Faculty of Veterinary Sciences, University of Pretoria and used for this study. The bacterial strains were stored at -80°C in Tryptic Soy broth (TSB). Brain Heart Infusion (BHI) broth and TSB were used for culturing of the biofilm assays. The bacteria were grown on BHI agar and TSA at 37°C for 18–24 h. The bacterial colonies were then cultured in BHI broth and TSB for at least 18–24 h at 37 °C.

2.3. Antibiofilm assay

2.3.1. Biofilm forming ability assay

The capacity of the bacterial species to form biofilm was evaluated using an adapted technique documented by Stefanović (2018). Overnight cultures of the test bacteria grown in BHI and TSB media were standardized to a 0.5 McFarland standard, equivalent to approximately 1.5×10^8 CFU/mL. The bacteria were further diluted 1:100 in culture media, resulting in an inoculum of around 1.5×10^6 CFU/mL.

Next, 100 μL of the diluted inoculum were dispensed into each well, followed by the addition of 100 μL of culture medium. These plates were then covered and incubated for 24 h. Quantitative determination of the bacterial biofilm formation (BF) was performed using crystal violet staining. The plates underwent a gentle wash with sterile distilled water three times to remove planktonic cells, followed by drying at 60°C for 45 min. Sessile cells were stained with 0.1 % crystal violet (100 μL) for 15 min and then rinsed to eliminate excess stain.

To destain the crystal violet-bound cells adhering to the wells, 150 μL of ethanol was added, and 100 μL of the destained ethanol was transferred into a new microplate. The absorbance was read at a wavelength of 590 nm. The biofilm-forming ability (BFA) was categorized as follows: (a) non-biofilm former if $OD_{test} \leq OD_c$, (b) weak biofilm former if $OD_c < OD_{test} \leq 2 \times OD_c$, (c) moderate biofilm former if $2 \times OD_c < OD_{test} \leq 4 \times OD_c$, and (d) strong biofilm former if $OD_{test} > 4 \times OD_c$. Here, OD_c represents the mean $OD_{media\ ctrl}$ + (3 × standard deviation of $OD_{media\ ctrl}$), and OD_{test} denotes the mean optical density of the tested bacterial strain ($OD_{test} - OD_{media\ ctrl}$).

Table 2
List of clinical isolates and ATCC strains and their laboratory codes.

Species	Types	Laboratory Codes
Streptococcus agalactiae	Clinical isolates	SAG 1 - 7
Streptococcus dysgalactiae	Clinical isolates	SDY 1 - 7
Streptococcus uberis	Clinical isolates	SUB 1 - 7
Streptococcus uberis	ATCC	SUB ATCC 700407
Escherichia coli	Clinical isolates	ECO 1 - 7
Escherichia coli	ATCC	ECO ATCC 25922

ATCC, American Type Culture Collection.

Only bacteria displaying a moderate to strong capacity for BF were considered for subsequent tests focused on inhibiting BF and eradicating preformed biofilms.

2.3.2. Inhibition of biofilm formation – prevention of initial bacterial cell attachment

To assess the potential of acetone and ethanol extracts in preventing bacterial cell mass formation and attachment, the method of Stefanović (2018) was employed. Plant extracts (100 μL) at three sub-MIC concentrations each (0.032, 0.016 and 0.008 mg/mL), and ciprofloxacin (0.02 mg/mL) as the positive control were added in twelve replicates to the wells of 96-well microtitre plates. Subsequently, 100 μL of a standardized bacterial culture (OD_{590nm} = 0.02, equivalent to 1.0×10^6 CFU/mL), in either BHI or supplemented TSB medium, was introduced into these wells and incubated at 37°C for 24 h. After this incubation period, the biofilm biomass was quantified using the crystal violet (CV) staining assay, as previously described for the biofilm formation assay. The percentage of biofilm inhibition was calculated using the following formula:

$$\text{Percentage } (\%) \text{ inhibition} = \frac{(OD_{\text{Negative control}} - OD_{\text{Experimental}}) \times 100}{OD_{\text{Negative control}}}$$

Biofilm inhibition was categorized on a scale from 0 to 100 %. Values below 0 % were recorded as 0 % BF inhibition, those falling between 0 % and 50 % indicated weak anti-biofilm activity, and values exceeding 50 % denoted effective biofilm inhibition. Any values surpassing 100 % were reported as 100 % BF inhibition.

2.3.3. Inhibition of development of pre-formed biofilms – assessment of destruction of biofilm mass

The ability of the extracts to hinder the ongoing development of biofilms or disrupt biofilms that had already formed was investigated. To achieve this, a 100 μL portion of standardized bacterial cultures was introduced at a concentration of 1.0×10^6 CFU/ml into each well of flat-bottomed 96-well microtitre plates. These plates were then incubated at 37°C for two different time periods: 24 h, which corresponds to the irreversible attachment phase, and 48 h, representing the mature biofilm stage. Importantly, during these incubation periods, the plates were kept still to facilitate the formation of multilayer biofilms. After the respective incubation durations, 100 μL portions of plant extracts at various concentrations (0.032, 0.016 and 0.008 mg/mL) were added to the wells of the 96-well microtitre plates, resulting in the desired final concentrations. These plates were further incubated at 37°C for an additional 24 h. Ciprofloxacin (0.02 mg/ml) was used as positive control, while solvents and sterile distilled water served as negative controls.

2.3.4. Crystal violet staining assay

To assess the biofilm biomass, a modified crystal violet (CV) staining assay was employed (Djordjevic et al., 2002) with adjustments outlined by Sandasi et al. (2008). In summary, the assay was initiated by washing the 96-well microtitre plates five times with sterile distilled water. Subsequently, they were air dried and then further dried in an oven at 60°C for 45 min. After this drying process, wells were stained with 100 μL of a 1 % crystal violet solution and incubated at room temperature for 15 min. Following incubation, plates were rinsed three times with sterile distilled water to eliminate any unabsorbed stain. At this point, biofilms could be detected as purple rings along the sides of the wells.

For a semi-quantitative assessment of biofilm formation, 125 μL of ethanol was added to destain the wells. Subsequently, 100 μL portion of the destaining solution was transferred to a fresh sterile plate and absorbance measured at 590 nm using a microplate reader (Bio-Tek Synergy). The mean absorbance of the samples was calculated

and the percentage inhibition of biofilm was determined using the following formula (Sandasi et al., 2008):

$$\text{Percentage } (\%) \text{ inhibition} = \frac{(OD_{\text{Negative control}} - OD_{\text{Experimental}}) \times 100}{OD_{\text{Negative control}}}$$

2.4. Anti-quorum sensing

2.4.1. Inoculum preparation

A sole colony of the *Chromobacterium* (*C.*) *violaceum* ATCC 12472 bacterial strain was first cultivated in Luria-Bertani (LB) broth. This initial culture was then used to inoculate 10 mL of LB broth and incubated overnight in a shaking incubator at 30°C with agitation at 0.76 x g. To create a working bacterial suspension, the culture was diluted with LB broth to achieve an absorbance of 0.1 ± 0.02 at a wavelength of 590 nm, which corresponds to a McFarland standard of 0.5 (equivalent to approximately 1.5×10^8 CFU/mL).

2.4.2. Quantitative detection of violacein inhibition in the presence of plant extracts

Stock solutions of plant extracts were prepared in DMSO at a concentration of 10 mg/mL for each extract. The positive control, vanillin, was similarly prepared in LB broth, reaching a concentration of 10 mg/mL. In 48-well plates, 500 μL from the 10 mg/mL samples of plant extracts were added into the first well, which was designated for extract samples. Then, 500 μL was drawn from the 10 mg/mL of vanillin and placed in the first well designated for the positive control. The wells for both extract samples and the positive control underwent serial dilutions, where 500 μL was pipetted from the first well, progressively diluted down to the last well, and the final 500 μL was discarded from the last wells. Then, 500 μL of the *C. violaceum* culture were introduced into each well, except for those designated as blanks (comprising sample blanks and positive control blanks). Subsequently, 500 μL of LB broth was added to all wells, ensuring a total volume of 1000 μL in each well. The blank wells contained only 1000 μL of media without samples. Following this, the concentrations of the samples and control in the wells ranged from 2.5 to 0.08 mg/mL.

The plates were covered with parafilm and then incubated for 24 h in an orbital shaker set at 140 rpm and maintained at 30°C. After the incubation period, the QSIA was evaluated based on two factors: growth (turbidity) and pigmentation (purple color). The minimum inhibitory concentration (MIC) value was determined as the lowest sample concentration that visibly inhibited the growth of *C. violaceum* (Ahmad et al., 2015). Similarly, the minimum quorum sensing inhibitory concentration (MQSIC) value was established as the lowest sample concentration that visibly hindered pigmentation.

2.4.3. Violacein quantification

After 24 h of incubation of the treated *C. violaceum* culture, 1 mL was transferred from each well into new, identical 48-well plates. These plates were then subjected to centrifugation at 1800 x g using an EBA Hettich centrifuge from Labotec for 20 min to precipitate the bacteria containing violacein. The resulting supernatant was discarded. Then, 1 mL of 100 % DMSO was added to the bacterial pellet that remained at the bottom of each well, and the plates were vortexed until the pellet was completely dissolved. Subsequently, the plates underwent another round of centrifugation at 1800 x g for 20 min to separate the bacterial cells from the solution. From each well, 200 μL of the supernatant was transferred into wells of 96-well microtiter plates in duplicate. The absorbance was measured at a wavelength of 595 nm. The percentage inhibition of violacein production was determined using the following formula:

$$\text{Percentage violacein inhibition} = \frac{(\text{meanOD}_{\text{control}} - \text{meanOD}_{\text{test}}) \times 100}{\text{meanOD}_{\text{control}}}$$

The concentration of each sample required to inhibit 50 % of violacein production was determined as the $MQSIC_{50}$ using a regression line between the % violacein inhibition and their respective concentrations.

2.5. Statistical analysis

The data was input, organized and analyzed in Microsoft Excel 365. The mean and standard error of means, or mean and standard deviations where appropriate, for the different assays, were determined. Percentages were also calculated. The $MQSIC_{50}$ and LC_{50} were determined using linear and non-linear regression curves where necessary.

3. Results

3.1. Biofilm forming ability of the bacterial isolates

This study evaluated the biofilm-forming ability of 29 bacterial strains using two different growth media, namely Brain Heart Infusion broth (BHI) and Tryptic Soy broth (TSB), following a 24 h incubation period. The results are summarized in Table 3 and Fig. 1. Among the bacterial strains cultivated in BHI, 68.97 % (20) demonstrated biofilm-forming abilities (BFA), albeit to varying degrees. Notably, 20 % (4) of these strains exhibited strong BFA, 35 % (7) were categorized as moderate biofilm formers, and the remaining 45 % (9) were considered weak biofilm formers. In contrast, when the same strains were grown in TSB, a higher percentage, specifically 93.10 % (27), displayed BFA. Within this group, 18.52 % (5) were identified as strong biofilm formers, 25.93 % (7) as moderate biofilm formers, and the majority, 55.56 % (15), fell into the category of weak biofilm formers.

Interestingly, each bacterial species exhibited distinct BFA in the two different growth media. All *Str. agalactiae* isolates were found to express their BFA in both BHI and TSB. However, three isolates (SAG2, SAG3, and SAG6) showed an enhancement in biofilm formation when cultivated in TSB compared to BHI, whereas one isolate (SAG1) displayed a decline in biofilm-forming ability in TSB. Similarly, in the case of *Str. dysgalactiae*, five strains (SDY1, SDY3, SDY4, SDY5, and SDY6) exhibited improved BFA in TSB, one remained unchanged, and one showed a reduction in biofilm formation. Among the *E. coli* isolates, five (ECO2–6) demonstrated enhanced biofilm-forming abilities when grown in TSB, while two isolates remained unaffected by the change in medium. Conversely, when assessing *Str. uberis* strains, four of them (SUB2, SUB4, SUB7, and SUB ATCC 700407) displayed unimproved biofilm-forming abilities in TSB compared to BHI. Furthermore, the remaining four *Str. uberis* isolates experienced a decrease in biofilm formation when cultured in TSB.

3.2. Anti-biofilm activity

To evaluate the anti-biofilm activities of eight different extracts at three sub-minimum inhibitory concentrations, this study focused on the isolates; SAG5, SDY5, SUB6 and ECO4, as these strains had the best biofilm-forming capabilities within their respective species. The choice between using TSB or BHI broth for the experiments was determined by the medium that better enhanced biofilm formation for each particular species.

The results of these investigations are presented in Table 4, where the percentage of biofilm inhibition is documented. In our analysis, any extract displaying an inhibition percentage exceeding 50 % was considered to possess substantial anti-biofilm activity. Conversely,

Table 3
Comparative biofilm forming ability of the organisms in Brain Heart Infusion broth (BHI) and Tryptic Soy broth (TSB).

Biofilm forming ability of the organisms in Brain Heart Infusion broth (BHI) and Tryptic Soy broth (TSB)				
Organism	BHI	TSB	Variance	Preferred Medium
SAG1	M	W	Neg	**TSB**
SAG2	M	S	Pos	
SAG3	W	S	Pos	
SAG4	M	M	Nil	
SAG5	S	S	Nil	
SAG6	W	M	Pos	
SAG7	S	S	Nil	
SDY1	N	W	Pos	**TSB**
SDY2	M	W	Neg	
SDY3	N	W	Pos	
SDY4	N	W	Pos	
SDY5	W	M	Pos	
SDY6	N	N	Nil	
SDY7	N	W	Pos	
SUB1	M	W	Neg	**BHB**
SUB2	W	W	Nil	
SUB3	S	M	Neg	
SUB4	W	W	Nil	
SUB5	M	W	Neg	
SUB6	S	M	Neg	
SUB7	W	W	Nil	
SUB ATCC	M	M	Nil	
ECO1	W	W	Nil	**TSB**
ECO2	N	W	Pos	
ECO3	N	W	Pos	
ECO4	W	S	Pos	
ECO5	W	M	Pos	
ECO6	N	W	Pos	
ECO7	N	N	Nil	

ATCC, American Type Culture Collection; ECO, *Escherichia coli*; SAG, *Streptococcus agalactiae*; SDY, *Streptococcus dysgalactiae*; SUB, *Streptococcus uberis*; M, moderate biofilm former; N, non-biofilm former; S, strong biofilm former, W, weak biofilm former; Nil, no difference; Neg, negative difference; Pos, positive difference; TSB, Tryptic Soy broth; BHI, Brain Heart Infusion broth.

extracts with inhibition percentages falling within the range of 0 to 50 % were categorized as having limited or poor anti-biofilm activity. It's important to note that values equal to or greater than 100 % were recorded as 100 %, signifying a complete inhibition of biofilm formation, while values less than or equal to 0 % were deemed to have no discernible anti-biofilm activity, consistent with the criteria established by Sandasi et al. (2011).

The study's findings reveal the biofilm inhibitory activity of various extracts against different bacteria (Table 4). All extracts exhibited over 50 % biofilm inhibition against *Str. agalactiae*, while a similar outcome was observed with *Str. uberis*, except for *Indigofera* (*I.*) *frutescens*, which did not inhibit biofilm formation. In contrast, only acetone extracts of *Searsia* (*S.*) *lancea* and *Antidesma* (*A.*) *venosum* demonstrated antibiofilm activity against *Str. dysgalactiae* and *E. coli*, with the remaining extracts failing to inhibit biofilm formation in these two bacterial strains at any concentration. *A. venosum* displayed the most effective antibiofilm activity against streptococcal species, while the acetone extract of *S. lancea* was the only one active against the *E. coli* strain. Contrarily, *I. frutescens* was active against only *Str. agalactiae*, while ciprofloxacin, at a concentration of 0.01 mg/mL, exhibited biofilm inhibition activity against all the bacteria.

In the biofilm eradication test conducted after 24 h as shown in Table 5, all plant extracts demonstrated good antibiofilm activity against 24 h preformed biofilms of *Str. agalactiae* and *Str. dysgalactiae*, except for the ethanol extract of *Erythrina* (*E.*) *caffra*, which showed poor activity against *S. dysgalactiae*. Additionally, all extracts showed good activity against *Str. uberis* and *E. coli*, except for *E. caffra* and the ethanol extracts of *S. lancea* and *A. venosum*, which exhibited no

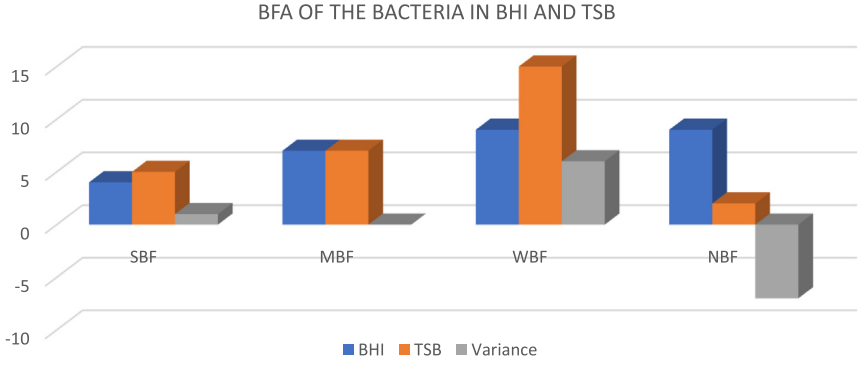

Fig. 1. Variations in biofilm forming ability of the organism in BHI and TSB.
BFA, biofilm forming abilities; SBF, strong biofilm former; MBF, moderate biofilm former; WBF, weak biofilm former; NBF, non-biofilm former; BHI, Brain Heart Infusion broth; TSB, Tryptic Soy broth.

Table 4
Percentage biofilm inhibition when extracts were introduced at 0 hour.

% Inhibition of biofilm formation at 0 hour								
	Streptococcus agalactiae		*Streptococcus uberis*		*Streptococcus dysgalactiae*		*Escherichia coli*	
Conc (mg/mL)	**Acetone**	**Ethanol**	**Acetone**	**Ethanol**	**Acetone**	**Ethanol**	**Acetone**	**Ethanol**
Searsia lancea								
0.016	100.00	100.00	84.22	100.00	0.00	0.00	23.58	0.00
0.008	100.00	87.88	77.96	48.84	0.00	0.00	19.44	0.00
0.004	100.00	29.29	20.00	23.54	0.00	0.00	14.00	0.00
Erythrina caffra								
0.016	100.00	76.67	90.56	74.32	0.00	0.00	0.00	0.00
0.008	100.00	50.48	64.49	25.02	0.00	0.00	0.00	0.00
0.004	100.00	14.76	14.26	21.25	0.00	0.00	0.00	0.00
Antidesma venosum								
0.016	100.00	100.00	100.00	100.00	92.58	0.00	0.00	0.00
0.008	100.00	100.00	100.00	100.00	73.43	0.00	0.00	0.00
0.004	100.00	100.00	100.00	100.00	70.63	0.00	0.00	0.00
Indigofera frutescens								
0.016	100.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
0.008	100.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
0.004	100.00	100.00	0.00	0.00	0.00	0.00	0.00	0.00
Ciprofloxacin								
0.010	73.26		100.00		75.32		87.33	

biofilm disruption activity. Ciprofloxacin displayed good activity against all 24 h preformed biofilms, except for *E. coli*, where it showed poor activity. *I. frutescens* was the only plant with good activity against all the tested organisms.

Interestingly, according to Table 6, the plants' activities appeared to improve as the biofilm matured, with few exceptions. All extracts of *S. lancea* displayed good biofilm disruption activity against all the 48 h preformed biofilms of the bacteria, without exception, unlike the other plants, which had one or two extracts with poor or no activity against biofilms of some organisms. Notable examples include the ethanol extract of *E. caffra* against *E. coli*, the ethanol extract of *A. venosum* against *Str. uberis* and *E. coli*, and the ethanol extract of *I. frutescens* against *Str. uberis*. In contrast, ciprofloxacin consistently showed good activity against all the tested bacteria.

3.3. Quorum sensing inhibition

The ability of the plant extracts to inhibit quorum sensing (QSI) was evaluated using the biosensor bacterium *C. violaceum*. The minimum concentration required for quorum sensing inhibition (MQSIC) was established as the lowest concentration that did not inhibit bacterial growth (measured by visible turbidity) while preventing the formation of a purple ring, signifying the absence of violacein pigment production. It was observed that only the ethanol extracts of *S. lancea* and *I. frutescens* exhibited QSI activity at 2.50 mg/mL (Table 7), effectively impeding violacein production without inhibition of cell growth. All other extracts did not inhibit violacein production at the tested extract concentration of 2.50 mg/mL and below. Furthermore, all the extracts exhibited lower MQSIC compared to vanillin (positive control) which had a MQSIC of 0.16 mg/mL and a minimum inhibitory concentration (MIC) concentration of 0.63 mg/mL. The minimum inhibitory concentration was determined as the lowest concentration at which there was neither growth nor turbidity, but rather the presence of a purple ring formation.

The plant extracts were evaluated against *C. violaceum* to determine the concentration at which 50 % violacein production inhibition occurred, known as the $MQSIC_{50}$ value. The $MQSIC_{50}$ values for all the plant extracts were deduced from the concentration versus % violacein inhibition standard graph, falling within the range of < 0.08 to > 2.50 mg/mL, as shown in Table 8. A lower IC_{50} value indicates a more potent ability to inhibit violacein production. The ethanol extracts of *S. lancea* demonstrated the lowest $MQSIC_{50}$ value (< 0.08 mg/mL)

Table 5
Percentage disruption of 24 h preformed biofilm.

% disruption of 24 h preformed biofilm								
	Streptococcus agalactiae		*Streptococcus uberis*		*Streptococcus dysgalactiae*		*Escherichia coli*	
Conc (mg/mL)	**Acetone**	**Ethanol**	**Acetone**	**Ethanol**	**Acetone**	**Ethanol**	**Acetone**	**Ethanol**
Searsia lancea								
0.016	86.29	100.00	100.00	0.00	100.00	100.00	92.13	0.00
0.008	80.43	90.04	100.00	0.00	100.00	100.00	66.25	0.00
0.004	78.79	88.17	91.03	0.00	100.00	100.00	26.72	0.00
Erythrina caffra								
0.016	100.00	100.00	0.00	0.00	27.08	100.00	0.00	0.00
0.008	100.00	81.17	0.00	0.00	21.33	100.00	0.00	0.00
0.004	100.00	60.95	0.00	0.00	10.19	100.00	0.00	0.00
Antidesma venosum								
0.016	96.48	85.90	100.00	0.00	100.00	100.00	100.00	0.00
0.008	85.14	75.62	100.00	0.00	100.00	100.00	87.15	0.00
0.004	70.57	58.38	100.00	0.00	100.00	100.00	12.16	0.00
Indigofera frutescens								
0.016	60.28	91.60	100.00	100.00	100.00	100.00	100.00	80.72
0.008	45.76	52.58	100.00	100.00	64.73	100.00	97.48	70.83
0.004	27.03	39.72	100.00	81.63	0.00	100.00	88.69	0.00
Ciprofloxacin								
0.010	79.40		100.00		72.54		49.65	

Table 6
Percentage disruption of 48 h preformed biofilm.

	% disruption of 48 h preformed biofilm							
	Streptococcus agalactiae		*Streptococcus uberis*		*Streptococcus dysgalactiae*		*Escherichia coli*	
Conc. (mg/mL)	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol	Acetone	Ethanol
Searsia lancea								
0.016	100.00	64.52	100.00	100.00	100.00	100.00	100.00	60.82
0.008	100.00	51.27	100.00	100.00	100.00	70.15	100.00	53.82
0.004	47.76	31.58	100.00	100.00	100.00	67.77	100.00	27.65
Erythrina caffra								
0.016	100.00	100.00	100.00	79.45	100.00	100.00	100.00	12.02
0.008	100.00	58.62	100.00	68.13	77.97	100.00	89.74	0.00
0.004	100.00	11.49	77.36	27.46	36.42	100.00	29.75	0.00
Antidesma venosum								
0.016	100.00	100.00	79.31	0.00	100.00	100.00	69.63	0.00
0.008	100.00	100.00	0.00	0.00	100.00	100.00	57.90	0.00
0.004	100.00	66.20	0.00	0.00	100.00	100.00	18.86	0.00
Indigofera frutescens								
0.016	100.00	73.22	100.00	0.00	100.00	100.00	100.00	100.00
0.008	100.00	53.18	100.00	0.00	100.00	92.21	100.00	100.00
0.004	100.00	49.33	100.00	0.00	100.00	78.88	24.26	0.00
Ciprofloxacin								
0.010	92.47		100.00		74.18		51.48	

Table 7
Minimum quorum sensing inhibition concentration (MQSIC), minimum inhibitory concentration (MIC) of the extracts against *Chromobacterium violaceum* and their $MQSIC_{50}$ (50 % inhibitory concentration) ± SD.

Plants	Extractant	MQSIC (mg/mL)	MIC (mg/mL)	$MQSIC_{50}$ (mg/mL)
Searsia lancea	Acetone	> 2.5	> 2.5	1.47 ± 0.37
	Ethanol	2.50 ± 0.00	> 2.5	< 0.08
Erythrina caffra	Acetone	> 2.5	> 2.5	0.68 ± 0.07
	Ethanol	> 2.5	> 2.5	0.45 ± 0.13
Antidesma venosum	Acetone	> 2.5	> 2.5	> 2.50
	Ethanol	> 2.5	> 2.5	0.75 ± 0.19
Indigofera frutescens	Acetone	> 2.5	> 2.5	> 2.50
	Ethanol	2.50 ± 0.00	> 2.5	0.10 ± 0.01
Vanillin		0.16 ± 0.00	0.63 ± 0.00	0.0004 ± 0.0000

MQSIC, minimum quorum sensing inhibition concentration; MIC, minimum inhibitory concentration; $MQSIC_{50}$, 50 % quorum sensing inhibitory concentration; mg/mL, milligram per millilitre; SD, standard deviation

Table 8
Selectivity index values of the most active extracts against *Chromobacterium violaceum* in terms of their MQSIC.

Plants	Extractant	Cell Type	LC_{50} (mg/mL) ± SD	QSSI	Mean QSSI	MQSIC (mg/mL)	% Yield (g/g)	TQSIA
Searsia lancea	Acetone	BD	0.10 ± 0.01	-	-	> 2.5	5.82	-
		Vero	< 0.075	-				
	Ethanol	BD	> 1	0.40	0.23	2.5 ± 0.00	15.79	6.32
		Vero	0.15 ± 0.04	0.06				
Erythrina caffra	Acetone	BD	> 1	-	-	> 2.5	3.51	-
		Vero	0.51 ± 0.09	-				
	Ethanol	BD	0.63 ± 0.04	-	-	> 2.5	4.77	-
		Vero	0.09 ± 0.01	-				
Antidesma venosum	Acetone	BD	0.65 ± 0.00	-	-	> 2.5	1.74	-
		Vero	< 0.075	-				
	Ethanol	BD	0.48 ± 0.02	-	-	> 2.5	3.03	-
		Vero	< 0.075	-				
Indigofera frutescens	Acetone	BD	0.61 ± 0.01	-	-	> 2.5	2.97	-
		Vero	0.33 ± 0.03	-				
	Ethanol	BD	0.46 ± 0.02	0.18	0.15	2.5 ± 0.00	8.41	3.36
		Vero	0.27 ± 0.04	0.11				

MQSIC, minimum quorum sensing inhibition concentration; QSSI, quorum sensing selectivity index; LC_{50}, 50 % lethal concentration; mg/mL, milligram per millilitre; BD, bovine dermis cells; Vero, Vero monkey kidney cells; g/g, gram per gram; TQSIA, total quorum sensing inhibition activity of the extracts; -, not determined; SD, standard deviation.

among the plant extracts, while acetone extracts of *A. venosum* and *I. frutescens* exhibited the least violacein inhibition ($MQSIC_{50}$ > 2.50 mg/mL).

Among the extracts, only two with definite minimum quorum sensing inhibition concentration (MQSIC) values had their quorum sensing selectivity index (QSSI) determined, as shown in Table 8. The ethanol extract of *S. lancea* exhibited QSSI values of 0.40 and 0.06 against BD and Vero cells, respectively, with a mean QSSI of 0.23. Contrarily, the ethanol extract of *I. frutescens* had QSSI values of 0.18 and 0.11 against BD and Vero cells, respectively, resulting in a mean QSSI of 0.15. Both extracts appeared to be less toxic to BD cells compared to Vero cells, indicating a degree of selectivity in their action.

Furthermore, the total quorum sensing inhibition activity (TQSIA) of these extracts was evaluated, which quantifies the volume (mL) to which a gram (g) of a plant extract can be diluted while retaining its quorum sensing inhibition activity, serving as a measure of the extracts' efficacy in quorum sensing inhibition. The calculated values for this study were 6.32 mL/g for the ethanol extract of *S. lancea* and 3.36 mL/g for the ethanol extract of *I. frutescens*.

4. Discussion

4.1. Biofilm forming ability of the bacterial isolates

Biofilm formation is a crucial aspect of microbial behaviour with significant implications across various domains, from healthcare to environmental science. The comparative analysis of biofilm formation data of these bacteria of mastitis origin in Brain Heart Infusion broth (BHI) and Tryptic Soy Broth (TSB) media revealed both consistencies and discrepancies among different organisms. While some organisms maintain consistent biofilm-forming abilities irrespective of the medium, others exhibit medium-dependent behavior. This study highlighted variations in the biofilm-forming ability (BFA) of mastitis bacteria in BHI and TSB. In TSB, the total number of strains capable of biofilm formation was 27 (93.10 %) while in BHI, it was 20 (68.97 %), which suggests that, at a holistic level, these two media support biofilm formations equivalently.

However, the distribution of BFA was different when BHI was compared to TSB. According to the findings of this study, when using BHI, four isolates were classified as strong biofilm formers, seven strains were moderate biofilm formers, nine were weak biofilm formers and the remaining organisms showed no biofilm formation. Inversely, when using TSB, five isolates were strong biofilm formers, seven strains were moderate biofilm formers and 15 were weak biofilm formers and the remaining two organisms were non-biofilm formers. This variation in BFA suggests that the choice of media can significantly impact the outcome when evaluating biofilm formation by mastitis bacteria (Ma et al., 2017; Bonsaglia et al., 2020).

It is imperative to note the variations in biofilm-forming abilities across organisms when cultivated in TSB. For example, 13 isolates across three species (*Str. agalactiae, Str. dysgalactiae* and *E. coli*) demonstrated enhanced biofilm formation in TSB compared to BHI, while six strains across three species (*Str. agalactiae, Str. dysgalactiae* and *Str. uberis*) exhibited diminished biofilm formation in TSB, further highlighting the media-dependent nature of their biofilm-forming potential. The content of the growth medium has been observed to impact bacteria's capacity to generate biofilms in controlled laboratory settings. Specifically, the inclusion of glucose in the growth medium, as seen in the standard TSB medium with its typical 0.25 % glucose content, has been noted to augment biofilm development (Stepanović et al., 2007), but this contrasts with the findings of Kaur et al. (2009) who observed that the inclusion of glucose did not promote the development of biofilms by *Str. agalactiae*.

Furthermore, there are instances of bacteria displaying similar behavior in both media. Examples include the eight strains across all the species tested consistently exhibited the same BFA in both BHI and TSB media, and two other isolates, SDY6 and ECO7, classified as non-biofilm formers, consistently exhibit no biofilm formation in either BHI or TSB. This uniformity suggests a robust biofilm-forming phenotype unaffected by the choice of medium, and demonstrates the resilience of their non-biofilm-forming phenotype across media, respectively. Therefore, it can be safely postulated that their BFA is influenced by their genetic make-up and other environmental factors beyond the scope of this study.

Remarkably, at the species level, this study revealed that *Str. agalactiae, Str. dysgalactiae* and *E. coli* exhibited superior BFA when grown in TSB as opposed to BHI after a 24-hour incubation period. Conversely, *Str. uberis* demonstrated the opposite trend, with a decline in BFA when grown in TSB, as it only diminished and did not enhance the BFA of any of the *Str. uberis* strains, suggesting it is an inappropriate medium for *Str. uberis* biofilm investigations in this study. However, it demonstrated enhancement of BFA of 71.43 % each of the *E. coli* and *Str. dysgalactiae*, and 42.86 % of the *Str. agalactiae* isolates, making it the medium of choice for the biofilm studies of these isolates. This variance underscores the profound impact of medium selection on biofilm formation for certain organisms. Certain research studies have indicated a favoritism towards either BHI or TSB. In the case of specific strains within *Staphylococcus, Vibrio,* and *Pseudomonas* species, it has been documented that some strains tend to produce higher quantities of biofilms in TSB, while others exhibit this tendency in BHI (Stepanović et al., 2007; Hoštacká et al., 2010; Nyenje et al., 2013). Several researchers have documented the in vitro biofilm forming abilities (BFAs) of pathogens causing bovine mastitis (Pedersen et al., 2021) but this study appears to be the first to report the differential BFAs of *Str. uberis, Str. dysgalactiae* and *E. coli* recovered from milk samples in BHI and TSB.

Bonsaglia et al. (2020) documented comparative BFAs of *Str. agalactiae* in both media, and they concluded that, when cultured in CO_2, the likelihood of biofilm development by *Str. agalactiae* was higher in isolates cultured in TSB as compared to BHI, with an odds ratio of 4.57. Considering the same aerobic environment as in this study, they reported BFA in 81.25 % of *Str. agalactiae* in TSB and 31.25 % in BHI. Though this study observed BFAs in 100 % of *S. agalactiae* grown in both media, TSB was found to enhance BFA in 42.86 % of *Str. agalactiae* as compared to when grown in BHI. In a separate investigation conducted by Ma et al. (2017), it was reported that 46.7 % of *Str. dysgalactiae* isolates (which were not associated with mastitis) exhibited moderate or strong BFAs in TSB, while 43.5 % showed similar abilities in BHI. The findings of these studies further emphasized the significant impact the choice of media can have on the outcome of biofilm formation investigations by mastitis bacteria.

4.2. Antibiofilm activity of the plant extracts

Biofilm formation is one of the virulence factors underlying the chronic and challenging nature of bovine mastitis. Pathogenic bacteria employ biofilm formation as an adaptive survival strategy when encountering host environments, using a protective extracellular matrix to shield themselves. This matrix serves as a formidable barrier against host immune responses, rendering microbial cells resistant to defenses and antibiotics, ultimately leading to chronic, hard-to-treat infections (Mah and O'Toole, 2001; Flemming and Wingender, 2010). Most of the bacterial isolates included in this study have been shown to demonstrate biofilm-forming and antimicrobial resistant capabilities. This underscores the significance of understanding biofilm mechanisms in combatting such virulent and antibiotic-resistant organisms in bovine mastitis (Sutherland, 2001).

The aim of the antibiofilm assay in this study was to investigate the effects of the plant extracts, at three different sub-minimum inhibitory concentrations (Liu et al., 2022; Liu et al., 2023) on the prevention of biofilm formation (0 h), prevention of biofilm maturation (24 h preformed biofilm) and disruption of matured biofilm (48 h

preformed biofilms) in the isolates. For uniformity, the same concentrations of the extracts were used irrespective of the differing MICs of each of the extracts against individual isolates included in this study. The results showed that all the plant extracts demonstrated antibiofilm activities against at least one of the isolates at different stages of the biofilm development.

All the plant extracts demonstrated good antibiofilm activity against *Str. agalactiae* and *Str. uberis* at all the stages of biofilm formation except *E. caffra* which had no antibiofilm activity against 24 h preformed biofilm, and *I. frutescens* with no activity against biofilm formation by *Str. uberis*. Furthermore, it appears that the 24 h preformed biofilm of *Str. uberis* was more resistant to the activities of the extracts than the matured (48 h) preformed biofilm. In contrast, a study reported that *A. venosum* demonstrated good antibiofilm activity against 24 h but poor activity against 48 h and 72 h (Shirinda et al., 2019). This study is the first to report the antibiofilm activities of these plants against *Str. agalactiae, Str. dysgalactiae, Str. uberis* and *E. coli* isolates. Further research is needed to unravel the biochemical processes which emerged into these patterns of susceptibility.

Prior research has indicated that sub-inhibitory concentrations of extracts can modify gene expression, leading to a decrease in biofilm formation (Liu et al., 2023). This reduction is achieved by inhibiting metabolic activity and the production of extracellular polysaccharides (Liu et al., 2023). Since the biomolecular and physiological processes at play at different stages of biofilm formation varies, there is a need for more detailed study of the mechanism of antibiofilm activities of these extracts at different stages of biofilm formation to fully understand the antibiofilm activities of these extracts and their applications in infectious disease management.

The antibiofilm activities of the extracts against *Str. dysgalactiae* and *E. coli* appeared to improve as the biofilms aged. They were able to eradicate preformed biofilms though they showed no effect on prevention of biofilm formation. This discovery is interesting because the potential of certain agents to eliminate preformed biofilms presents an intriguing opportunity for addressing advanced infections (Martínez Chamás et al., 2023), although the finding of this work is contrary to previously reported findings which suggested it is easier to prevent biofilm formation than eliminating the preformed biofilm (dos Santos Ramos et al., 2021; Erhabor et al., 2021; Adeyemo et al., 2022; Martínez Chamás et al., 2023). In agreement with this work, Adeyemo et al. (2022) reported poor biofilm formation inhibition by the acetone extract of *S. lancea* against *E. coli* (ATCC 35218).

The acetone and ethanol extracts of *A. venosum* consistently showed 100 % inhibition against all tested organisms and concentrations. *Antidesma venosum* and *S. lancea* appeared to show superior antibiofilm activity when compared with other plants in this study. This is corroborated by reports which show that both *S. lancea* and *A. venosum* demonstrated good antibiofilm formation activity against *E. coli* (ATCC 25922) and *Clostridium perfringens,* respectively (Shirinda et al., 2019; Adeyemo et al., 2022).

For some of the extracts, the findings indicated a substantial inhibition of bacterial biofilm formation by the extracts at all three minimum inhibitory concentrations (MICs), with no notable variance in biofilm inhibition observed among the three sub-MICs (Liu et al., 2023). Moreover, when the percentage biofilm inhibition of the extracts at the three different concentrations were different, the values appeared to be concentration dependent, i.e., the activities increased with concentration. This is similar to previous findings which recorded improved biofilm inhibition as the concentration increases (Liu et al., 2022; Liu et al., 2023).

The biofilm inhibition results obtained in this study suggest that the selected plants are potential sources of good alternative therapy for prevention and eradication of persistent infection in bovine mastitis. Therefore, they should be further investigated to determine their probable mechanisms of biofilm inhibition.

4.3. Quorum sensing inhibition

Quorum sensing (QS) is an essential intercellular communication system in bacteria which synchronizes gene expression based on shifts in population density. Most bacteria, including both Gram-positive and Gram-negative bacteria, link QS with virulence expression such as biofilm formation, sporulation, toxin synthesis, and swarming. Gram-negative bacteria use N-acyl homoserine lactones, while Gram-positive bacteria use autoinducing peptides to regulate QS mechanism. QS activation results in heightened resistance against the host immune system and antimicrobial agents (Durán et al., 2016; Poli et al., 2018; Cheng et al., 2022). *Chromobacterium violaceum* and its QS-controlled purple pigment, violacein, have implications for bioactive material research. *C. violaceum* is used as a model organism to study QS. It is regulated by LuxR-LuxI and CviR-CviI homologous systems, along with N-hexanoyl-L-homoserine lactone. QS disruption is a potential target for controlling infectious agents (Vattem et al., 2007). Hence, focusing on this mechanism presents an alternative approach to limiting the spread of infectious agents.

Consequently, this research delved into the potential of plant extracts to disrupt the QS signaling pathway. The plant extracts exhibited the ability to interfere with QS mechanisms by inhibiting the production of violacein. The ethanol extracts of *S. lancea* and *I. frutescens* inhibited violacein production at MQSIC value of 2.50 mg/mL. Although other extracts did not completely inhibit QS in *C. violaceum* at the highest concentration used, the $MQSIC_{50}$ results indicated that all the plant extracts are able to inhibit QS in a concentration dependent manner. This implies that at higher concentrations the plant extracts may completely inhibit QS. Moreover, it should be noted that the MQSIC values were observed to be below their corresponding MICs. This suggests that the quorum sensing inhibition (QSI) observed was not due to inhibition of cell growth; instead, it indicates that the plant extracts may modulate virulence factors by suppressing the formation of violacein pigment at concentrations below the MQSIC (Adeyemo et al., 2022). Vanillin, the positive control, exhibited a lower MQSIC value than all the plant extracts.

The QSI activity (QSIA) of most of the plants evaluated in this study has not been previously documented. This study therefore appears to be the first to report on QSIA of these plant extracts except the acetone extract of *S. lancea*. The only available study documented the QSIA of acetone extracts of *S. lancea, S. pendulina, S. leptodictya*, and *S. batophylla*. Contrary to the results obtained in this study the acetone extract of *S. lancea* had MQSIC of 0.08 mg/mL (Adeyemo et al., 2022), which is a better activity compared to the value obtained in this study. Geolocation of a plant, techniques, seasonal changes and other factors have been reported to be responsible for such variance in activity of plants (Pandey et al., 2011).

Some of the plant species have been reported to contain flavonoids, alkaloids and phenolics, and phytochemicals belonging to these groups have been documented to exhibit significant QSIA (Vasavi et al., 2014; Karbasizade et al., 2017; Cheng and Han, 2020). Further research will decipher their phytochemical contents and the specific compounds responsible for their QSIA.

The lowest concentration required to inhibit 50 % of violacein production ($MQSIC_{50}$) against *C. violaceum* was obtained from the ethanol extract of *S. lancea*. Though the value obtained in this study is higher than that reported by (Adeyemo et al., 2022) for the acetone extract of *S. lancea*, both findings suggest that both ethanol and acetone extracts from *S. lancea* significantly suppressed QS in *C. violaceum* without exerting toxic effects on the bacteria. Therefore, the plant should be further investigated to better understand its QSIA and its applications in the management of bovine mastitis.

Furthermore, the TQSIA values provided insight into the efficacy of these extracts in QSI, with the ethanol extract of *S. lancea* having a TQSIA of 6.32 mL/g and the ethanol extract of *I. frutescens* having a

TQSIA of 3.36 mL/g. This signifies the ethanol extract of *S. lancea* to be the most efficacious of all the plant extracts. This is very important in bioprospecting towards development of these extracts on an industrial scale.

Moreover, the quorum sensing selectivity index (QSSI) was calculated to evaluate the QSIA of plant extracts against Vero and bovine dermis (BD) cells, providing a quantitative measure of their effectiveness without harming bacteria or mammalian cells (Ogbuadike et al., 2023). The results demonstrated that the ethanol extract of *S. lancea* exhibited notable QSIA, with a mean QSSI of 0.23. Additionally, the ethanol extract of *I. frutescens* displayed moderate QSIA, with a mean QSSI of 0.15. Though these values are less than the safe cut off mark (1), the results indicate that both extracts appeared to be less toxic to BD cells compared to Vero cells, indicating a degree of selectivity in their action.

In conclusion, this study evaluated the anti-QS activity of various plant extracts against the QS mechanism in *C. violaceum* and, mammalian Vero and bovine dermis (BD) cells, utilizing important metrics such as MQSIC, $MQSIC_{50}$, QSSI and TQSIA. Overall, these findings suggest that *S. lancea*, in particular, holds promise as a potential candidate for further exploration and development of anti-quorum sensing agents. Further research is warranted to elucidate the underlying mechanisms and therapeutic potential of these plant extracts in combating quorum sensing-mediated bacterial infections in bovine mastitis.

5. Conclusions

The findings of this study underscore the nuanced interplay between microbial characteristics and environmental factors, such as growth medium, shedding light on the complexity of biofilm formation mechanisms. Understanding these distinctions is pivotal for diverse applications, ranging from infection control in healthcare to industrial biofilm management.

The ability of bacteria to form biofilms, which relies on QS, is a well-recognized crucial virulence factor that enables their survival and persistence in various environments and host organisms. Discovering plant extracts that exhibit effective QSI and demonstrate significant activity against bacterial biofilms at various developmental stages could offer an alternative approach to managing mastitis, particularly in cases of chronic infections. Consequently, the capability of these studied plant extracts, especially *S. lancea*, to inhibit QS and biofilm formation may play a pivotal role in reducing bacterial biofilm development and, subsequently, in alleviating infections and curbing the emergence of antimicrobial resistance. The choice of the most suitable extract would depend on the specific organism and the desired concentration for biofilm inhibition. Nonetheless, further research is recommended to elucidate the precise mechanism underlying the inhibition of quorum signaling and to identify the specific compounds responsible for the observed antibiofilm and QSI activities.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ayodele O. Akinboye: Conceptualization, Data curation, Formal analysis, Writing – original draft. **Fikile N. Makhubu:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Joanne Karzis:** Methodology, Resources, Writing – review & editing. **Inge-Marie Petzer:** Methodology, Resources, Supervision, Writing – review & editing. **Lyndy J. McGaw:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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