



Antibiotic resistance and biofilm formation of thermotolerant *Kocuria* and *Micrococci* isolated from the milk samples of healthy cows

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Abstract

The current study has focused on the identification of antibiotic resistance and biofilm formation among the thermotolerant bacteria isolated from the raw milk samples such as *Micrococci* and *Kocuria* to predict its risk with food safety and public health. The antibiotic susceptibility testing conducted in the study has revealed the selected bacterial isolates to have multidrug resistance, particularly against clindamycin and ampicillin. This indicates the risk with antibiotic resistance gene dissemination through the dairy products. The emergence of biofilm formation among the selected isolates has also been further confirmed through various methods which highlighted their capacity to persist and potentially contaminate the dairy processing environment. The presence of *aap* gene associated with the biofilm formation could also be demonstrated among the selected isolates which underscores the organisms to have the diverse genetic basis for the same. These findings emphasise the need for continuous surveillance on dairy herd health for the development of strategies to mitigate the impact of antibiotic resistant bacteria on food safety. The results of the study hence suggest the need for advanced research on AMR genetic determinants and gene transfer dynamics among non-clinical bacterial isolates from dairy sector in a one health perspective.

Keywords *Micrococci* · *Kocuria* · AMR · Cow milk · Biofilm

Introduction

Antibiotic or antimicrobial resistance (AMR) is a significant global concern, undermining the effectiveness of existing antibiotics against infectious diseases. While many studies have already been focused on the AMR in relation to the clinical infections, the non-clinical bacterial isolates are also having the potential risk with AMR gene transmission to human pathogens (Samtiya et al. 2022). Bacterial species such as those belongs to *Bacillus*, *Micrococcus*, and *Corynebacteria*, have already been known to survive the pasteurisation. These thereby generate AMR concerns in food safety as the thermotolerant bacteria can withstand the various processing methods, involving temperature and

chemical treatments (Mittal and Bharadwaj 2021). Also, the antibiotic resistance transmission risk between both the closely related and phylogenetically distant bacterial species, such as from Gram-positive to Gram-negative demands detailed study (Patangia et al. 2023). This is particularly relevant for bacteria associated with the food items such as raw milk as there are only limited research data available on the same. Hence, *Micrococci* and *Kocuria* spp. were selected in the study to identify their antibiotic resistance and biofilm formation due to their presence in milk (Sahoo et al. 2024).

Thermotolerant bacteria are of concern if they have genetic basis of AMR. When present as biofilms, these bacteria can replicate rapidly. Hence the presence of AMR genes in them poses the risk of transfer of resistance to other microorganisms through the dairy products (Lee et al. 2024; Elias et al. 2017; Gleeson, O'Connell & Jordan, 2013). Therefore, it is essential to identify these bacteria from milk to characterise their AMR nature and also biofilm formation through advanced imaging techniques like Field Emission Scanning Electron Microscopy (FESEM) (Baishya et al. 2021). Hence, the current study has been aimed to investigate the antibiotic resistance and biofilm formation in *Micrococci* and *Kocuria*

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spp. to evaluate their persistence and potential threats to food safety and public health.

Methodology

Sample collection

In the study, raw milk samples ($n = 16$) from the healthy cows were collected from various farms in Kottayam district, Kerala, India. The sample was collected after cleaning the udders with lukewarm water and then 0.5% iodine teat dip was applied onto each teat. The iodine dip was then scrubbed off using clean paper towel and further the teats were scrubbed with 70% isopropyl alcohol. During the milk sample collection, the fore-stream milk samples were discarded and only the mid-stream milk was taken into sterile pre-labelled vials. The collected milk samples were immediately kept under refrigerated conditions and transported to the laboratory for further analysis (Coenye 2023).

Methylene blue reduction test (MBRT)

For this, 5 mL of the milk sample was taken in a sterile test tube followed by the addition of 0.5 mL of methylene blue reduction test reagent (0.005%) and kept under room temperature to observe the colour change from blue to white (Oluchi et al. 2022).

Isolation and identification of bacterial species from raw milk samples

For this part of the study, 10 μ L the milk samples were streaked on Brain heart infusion agar plates followed by the incubation for 18–24 h to get the isolated colonies of bacteria (Oluchi et al. 2022). These were further purified on nutrient agar plates for the storage and analysis. The isolated bacteria were further subjected to Gram staining by using the K001- HiMedia kit, as per the manufacturer's instructions and identification of bacterial isolates was done by using VITEK® 2 automated system. For this, bacterial suspension with a turbidity equivalent to 0.5 McFarland standard was made with 0.45% sodium chloride solution in a VITEK® 2 instrument (BioMérieux). The Gram positive (GP) identification card for the bacteria was included with the following biochemical reactions such as; phosphatidylinositol phospholipase C, arginine dihydrolase, β -galactosidase, α -glucosidase, alanine-phenylalanine-proline arylamidase, l-aspartic acid arylamidase, β -galactosidase, α -mannosidase, alkaline phosphatase, l-leucine arylamidase, proline arylamidase, β -glucuronidase, α -galactosidase, l-pyrogutamic acid arylamidase, alanine arylamidase, tyrosine arylamidase, and urease (Ziougou et al. 2023).

Antibiotic susceptibility test

The antibiotic susceptibility test was performed for the selected bacterial isolates as per the Kirby Bauer disc diffusion method (Wanja et al. 2020). The inoculum for the antibiotic susceptibility test was prepared in cation- adjusted Mueller Hinton broth by adjusting the turbidity comparable to 0.5 McFarland standard before streaking and a sterile cotton swab was used to perform the lawn streaking. The antibiotics such as gentamicin (10 mcg), penicillin (10 mcg), clindamycin (2 mcg), chloramphenicol (30 mcg), ampicillin (10 mcg), vancomycin (30 mcg), and co-trimoxazole (25 mcg) were tested against each of the identified *Micrococci* and *Kocuria* strains as per the CLSI guidelines by measuring the zone of inhibition by the isolates against the antibiotics used (Marques et al. 2023).

Detection of biofilm formation

Identification of biofilm formation on Congo red agar plates

Here, the selected bacterial isolates were subjected to qualitative biofilm production analysis by streaking onto the surface of sterile Congo Red Agar plates. The plates were further incubated for 24 h and observed for the colour of the colonies formed (Arciola et al. 2005).

Tissue culture plate assay (TCP) for the quantitative analysis on biofilm

Here, a loop-full of bacterial culture was inoculated into 5 mL Tryptic soy broth (TSB) containing with 1% glucose and incubated for 24 h. The overnight grown culture was then diluted to 1:100 ratio with the fresh TSB and 0.2 mL of this was then inoculated into individual wells of sterile polystyrene 96 well plates followed by the incubation for 24 h. After the incubation, the content of tissue culture plate was removed by gentle tapping, and washed with PBS (pH 7.2) for four times to remove the free-floating planktonic bacteria. It was then stained with 200 μ L of crystal violet (0.1%) and kept for 15 min at room temperature. Each of the well was then washed thrice with PBS to remove the excess stain. The plates were further dried and 200 μ L of 90% ethanol was added into each of the well and shaken for few min. The optical density (O.D.) reading was then taken at 595 nm using ELISA plate reader (Biorad Imark) (Mathew et al. 2024).

Field emission scanning electron microscopy (FESEM) analysis of biofilm forming bacterial species

For this, a loop-full of isolated bacterial colony was inoculated into 5 mL of TSB supplemented with 1% glucose and



incubated for 24 h. The overnight grown culture was diluted 1:100 ratio with fresh TSB and 0.2 mL of this diluted culture was inoculated into individual wells of sterile polystyrene 24-well plates. Then clean autoclaved glass pieces of 1 × 1 cm were placed aseptically at the bottom of each well and the plate was incubated for 18–20 h. After the incubation, each of the glass piece was removed from the wells using sterile forceps and rinsed gently with sterile PBS three times. The glass piece was then fixed with 2.5% glutaraldehyde for 1–2 h at 4 °C, and dehydrated with graded ethanol series such as 10%, 30%, 50%, 70%, 90% and 100% ethanol and air dried and the glass slide was finally observed under FESEM using MAIA 3 TESCAN (Nguyen et al. 2015).

Detection of biofilm associated gene

For this, the genomic DNA was isolated from the selected bacterial isolates by using Nucleospin Microbial DNA isolation kit (MN Catalogue no.740235) as per the manufacture's instruction. The eluted DNA was subjected to agarose gel (0.8%) electrophoresis to visualise the genomic DNA by using the Gel Doc EZ Imaging System (Biorad, India). The genomic DNA was further used as template for the PCR for the detection of *aap* gene specific for the biofilm formation. The primers used were *aap*-F (5'-AAACGGTGGTATCTTACGTGAA-3') and *aap*-R (5'-CAATGTTGCACCATCTAAATCAGCT-3') (Ahmad et al. 2022). The PCR was carried out with the following conditions; initial denaturation for 5 min at 94 °C, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 60 s, with final extension at 72 °C for 7 min. The amplified product was run on 1.5% of agarose gel and visualized under Gel Doc EZ Imaging System (Biorad, India) (Soumya et al. 2020). A one-way ANOVA analysis of the triplicate values generated for each of the isolates from the respective O.D. values was carried out by using GRAPES 1.0.0 (Gopinath et al. 2020).

Results

Methylene blue reduction test (MBRT) of selected milk samples

In this study, out of the 16 milk samples tested, three were found to have a colour change from blue to white within 2 h, while the same occurred for other samples after 3 h only. The observation on the test samples were confirmed by comparing with the colour change of boiled milk, which served as the negative control (Supplementary Fig. 1).

Isolation and identification of microorganisms from the selected milk samples

In the study, the isolated bacterial colonies (n = 52) that appeared on the BHI agar plates were further purified on nutrient agar plates and subsequently stored under the refrigerated conditions for further analysis.

Here, 24 bacterial isolates which were appeared as violet cocci in tetrads and octads (Supplementary Fig. 2) were selected for the species identification and the AST analysis by using VITEK® 2 Automated system.

From the results obtained, 16 bacterial isolates were identified as *Micrococcus* sp. and 8 as *Kocuria* sp. Here, the strains M12.2, M16.3, M17.3, M23.1, M24.3, M28.2, M28.3, M30.2, M37.2 and M37.4 were coming under the *Micrococcus* sp. At the same time the strains M15.1, M16.2, M25.3, M27.2, M29.4 and M34.1 were *Micrococcus luteus*. Also the strains M15.3 and M33.3 were identified as *Kocuria rosea* and M17.2, M25.5, M30.1 and M35.1, as *Kocuria kristinae*. Bacterial strain labelled as M26.5 was then identified as *Kocuria varians* whereas M29.1 was identified as *Kocuria rhizophila*.

Antibiotic susceptibility testing (AST)

Here, the zone of inhibition for each isolate against the tested antibiotics was measured, and based on the diameter of zone of inhibition obtained, the isolates were categorised as resistant or susceptible according to the CLSI guidelines (Fig. 1). From the results obtained, all the isolates were found to have highest resistance to clindamycin, followed by ampicillin. In contrast, the lowest resistance was observed against the gentamicin.

Detection of biofilm formation

Identification of biofilm formation on Congo red agar plates

Here, out of the 24 isolates, eight were appeared as moderate slime producers after the incubation period and were M15.1 (*Micrococcus luteus*), M17.2 (*Kocuria kristinae*), M17.3 (*Micrococcus* sp.), M37.4 (*Micrococcus* sp.) M23.1 (*Micrococcus* sp.), M37.2 (*Micrococcus* sp.), M30.1 (*Kocuria kristinae*) and M28.3 (*Micrococcus* sp.). The colonies that exhibited a slight crystalline texture and caused the agar medium to turn black around the streak lines were identified as biofilm producers (Fig. 2).

Biofilm formation analysis by the tissue culture plate method

Here, all the 24 isolates were found to be biofilm producers as the O.D. values observed were between the range of

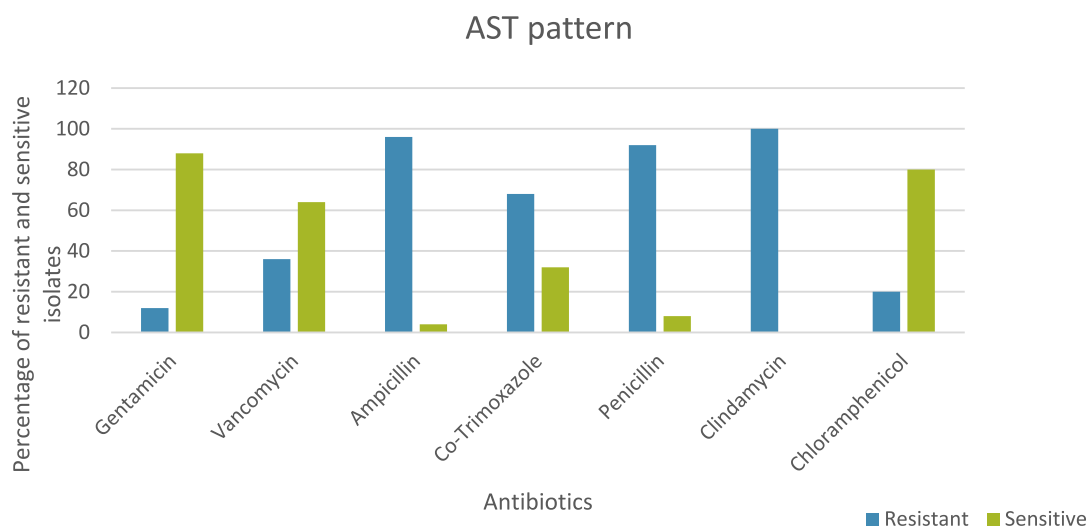
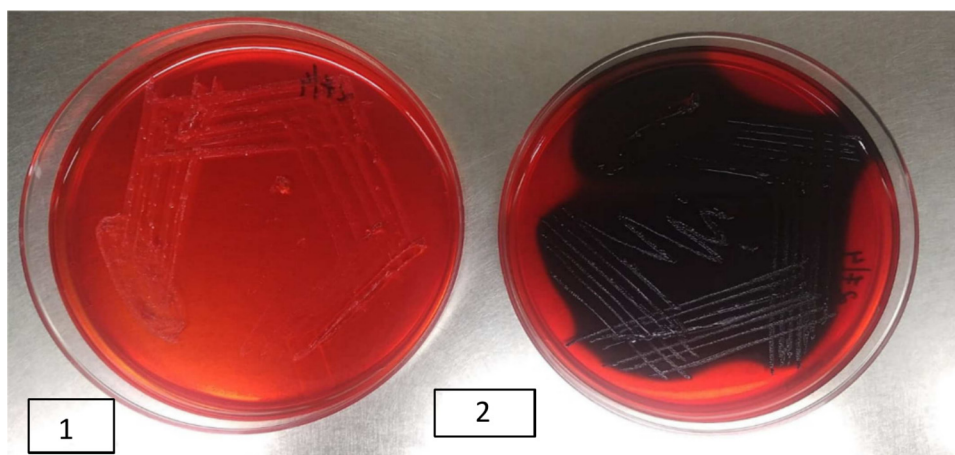


Fig. 1 Antibiotic resistance and sensitivity pattern of 24 bacterial isolates selected for the study

Fig. 2 Biofilm formation of M37.2 (*Micrococcus luteus*) (2) isolate on Congo red agar plate along with ATCC control strain of *E. coli* (1)



0.08 and 0.136. However, the strains such as M37.2 (*Micrococcus luteus*) and M37.4 (*Micrococcus* sp.) were found to have more biofilm formation capabilities compared to the rest due to the O.D. values of 0.136 and 0.133 respectively. The analysis of the completely randomized design (CRD) indicated a significant difference between at least one pair of treatments, as evidenced by a P-value < 0.05. Further multiple comparisons using the LSD test identified the best treatments (Fig. 3).

Field emission scanning electron microscopy (FESEM) analysis of biofilm

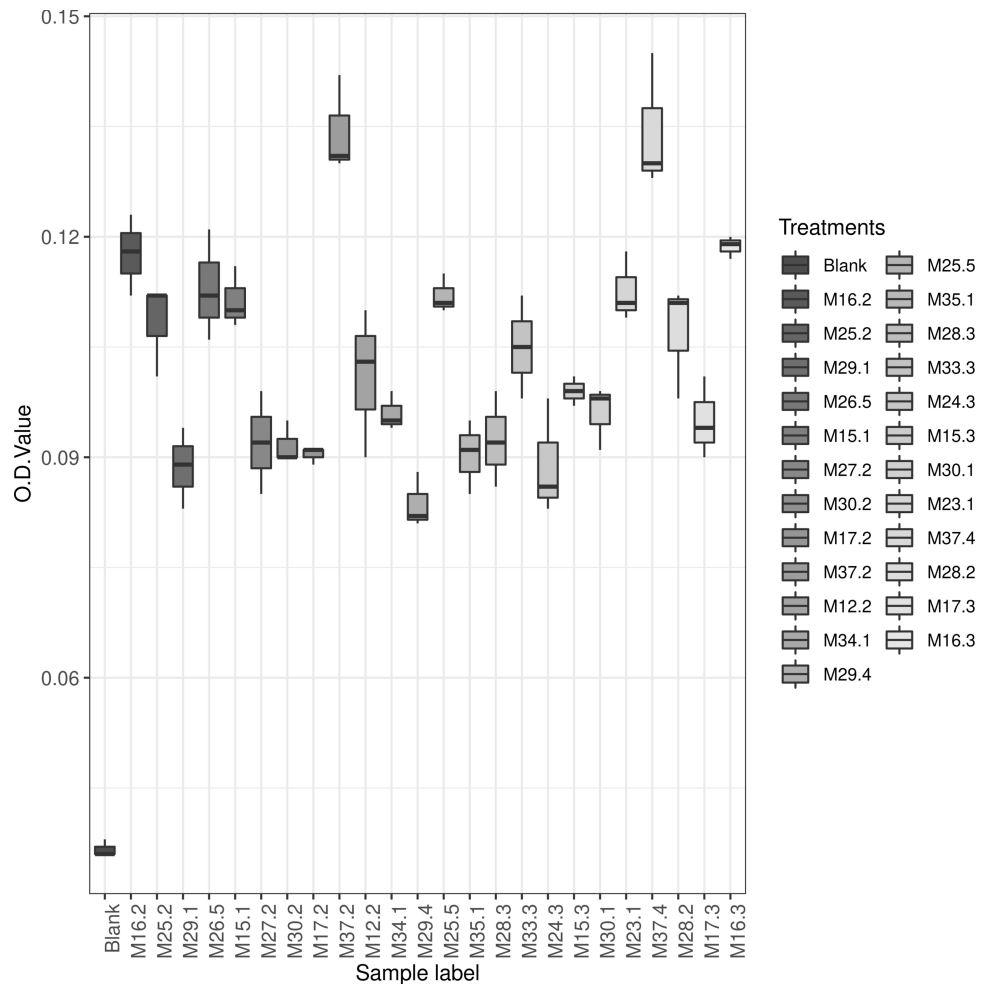
The bacterial strain M37.2 which showed the enhanced biofilm formation was also subjected to biofilm observation under

the FESEM at the magnifications of 5 μm and 2 μm (Fig. 4). At the 5 μm magnification, the overall architecture of the biofilm formed by the bacteria was more apparent where the cocci formed a structured community. Whereas at the 2 μm magnification, the biofilm exhibited a dense aggregation of cocci, with individual bacterial cells clearly distinguishable. The morphology showed a characteristic clustering pattern typical of cocci, indicating effective adherence to the substrate.

Detection of biofilm associated gene

Out of the 24 isolates selected for the study, *aap* gene related to the biofilm formation could be amplified from the isolate M37.2 due to the formation of a PCR amplicon with 466 bp size (Fig. 5) when compared with the positive control as demonstrated by the agarose gel electrophoresis.

Fig. 3 The statistical analysis of the biofilm formed by selected bacteria as demonstrated through the respective O.D. values generated from TCP assay where the grouping was based on least significant difference (LSD) test



Discussion

Micrococci and *Kocuria* spp. are different but closely related genera of Gram-positive bacteria that are commonly found in various environments, including the soil, water, and on human skin. They are considered as thermophilic bacteria due to their existence in milk and dairy products even after the pasteurization (Lee et al. 2024). The thermophilic bacteria, which escapes the pasteurization of milk may not often cause serious threat to humans but under certain conditions such as in the case of immunocompromised individuals, they can cause infections as well as mediate the transfer of antibiotic resistance genes to other bacterial pathogens. They also have the potential to adversely affect the quality and shelf life of dairy products. Both these genera have also been reported to possess the biofilm-forming ability, due to their capacity to get attached on to the surfaces to result in complex microbial communities. Biofilms are structured communities of bacteria that are embedded in a self-produced matrix of extracellular polymeric substances (EPS) (Mahto et al. 2022).

In this study, the milk sample was collected from various farms of Kottayam district of Kerala. The microbial load of the samples was tested by the methylene blue dye reduction test, which is generally used to check the microbiological quality of raw and pasteurized milk. The blue color of the dye solution added in to the milk is decolorized when the oxygen present in the milk is exhausted owing to the microbial activity (Oluchi et al. 2022). The earlier the decolorization, the higher the milk's bacterial load and hence this test is being commonly performed at dairy reception docks, processing units, and milk chilling facilities to accept or reject the raw and processed milk. In this study, three of the milk samples showed the discoloration of blue to white within 2 h indicating the milk is of low quality whereas the rest of the samples showed the color change after 3 h indicating fair quality of the selected milk. Even though the microbial load was higher in three samples, the targeted bacteria were able to get isolated from all the milk samples. The bacteria isolated from these milk samples were subjected to automated species identification by the VITEK® 2 automated system and were identified as strains of *Micrococci* and

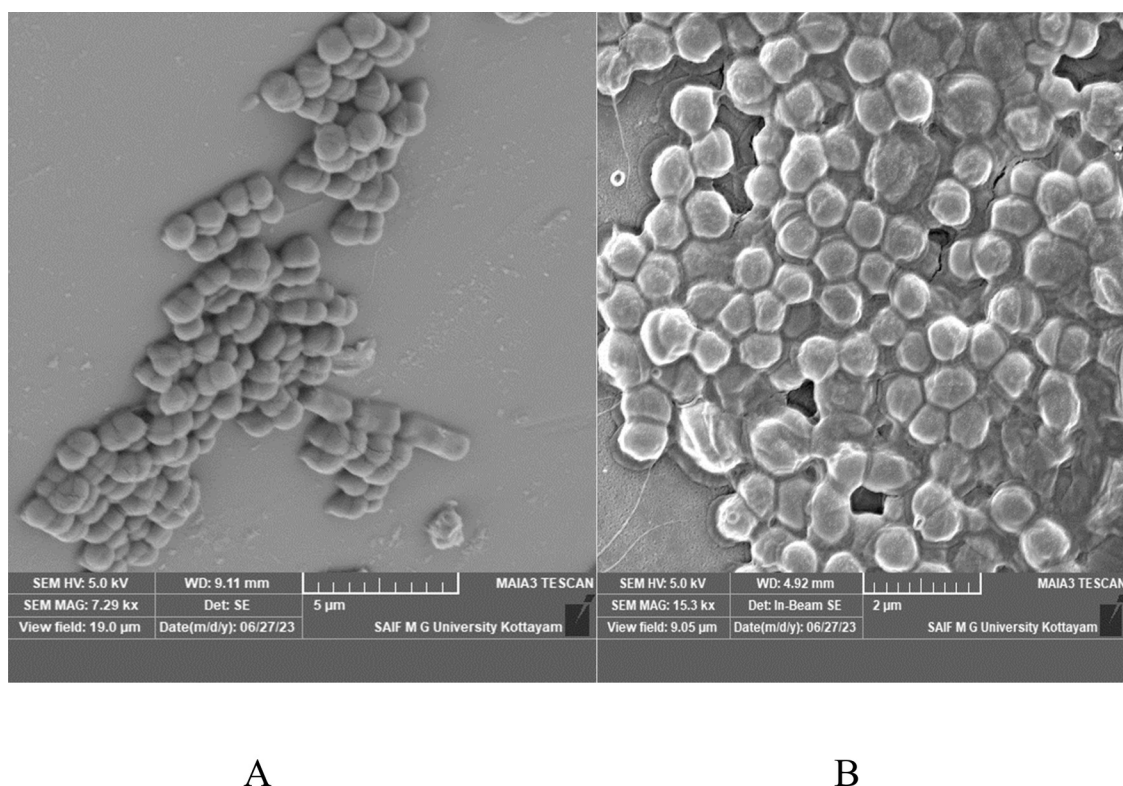


Fig. 4 The bacterial strain M37.2 showing the formation of biofilm under the magnification of (A) 5 µm and (B) 2 µm on the surface of glass piece after the incubation period

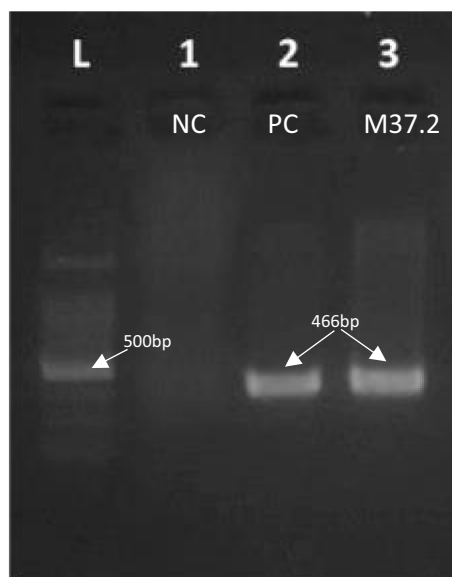


Fig. 5 Agarose gel electrophoresis image of the PCR amplicon generated after the gene screening of *aap* (466 bp) from the isolate M37.2 in well number (3). L represents Ladder DNA, PC in well number (1) represents Positive Control and NC in well number (2) represents Negative Control

Kocuria spp. In this study, the targeted bacterial species were identified after the Gram staining with the help of GP card for bacteria. As the maximum incubation time required for the automated system is 18 h, this method of identification enables the rapid bacterial identification along with the AST analysis, which could help to have an understanding on the AST pattern of thermophilic bacteria from the milk and dairy products (Qin et al. 2024). The susceptibility of *Micrococcus* and *Kocuria* spp. to the common antibiotics was tested by using the Kirby Bauer disc diffusion method according to the CLSI guidelines, which included 7 antibiotics from different categories. Here, the highest resistance was shown against the antibiotics clindamycin and ampicillin followed by co-trimoxazole which is in par with the previous reports on AST pattern of the same genera (Chimbekujwo et al. 2022, Perrin-Guyomard et al. 2005, Dib et al. 2012 and Živković Zarić et al. 2019). Although a detailed study to identify intrinsic resistance mechanisms in *Micrococci* and *Kocuria* is critical. The formation of biofilm on CRA plates by the isolates M15.1 (*Micrococcus luteus*), M17.2 (*Kocuria kristinae*), M17.3 (*Micrococcus* sp.), M37.4 (*Micrococcus* sp.), M23.1 (*Micrococcus* sp.), M37.2 (*Micrococcus* sp.), M30.1 (*Kocuria kristinae*) and M28.3 (*Micrococcus* sp.) indicated the CRA assay to be effective for identifying the slime and biofilm production



phenotypically as described before (Soumya et al. 2017). The study report by Ali & Riaz have also been supported the use of CRA plate experiment to be a reliable method for assessing the biofilm production by bacteria (Ali & Riaz 2024). The TCP assay further showed the strains M37.2 (*Micrococcus* sp.) and M37.4 (*Micrococcus* sp.) to have the ability to form strong biofilm. In a previous study, the residual milk leftovers in milking equipment have been reported to be an ideal environment for the growth of thermotolerant bacteria such as *Micrococci* and *Kocuria* (Dubey, Raj & Kumar, 2022; and Machado et al. 2020). Thermotolerant bacteria associated with these residues could dislodge and enter the raw milk during the milking, resulting in an increase in the Standard Plate Count (SPC) which thereby decrease the quality of milk (Ribeiro-Júnior et al. 2020). Further validation of the biofilm formation by the thermotolerant bacteria isolated in this study was carried out by the PCR based screening for the accumulation associated protein (*aap*) gene. The result revealed the isolate M37.2 to have the presence of *aap* gene due to the 466 bp size PCR product formed in accordance with our previous report (Soumya et al. 2020). Even though no available scientific literature shows the presence of *aap* gene in *Micrococci* as of now, the occurrence of the same has already been reported in the case of *Staphylococcus* spp. in many previous studies (Dalvand et al. 2023, Mottaghiyan et al. 2019 and Soumya et al. 2017). Hence the results of the study provide novel insight into the thermotolerant bacteria.

Conclusion

Biofilm formation by the thermotolerant bacterial strains can lead to various issues in the food chain. Once established, the biofilms are more resistant to antimicrobial agents, making them challenging to treat. Therefore, understanding the genetic basis of biofilm formation of these bacterial strains is crucial for developing strategies to prevent and manage the biofilm associated challenges. The specific genes involved in the biofilm formation by bacteria may vary among different strains and species. While numerous studies have investigated the genetic determinants of biofilm formation in other bacteria, such as *Staphylococcus aureus* and *Escherichia coli*, there is relatively limited research specifically focused on *Micrococcus* and *Kocuria* species. More studies are needed to identify and characterize the genes and mechanisms involved in the biofilm formation by thermotolerant bacteria to gain a deeper understanding on this process. Further research is needed to elucidate the genetic factors and mechanisms underlying biofilm formation in *Micrococcus* species, which will contribute to the development

of effective strategies for preventing and managing biofilm associated risks.

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Availability of data and materials All data generated or analysed during this study are included in this article.

Declarations

Conflict of 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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