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Antimicrobial Activity of Turmeric against Bio-Film Forming Staphylococcus Aureus Isolated from Dairy Milk

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Abstract- Staphylococcus aureus is a well-known pathogen capable of producing enterotoxins during bacterial growth in contaminated milk, causing mastitis in dairy cattle and the ingestion of such preformed toxins is one of the major causes of food poisoning around the world. The capacity of S. aureus to produce biofilms increases its antibiotic resistance, posing a considerable challenge to dairy hygiene and public health. This research explored the antimicrobial activity of turmeric (Curcuma longa) extracts towards biofilm-producing S. aureus isolated from dairy milk. Milk samples were collected from dairy farms and screened to microbiological analysis for the isolation and identification of S. aureus by using selective culture media, morphological, and biochemical methods. The biofilm-forming ability of the isolates was determined by using Congo Red Agar method and the microtiter plate assay. Ethanolic turmeric extract was prepared using appropriate maceration extraction methods and tested for antimicrobial activity

Keywords— Antimicrobial activity, Bio-Film, Congo red Agar, Dairy Milk, microtiter plate, turmeric extract

I. INTRODUCTION

Milk and dairy products are highly susceptible to microbial contamination due to their nutrient-rich composition. One of the major concerns in the dairy industry is the formation of biofilms-structured microbial communities encased in a self produced matrix that adhere to surfaces like milking equipment and storage tanks. These biofilms are resistant to antibiotics, disinfectants, and thermal treatments, making them difficult to eliminate and contributing to persistent contamination, food spoilage, reduced shelf life, and serious public health risks (Marchand, S. 2012;, Diarra, C. 2023). Milk is an ideal environment for bacteria to grow rapidly and can spread bacteria which are harmful for people's health. Although it has numerous health benefits and nutritional significance, milk can be used as an outlet for the spread of diseases infections, which happens commonly (Khairullah et al., 2020; Widodo et al., 2020). Efforts to increase the availability of milk must be supported by improvements to the quality and safety of dairy products because a food's high content of nutrients is worthless if it is harmful to human health (LeJeune et al., 2009).

Impact of Biofilms in Dairy Processing plants

The utilisation of milk as a culture medium for spoilage and pathogenic bacteria leads to the contamination of dairy products and milk production facilities. Microorganisms contaminate dairy products in production facilities through vegetative cells, spores, or detached biofilm clusters that adhere to the stainless-steel components of supplementary equipment used in dairy production. The predominant processing lines in dairy manufacturing facilities are fabricated from stainless steel, as stainless steel components completely adhere to all relevant regulations for food contact applications. Despite stainless steel being the preferred material in the dairy business, the production, adhesion, and contamination of biofilms remain unavoidable. Studies have established that dead ends, corners, cracks, crevices, gaskets, valves, and joint are all suitable areas for bio-film formation and, therefore, it is essential to use surfaces with

minimal cracks and crevices to reduce bacterial adherence and bio-film formatioStorgards et al., 1999; Bremer et al., 2009).

Bio-film-Forming Pathogens of Concern in Dairy

Staphylococcus aureus: Staphylococcus aureus is gram-positive bacteria may grow in both oxygen-rich and oxygen-poor conditions, classifying it as a facultative anaerobe. It is considered a risk key pathogen for health hazards in human as well as veterinary medicine. It is known to be an important reason why cows get mastitis (Dufour et al., 2012). S. aureus is a big public health threat because it can make food sick when it comes from animals, like milk and other dairy items (Hornik et al., 2021). The bacteria make heat-resistant enterotoxins (SE) that can cause staphylococcal food poisoning, which is a common cause of foodborne illness around the world (Fisher et al., 2018; Cvetnić et al., 2021). Besides causing dangerous illnesses, S. aureus is the cause of many other serious invasive diseases, such as infections that come from being in the hospital, infections that happen after surgery, infections in joints that have prosthetics, and infections in the respiratory tract (Cheung et al., 2021). When S. aureus makes biofilm, it gets harder to treat because this structure makes drugs less effective and infections last longer. Antibiotic resistance is becoming a bigger issue. Because of this, we need to find new ways to kill germs. Black cumin, also known as Nigella sativa is a plant used in medicine. It is well known for having a wide range of pharmacological qualities, including antifungal, antibacterial, and anti-inflammatory effects (Gholamnezhad et al., 2016). The use of nanoparticle carriers has been proposed to enhance the efficacy of antimicrobial agents as well as drug delivery particularly against bio-film-forming bacteria (Zhou et al., 2018).

Curcumin-Mediated Inhibition of S. aureus

Curcumin inhibits the growth of both Gram-positive and Gram-negative bacteria (S. C. Gupta et al., 2012; P. Tyagi et al., 2015). S. aureus is one of the Gram-positive strains that susceptible to curcumin-mediated inhibition. S. aureus is a pathogen that causes various infections including infective endocarditis (IE), bacteremia, skin and soft tissue, osteoarticular, and pleuropulmonary infections (S.H. Mun et al., 2014). Mun et al., (2014) showed that the minimal inhibitory concentrations (MICs) of curcumin against 10 strains of S. aureus (including 2 ATCC MSSA and MRSA standard strains, 4 MRSA clinical isolates, and 4 MRSA from culture collection) ranged from 125 to 250 μg/mL while a study by Wang et al. (2016) showed the MIC of 256 μg/mL against MSSA. Using a broth microdilution assay, our group (S.Y. Teow 2015) also showed that 250 μg/mL curcumin was required to kill the two ATCC MSSA (#25923) and MRSA (#43300) strains. The activity was time and dose-dependent, and 100% killing was achieved at 50 μM (equivalent to 18.42 μg/mL), after 2 hr exposure (Tyagi et al., 2015). This study explores turmeric's antimicrobial potential against biofilm-forming Staphylococcus aureus isolate from dairy milk, offering a natural solution for mastitis control, reducing antibiotic resistance, improving dairy safety, and promoting eco-friendly livestock management.

II. METHODOLOGY

Sample of milk was collected aseptically from local dairy farms and markets. Each sample was transported to the laboratory under refrigerated conditions and processed within 4-6 hours of collection. All collection and handling were performed using an aseptic process and sterile items, flaming and refrigerated (4°C ± 2°C). The laboratory work of this study was done in Department of Biotechnology, Keral Verma Subharti College of Science, Swami Vivekanand Subharti University Meerut. Each sample was serial diluted in sterile normal saline. Aliquots were cultured on selective media, Mannitol Salt agar, to isolate a wide spectrum of bacteria. Plates were incubated at 37 °C for 24–48 h. Pure isolates were obtained by sub culturing distinct colonies. Pure isolates were gram stained and viewed under microscope to identify the cell morphology and gramme reaction. Preliminary identification was done using biochemical tests such catalase oxidase, indole synthesis, methyl red, Voges Proskauer, and mannitol fermentation test.

Biofilm Formation Assay

The pathogenic strains were grown in Luria-Bertani liquid medium in tubes overnight at 35°C (5 mL). 100

µL of overnight cultures were inoculated into the wells of 96 well polystyrene flat bottom microtiter plates and incubated without shaking at 37°C for 18 hours. The optical density (OD) of each well was measured at an absorbance of 620 nm using a spectrophotometer. The plates were rinsed 3 time with sterile saline to remove any securely attached bacteria and then allowed to air dry for 20 min after the rinsing process. To all wells, 130 µL of 1% crystal violet was applied and left for 15 minutes to react to stain the adhering bacterial cells. The crystal violet was washed from the wells with distilled water and left to air dry for 20 minutes. After that, 130 µL of 96% ethanol was added to each well to dissolve the crystal violet that remained in the biofilm matrix. The OD of the stained and attached bacterial cells in the test and control wells was measured at 540 nm. Biofilm formation index was measured b− CW)/G

Where BFI is the Biofilm Formation Index, AB is the OD at 540nm of attached and stained bacteria, CW is the OD of stained control wells without bacteria at 540nm, and G is the OD of suspended bacterial growth at 620nm. All assays were performed in triplicate (Naves et al., 2008). BFI values of >1.10 indicated strong biofilm formation, 0.70–1.09 indicated moderate biofilm formation, and <0.35 indicated no biofilm formation.

Extraction of Turmeric

Turmeric powder [10g] was macerated (100 mL of 90% ethanol) in a closed glass container. The mixture was kept in orbital shaker incubator at 37 °C temperature for 72 hours. After maceration, the mixture was filtered through Whatman No. 1 filter paper. The filtrate was concentrated at 40 °C temp using a rotary evaporator and stored at 4 °C in amber vials until further analysis. The ethanolic extracts were subjected to phytochemical screening for qualitative detection of phytoconstituents. The extracts were subjected to phytochemical tests for determination of plant secondary metabolites such as alkaloid, flavonoid, tannin, saponin, and triterpenoid/steroid (Sinaga SM et al., 2018; Simanjuntak, HA. 2020).

Antimicrobial Activity of Turmeric

The antibacterial activity of turmeric (Curcuma longa) ethanolic extract against Staphylococcus aureus was determined by agar well diffusion technique. The microbes were suspended in sterile normal saline and adjusted to the 0.5 McFarland turbidity requirements. Mueller–Hinton Agar (MHA) was made and placed into sterilised Petri plates. Once solidified, 100 µL of the standardised bacterial solution was spread evenly across the surface of the agar with a sterile L-shaped spreader. Turmeric extract was diluted in 10 % dimethyl sufoxide (DMSO) to reach the concentrations of 500, 250, 125, 62.5 and 31.2 mg/mL. Aseptic punching of wells of diameter 6 mm was done in the inoculated agar plates and 100 µL of each extract concentrations were dispersed in respective wells. Ciprofloxacin solution was used as the positive control while 10% DMSO was the negative control. The infected plates were let to stand for 30 min at room temperature to allow diffusion of the test solutions then incubated at 37°C for 24 h. The antibacterial activity was determined by measuring the diameter of the inhibitory zone around each well. All tests were performed in triplicate and findings are presented as mean inhibition zone diameter ± standard deviation (SD).

III. RESULTS AND DISCUSSION

Staphylococcus aureus was successfully isolated from dairy milk samples collected from local dairy farms. The isolates formed characteristic yellow colour colonies on Mannitol Salt Agar, indicate mannitol fermentation. Identification of Staphylococcus aureus is confirmed by morphologically by gram staining and biochemical analysis indicates the presence of Staphylococcus aureus species (Table 1).

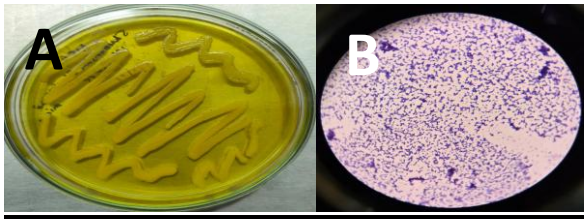

Figure 1: (A) S. aureus show yellow colonies on Mannitol Salt Agar (B) Gram staining of S. aureus

Table: 1. Biochemical analysis of Staphylococcus aureus

Biochemical Test	Result	Observation
Gram Staining	Gram Positive	Gram-positive cocci in clusters
Catalase Test	Positive	Bubble formation after adding H_2O_2
Oxidase Test	Negative	No purple color change
Coagulase Test	Positive	Plasma clot formation
Indole Test	Negative	No red ring
Methyl Red (MR) Test	Positive	Red color after reagent
Voges–Proskauer (VP) Test	Positive	Pink color
Mannitol Fermentation Test	Positive	Yellow color in MSA medium

The biofilm-forming capabilities of the identified isolates were assessed using Congo Red Agar method and microtiter plate. Staphylococcus aureus bio-film show black colour colonies with a dry crystalline consistency on congo red agar plates. The microtiter plate assay demonstrated that untreated Staphylococcus aureus showed strong biofilm formation with an OD value of 1.057.

The extent of biofilm formation was measured based on the following formula:
BFI = (AB − CW)/G
BFI= 0.142-0.013/0.122
BFI= 0.129/0.122
BFI=1.057

These results highlight the varying degrees of biofilm formation among the isolated dairy-associated microbes, emphasizing the need for further investigation into their implications for food safety and hygiene practices in dairy processing. The isolation of biofilm-forming pathogens from dairy products underscores the critical importance of stringent hygiene practices in dairy processing and handling. The strong biofilm-forming ability of Staphylococcus aureus is particularly concerning, given their well-documented association with foodborne illnesses. The presence of these microbes in dairy products not only poses a risk to consumer health but also highlights the necessity for effective monitoring and control strategies to prevent biofilm formation. Implementing such strategies is essential to ensure food safety and maintain the quality of dairy products.

Extraction & Phytochemical Screening: About 10 g of turmeric powder was soaked in 100 mL ethanol in a conical flask and kept at room temperature for 48–72 hours with occasional shaking to allow extraction of bioactive compounds. The mixture was then filtered using whatman filter paper and the filtrate was concentrated using a rotary evaporator or water bath at 40–50°C to remove the solvent. The resulting crude turmeric extract was stored at 4°C for further antimicrobial and biofilm inhibition studies against Staphylococcus aureus. Phytochemical screening results of turmeric ethanolic extract showed different phytochemical compounds (Table 2).

Table: 2. Phytochemical screening of rhizome turmeric ethanolic extract

No.	Screening	Reagent	Ethanolic Extract
1	Alkaloides	Mayer	Positive
2	Flavonoids	Lead Acetate	Positive
3	Saponins	Foaming Test	Positive
4	Tannins	FeCl3 1%	Positive
5	Triterpenoids	Sakowski	Positive

Antibacterial activity against Staphylococcus aureus

Antimicrobial efficacy of selected ethanolic against Staphylococcus aureus using the agar well diffusion method at different concentrations ranging from 31.2 to 500 mg/mL. The results demonstrated a concentration-dependent inhibitory effect, with higher zones of inhibition observed at increased extract concentrations (Table 3). The highest antibacterial activity was recorded at 500 mg/mL, producing a mean inhibition zone of 17.63 ± 1.60 mm, followed by 250 mg/mL (13.92 ± 1.55 mm) and 125 mg/mL (11.33 ± 0.33 mm). Lower concentrations

exhibited comparatively reduced inhibitory effects, with inhibition zones of 8.85 ± 0.51 mm and 9.83 ± 0.38 mm at 62.5 mg/mL and 31.2 mg/mL, respectively. The positive control, Ciprofloxacin, produced a significantly larger inhibition zone of 24.80 ± 0.61 mm, indicating superior antibacterial efficacy compared to the herbal extract. No inhibitory activity was observed for the negative control (DMSO), confirming that the solvent did not contribute to bacterial growth inhibition. O

Table: 3. Antimicrobial result of rhizome turmeric ethanolic extract

Sample Concentraction	Zone of Inhibiton (mm) Staphylococcus aureus
500 mg/mL	17.63 ± 1.60
250 mg/mL	13.92 ± 1.55
125 mg/mL	11.33 ± 0.33
62.5 mg/mL	8.85 ± 0.51
31.2 mg/mL	9.83 ± 0.38
Ciprofloxacin	24.80 ± 0.61
DMSO	0

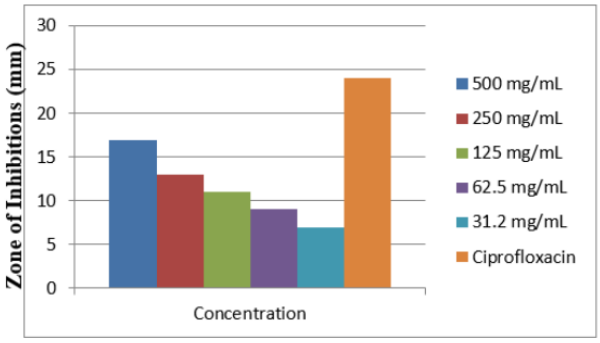

Figure: 1 Antimicrobial activity of turmeric against S.aureus

IV. CONCLUSION

The higher frequency of biofilm-forming bacteria in conventionally processed dairy products when compared to pasteurised dairy products shows the improved ability of these microbes to directly or indirectly damage and affect consumers. This difference may be attributed to the regular monitoring and follow-up of the food hygiene and safety authorities. Furthermore, the high tendency of the population to purchase traditional dairy products needs supply information about their further processing (i.e., heating time and temperature), as the absence of these details can increase the likelihood of human infection and poisoning. The present results show that adequate hygiene measures, such as constant hygienic supervision and training of the operators and employees involved in the production of traditional items, are important. Moreover, the regular washing and disinfection of equipment and supplies might prevent the potential biofilm formation of S. aureus in milk and dairy products. The present investigation rev

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