Characterization of Biofilm-Forming Potential of Dairy-Associated *Salmonella enterica* and *Staphylococcus aureus*

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Abstract: The presence of biofilm-forming bacteria in milk and dairy products poses a significant threat to food safety, public health, and product quality. Biofilms enable microbes to adhere to surfaces and persist in processing environments, leading to contamination and increased resistance to cleaning agents and antibiotics. This study aims to isolate and identify biofilm-forming microbes from raw milk and commonly consumed dairy products, such as yogurt and cheese. A total of 12 samples were collected from local markets and dairy farms under sterile conditions. Bacterial isolation was conducted using selective media, followed by morphological and biochemical characterization. Molecular identification through 16S rRNA gene sequencing confirmed the presence of key dairy-associated pathogens, including *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella spp.* The biofilm-forming ability of each isolate was assessed using the microtiter plate crystal violet assay.

The results revealed a high prevalence of moderate to strong biofilm producers among the isolated microbes. These findings underscore the importance of monitoring biofilm-forming bacteria in dairy products and implementing effective control strategies to reduce contamination and ensure product safety.

Keywords— Biofilm-forming bacteria, Dairy Products, *Staphylococcus aureus*, *Listeria monocytogenes*, 16S rRNA gene sequencing.

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[1,2].

Staphylococcus aureus is a pathogenic bacterium frequently associated with foodborne illnesses and dairy contamination. It can survive pasteurization, form biofilms, and produce heat-stable enterotoxins. In China, 53.7% of food poisoning cases in 2015 were linked to *S. aureus*[3,4] and in the U.S., it causes around 241,000 cases annually[5]. Studies in China have shown that 96.7% of isolates from pasteurized milk products could form biofilms, with 66.7% carrying virulence factors[7]. In Indonesia, commercially packed milk—popular among students—has also been found to harbor biofilm-forming *S. aureus*, posing a potential health risk.

Escherichia coli is a Gram-negative, facultative anaerobic bacterium commonly found in the intestines of humans and animals. However, its presence in milk and dairy products indicates fecal contamination and the potential presence of pathogenic strains. These include several pathotypes such as EPEC, ETEC, EAEC, and EHEC[26], some of which produce Shiga toxins leading to serious illnesses. *E. coli* can also form biofilms, enhancing its survival in dairy processing environments and increasing its resistance to sanitization measures [8,9].

Salmonella is a Gram-negative, rod-shaped pathogen capable of forming biofilms on dairy processing surfaces. It includes over 2700 serovars, with *Salmonella enterica* subsp. *enterica* being the most common in human and animal infections[10,27]. The pathogen can be shed in raw milk and is a major contributor to salmonellosis outbreaks, especially through the consumption of unpasteurized dairy products[11,12,17]. Its ability to form biofilms complicates eradication and heightens its public health impact.

Biofilm-forming bacteria such as *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella* pose serious challenges in the dairy industry[20,21]. Their ability to persist on equipment and resist conventional cleaning methods elevates the risk of contamination and foodborne illness. This study aims to isolate and characterize biofilm-forming microbes from milk, cheese, and yogurt to inform improved hygiene practices and safety measures in dairy production.

II. MATERIAL AND METHODS

Samples of milk, cheese, and yogurt, were collected aseptically from local dairy farms and markets. Each sample was transported to the laboratory under refrigerated conditions and processed within 24 hours of collection. Aseptic technique was used throughout all sampling and handling procedures by using sterile materials, flaming, and refrigeration (4°C ± 2°C). The laboratory work of the present study was carried out at the Department of Biotechnology, Keral Verma Subharti College of Science, Swami Vivekanand Subharti University, Meerut.

Serial dilutions of each sample were prepared using sterile saline solution. Aliquots were plated on selective and differential media[13], including MacConkey agar[13,14], Mannitol Salt agar, and Xylose Lysine Deoxycholate agar, to isolate a broad range of bacteria[15]. Plates were incubated at 37°C for 24–48 hours. Distinct colonies were sub-cultured to obtain pure isolates.

Pure isolates were subjected to Gram staining and observed under the microscope to determine cell morphology and Gram reaction. Biochemical tests, such as catalase oxidase[15], indole production[16], methyl red[17], Voges-Proskauer[18], citrate utilization, and triple sugar iron tests[19], were performed for preliminary identification.

Genomic DNA was extracted from selected isolates using a standard phenol-chloroform extraction method. The 16S rRNA gene was amplified using universal primers and sequenced[22]. Sequences were compared with known sequences in the NCBI database using BLAST for species-level identification.

The ability of isolates to form biofilms was assessed using the microtiter plate assay with crystal violet staining[23,24]. Overnight cultures were diluted and inoculated into 96-well polystyrene plates and incubated at 37°C for 24 hours. Wells were washed, stained with 0.1% crystal violet, and the absorbance was measured at 570 nm[25] to quantify biofilm formation.

Microorganism	Gram Stain	Disease Potential	Biofilm Traits	Notable Sources
Staphylococcus aureus	Gram-positive	Food poisoning, enterotoxemia	Heat-stable enterotoxins, strong biofilm	Pasteurized milk, cheese
Escherichia coli	Gram-negative	Diarrhea, HUS, colitis	Multiple pathotypes, shiga toxins, biofilm	Raw milk, soft cheese
Salmonella spp.	Gram-negative	Salmonellosis (GI infection)	Forms persistent biofilms, zoonotic	Raw milk, dairy plants

Table 1: Characteristics of Key Biofilm forming bacteria in dairy products

III. RESULTS AND DISCUSSION

In this study, a total of 12 bacterial isolates were successfully obtained from various dairy samples collected from local markets and dairy farms. The initial morphological and biochemical analyses (Table 1 and Table 2) indicated the presence of several pathogenic species, specifically *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella spp*. These findings were further validated through molecular identification techniques, where 16S rRNA gene sequencing revealed high similarity to known strains of these bacteria, confirming their identity and establishing their relevance in the context of dairy contamination.

S.N.	Isolates number	Colony diameter (mm)	Colony Characteristics
1.	R-1	1.0	Round, regular periphery, smooth, opaque, raised and dry.
2.	R-2	1.5	Oval, regular periphery, smooth, raised, opaque, discrete and dry.
3.	R-3	2.0	Slimy, deep & round, irregular periphery, translucent, mucoid and wet.
4.	R-4	3.0	Flat, irregular periphery, slimy, translucent and wet.
5.	E-1	0.5	Elongated, smooth, discrete, regular periphery, opaque and dry.
6.	E-2	1.0	Round, smooth, discrete, regular periphery, opaque and dry.
7.	E-3	4.0	Flat, mucoid, translucent, irregular periphery, slimy, rough and wet.
8.	E-4	2.0	Discrete, flat, irregular periphery, opaque and dry.
9.	S-1	1.0	Discrete, elongated, irregular periphery, raised and dry.
10.	S-2	1.0	Round, discrete, opaque, regular periphery and dry.
11.	S-3	3.0	Elongated, slimy, irregular, raised periphery, translucent, mucoid and wet.
12.	S-4	1.5	Flat, mucoid, smooth, regular periphery, opaque and dry.

Table 2: Characteristics of Colony Morphology of Selected Isolates

S.N.	Isolates	24 h.	48 h.	72 h.	96 h.	120 h.
1.	R-1	0.21	0.28	0.39	0.46	0.54
2.	R-2	0.26	0.31	0.38	0.48	0.51
3.	R-3	0.28	0.39	0.51	0.46	0.36
4.	R-4	0.24	0.41	0.56	0.42	0.32
5.	E-1	0.23	0.32	0.41	0.49	0.46
6.	E-2	0.26	0.31	0.38	0.47	0.53
7.	E-3	0.27	0.38	0.49	0.51	0.37
8.	E-4	0.24	0.30	0.39	0.46	0.41
9.	S-1	0.25	0.33	0.38	0.47	0.54
10.	S-2	0.23	0.29	0.36	0.44	0.51
11.	S-3	0.39	0.48	0.59	0.51	0.42
12.	S-4	0.31	0.38	0.47	0.56	0.49

Table – 3: Growth profile of selected isolates under controlled conditions (Absorbance at 570 nm)

The biofilm-forming capabilities of the identified isolates were assessed using the microtiter plate crystal violet assay. Among the isolates, *Staphylococcus aureus*, *Salmonella enterica* and *Listeria monocytogenes* exhibited strong biofilm-forming abilities, with significant absorbance values at 570 nm (Table 3), indicating a significant capacity for biofilm development. In contrast, *Escherichia coli* and *Salmonella enterica.* demonstrated moderate biofilm formation, suggesting that while they may not form biofilms as robustly

as the former two species, they still possess the potential to contribute to biofilm-related issues in dairy environments.

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*, *Salmonella enterica* and *Listeria monocytogenes* are 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.

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Author Profile

Dr. Ravindra Kumar Jain is an accomplished academician and researcher with over 23 years of experience in teaching, research, and academic administration in the field of Biotechnology. Currently serving as Professor at Swami Vivekanand Subharti University, Meerut, he has previously held key positions including Dean Academics and Head of the Department at Anand Engineering College, Agra. Dr. Jain holds a Ph.D. in Botany and a Post Graduate Diploma in Nano Biotechnology, and has qualified the CSIR-NET in Life Sciences. His research contributions include 21 published papers (with 9 indexed in SCI/Scopus), 4 authored books, 2 patents granted, and supervision of more than 60 undergraduate projects and Ph.D. theses. He is a Fellow of the Linnean Society of London and actively contributes as a reviewer and editorial board member for reputed journals. Dr. Jain has participated in over 60 seminars and 23 faculty development programs conducted by premier institutions such as IITs, IIMs, and IISc, and has coordinated skill development initiatives under PMKVY. His core research interests lie in plant-microbe interactions and microbial biotechnology.