DETECTION OF MULTIDRUG-RESISTANT MILK-ASSOCIATED PSYCHROTROPHIC *Pseudomonas* spp. USING LAB-BASED AND AGENT-BASED MODELLING APPROACHES IN PAKISTAN

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ABSTRACT

Pseudomonas spp. are opportunistic bacteria capable of contaminating milk and exhibiting antimicrobial resistance (AMR), posing potential public health concerns. Among them, *Pseudomonas aeruginosa* is an important opportunistic pathogen associated with antimicrobial resistance and public health concerns. To investigate selected virulence-associated markers, *toxA* and *algD* genes were targeted. Antimicrobial resistance and transmission dynamics were assessed using agent-based simulations. A total of 100 milk samples were collected from retail shops, farms and commercially packaged brands. Samples were cultured on Cetrimide agar, presumptively identified based on biochemical characteristics, examined for fluorescence and subjected to PCR for *toxA* and *algD* genes. AMR was evaluated against nine antibiotics using the Kirby–Bauer disc diffusion method and milk handling practices were assessed. Two agent-based models were developed to simulate AMR emergence during milk aggregation under varying horizontal gene transfer probabilities and AMR persistence associated with biofilm formation and cleaning frequency. All isolates were presumptively identified as *Pseudomonas* spp. and neither *toxA* nor *algD* was detected. Resistance was highest to aztreonam (6/10, 60% retail; 2/5, 40% packaged; 4/5, 80% farm), followed by meropenem, piperacillin, and gentamicin, while ceftazidime, amikacin and imipenem showed high susceptibility. Multidrug resistance (MDR) occurred in 5/10 (50%) retail isolates and 2/5 (40%) farm isolates, with none detected among the 5 packaged milk isolates. Model 1 simulations indicated that increasing the assumed horizontal gene transfer probability accelerated simulated AMR emergence during milk aggregation. Model 2 simulations indicated that biofilm formation enhanced the simulated persistence and maintenance of AMR. Poor hygiene practices in retail milk were associated with MDR among the tested retail isolates. Pasteurization and proper hygiene may reduce contamination and limit the spread of resistant strains.

Keywords: *Pseudomonas aeruginosa*, *Pseudomonas* spp., Bulk milk tanks, Multidrug resistance, Public health, Agent-based Modeling

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INTRODUCTION

Pseudomonas spp. are Gram-negative bacteria widely distributed in environmental, plant and animal niches (Badawy *et al.*, 2023, Shimokawa *et al.*, 2025). *Pseudomonas aeruginosa* is an important opportunistic pathogen associated with human infections, particularly in healthcare settings (Li *et al.*, 2022). Its large and versatile genome contributes to its ability to adapt to diverse environmental conditions. *Pseudomonas* spp. are psychrotrophic bacteria capable of growing at refrigeration temperatures, allowing them to persist and multiply in refrigerated milk during cold storage. Some *Pseudomonas* spp. produce extracellular proteases and lipases that may retain activity after certain heat treatments and degrade milk components, affecting quality and shelf life. (Wiedmann *et al.*, 2000, Machado *et al.*, 2015, Chang *et al.*, 2024).

Raw milk contains almost all the essential nutrients, but it can also be easily contaminated by microorganisms (Hassan *et al.*, 2025). Pakistan faces risks from informal supply chains, adulteration and seasonal dilution (Afzal *et al.*, 2011). Reports from Lahore indicate widespread milk quality issues, highlighting public health concerns (Ibrahim *et al.*, 2023). The pathogenic potential of *P. aeruginosa* is associated with multiple virulence factors, including exotoxin A, encoded by *toxA* and alginate production associated with *algD* (McIntosh *et al.*, 1992, Iglewski, 1996, Ibrahim *et al.*, 2022). In the present study, *toxA* and *algD* were selected as specific virulence-associated markers for molecular

screening and were not intended to represent the complete virulence profile of the isolates. The motility and type IV pili of *P. aeruginosa* enhance adhesion to surfaces (O'Toole and Kolter, 1998). Antimicrobial resistance (AMR) is a major global public health concern (Bhatnagar, 2024). Antimicrobial resistance to multiple antimicrobial classes further complicates the control of *Pseudomonas* spp. (Abd El-Baky *et al.*, 2020). The presence of antimicrobial-resistant *Pseudomonas* spp. in milk may represent a potential One Health concern because milk can serve as a vehicle for exposure to resistant bacteria, although detection alone does not establish foodborne transmission or human infection.

Agent-based modeling (ABM) is a computational approach that simulates complex systems by representing individual components as "agents" with specific characteristics. In the USA, machines and workers were taken as agents for food factories (Ivanek *et al.*, 2004, Sullivan *et al.*, 2021). In a dairy supply chain, milk batches can be modeled as agents carrying AMR genes, allowing exploration of how interactions during aggregation and transport may influence AMR emergence. Laboratory data provide observed patterns of contamination and resistance, while ABM can be used to explore potential AMR dynamics under different simulated conditions. Infrastructure surfaces can be modeled as agents to represent biofilm growth and its role in maintaining AMR over time. By manipulating agent properties, such as removing surfaces or altering bacterial interactions, ABM enables assessment of AMR dynamics, including emergence, persistence, and decay. This approach is especially valuable in developing countries like Pakistan, where financial constraints make it difficult to study and monitor all stages of milk production and distribution in real time from farms to retail shops, including bulk tanks and packaged milk.

However, the dynamics of AMR emergence and persistence across the Pakistani milk supply chain, particularly during milk aggregation and on dairy-associated surfaces, remain poorly understood. The present study aimed to detect *Pseudomonas* spp. in milk samples collected from Lahore and Kasur, to evaluate the presence of *toxA* and *algD* virulence-associated marker, and to determine antimicrobial resistance profiles. Based on the observed AMR data, two agent-based models were developed. ABM 1 simulated milk batches as agents to examine the emergence of AMR during aggregation processes, while ABM 2 modeled dairy supply chain surfaces as agents to investigate biofilm-mediated persistence of antimicrobial resistance.

MATERIALS AND METHODS

Study Design and Sample Collection A cross-sectional research design was used in Pakistan. A total of 100 milk samples were obtained in the summer months from July to September 2025 from Lahore and Kasur district. Retail milk samples were collected in Lahore, while farm bulk milk samples were collected from Kasur district. Of these, 46 samples were collected from retail shops in Lahore, 34 samples were collected from farm bulk milk tanks in Kasur district, and 20 samples were commercially packaged milk (pasteurized and UHT) from Lahore. The geographic distribution of the sampling locations in Lahore and Kasur district is presented in Figure 1. Samples were collected using convenience sampling based on accessibility and availability. Therefore, the findings may not be representative of the entire Pakistani dairy supply chain but reflect only the sampled locations. Milk from these farms is commonly transported to retail shops in Lahore. From each bulk tank, approximately 15 to 20 mL of milk was collected in sterile Falcon tubes. The tubes were gently immersed in the bulk milk tanks, sealed immediately after collection, and handled aseptically.

All bulk milk samples were placed in an icebox and maintained at 4°C. Samples were transported to the Epidemiology and Public Health Laboratory, University of Veterinary and Animal Sciences, Lahore, on the same day for further analysis. During sampling, visual observations and brief interviews were conducted with handlers or supervisors to record information related to cleaning frequency and hygiene practices of bulk milk tanks for risk assessment of factors that may contribute to antimicrobial resistance.

Additionally, 20 commercially available packaged milk packs were included in the study. Pasteurized milk samples were stored at 4°C. Ultra-high-temperature (UHT) milk packs were kept at ambient conditions as per the manufacturer's instructions. Only unopened, unexpired and properly sealed milk packs were selected for analysis.

Microbiological Analysis All culture media were prepared following the manufacturer's guidelines and sterilized at 121°C for 15 minutes. Buffered Peptone Water (BPW; Neogen, UK; Cat. No. UK2119801177) was used for enrichment and Cetrimide Agar (Biolab, Hungary; Cat. No. ECAB290321075P) for isolation of *Pseudomonas* spp. For enrichment, 1 mL of each milk sample was inoculated into 9 mL BPW and incubated for 24 hours at 37°C (Ibrahim *et al.*, 2022). After enrichment, streaking was done onto Cetrimide Agar and incubated at 37°C for 24 hours. Greenish to blue-green pigmented colonies were considered presumptive *Pseudomonas* spp. Gram staining was performed using standard procedures and Gram-negative rods were observed (Paray *et al.*, 2023). Presumptive identification of *Pseudomonas* spp. was based on catalase, oxidase, Triple Sugar Iron (TSI) and citrate utilization tests following standard methods (Hemraj *et al.*, 2013, Roy *et al.*, 2024). Fluorescent activity was assessed under UV light after incubation. Blue fluorescence was recorded as positive (Jawher and Hasan, 2023).

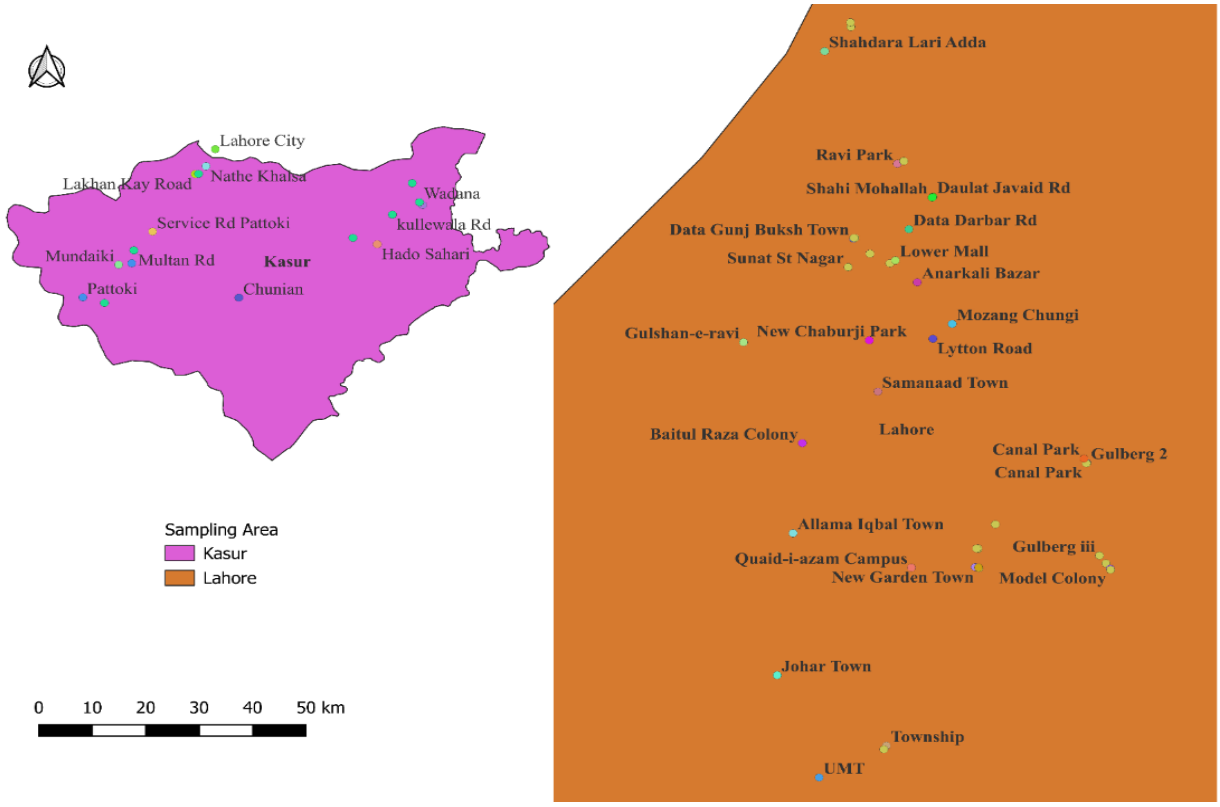


Fig. 1 Map showing the sampling locations of milk samples collected from Lahore and Kasur districts, Pakistan (prepared using QGIS 3.40.4)

Molecular Analysis Isolates were preserved in nutrient broth (Oxoid, UK; Cat. No. 3464135) with 20% glycerol and stored at –70°C. For revival, frozen cultures were inoculated into buffered peptone water at 37°C for 24 hours, followed by subculturing onto Nutrient Agar (Merck, India; Cat. No. JK3JF63356). DNA was extracted by the boiling method (Omar *et al.*, 2014). Briefly, bacterial pellets were washed, mixed thoroughly in nuclease-free water, boiled at 100°C for 10 minutes, centrifuged, and the supernatant containing DNA was preserved at –70°C for PCR analysis. Presumptive *Pseudomonas* spp. isolates were screened by qualitative PCR targeting the virulence-associated genes *toxA* and *algD*. Each 25 µL reaction contained 2X Hot Start Master Mix, 1 µL reverse primer, 1 µL forward primer, 8.5 µL nuclease-free water, and 2 µL DNA template.

For *toxA*, the forward primer F: GACAACGCCCTCAGCATCACCAGC and reverse primer R: CGCTGGCCCATTCGCTCCAGCGCT amplified a 396 bp fragment (Elshafiee *et al.*, 2019). For *algD*, the forward primer F: TTCCCTCGCAGAGAAAACATC and reverse primer R: CCTGGTTGATCAGGTCGATCT amplified a 520 bp fragment (Ullah *et al.*, 2016). PCR cycling and agarose gel electrophoresis were carried out as previously described by the respective studies. Amplified products were visualized under UV light using a 100 bp DNA ladder.

Antimicrobial Susceptibility Antimicrobial resistance of *Pseudomonas* spp. was tested by the Kirby-Bauer disc diffusion method (Swetha *et al.*, 2017). It includes nine antibiotics from seven classes: Piperacillin, Amikacin, Aztreonam, Ceftazidime, Piperacillin–Tazobactam, Meropenem, Imipenem, Gentamicin, and Levofloxacin. Preserved samples were revived, grown on Nutrient agar (Merck, India; Cat. No. JK3JF63356), and bacterial suspensions were adjusted to the 0.5 McFarland standard (Lalitha, 2004). Suspensions were spread on Mueller–Hinton agar. After application of antibiotic discs, plates were incubated at 37°C for 16–18 hours. Zones of inhibition were measured per CLSI standards. Isolates resistant to one antibiotic were considered antimicrobial-resistant. Those isolates resistant to three or more classes were classified as multidrug resistant (MDR).

Agent-Based Modeling The agent-based model represents a virtual dairy supply chain from farm to consumer, highlighting key causal links and intervention points (Fig. 2). Milk batches move through multiple stages and contact

various surfaces, influencing contamination and AMR dynamics. This framework guides the simulation of *Pseudomonas* spp. persistence and spread within the supply chain. To complement laboratory data, two agent-based models were developed in Python (version 3.10) using the Mesa framework (version 3.03). ABM 1, in which the primary agent is a milk batch agent, simulated the emergence of multidrug resistance in milk during aggregation and storage via probabilistic horizontal gene transfer (HGT) (Fiaz and Awan, 2026a). Milk batch agents were characterized by bacterial load, number of AMR genes, and supply chain pathway. Bacterial load increased by a fixed growth rate of 0.4 per simulation step. HGT probability was varied (0.0–0.20) to assess mechanistic effects and calibrate with laboratory data. The effective HGT probability was calculated from the specified HGT probability and a bacterial density-based contact factor capped at 1.0. For each value, 50 Monte Carlo simulations were performed. Each value included three stages, from farm production, transport, or aggregation to storage or branching. The model consisted of 30 farms with 6 milk batch agents per farm (180 agents in total), with an initial bacterial load of 10^3 to 10^4 and 10% of farms assigned a higher initial AMR burden. A bacterial density threshold of 5×10^5 was used for HGT. Model behavior was evaluated by toggling horizontal gene transfer on/off, performing HGT parameter sweeps and comparing AMR between raw and processing pathways. For reproducibility, the complete model implementation, simulation scripts, and analysis and visualization scripts are publicly available in the archived software release (Fiaz and Awan, 2026a). ABM 2, in which surfaces are primary agents, simulated persistence of resistance on dairy infrastructure surfaces through biofilm growth, colonization, and shedding. Each surface agent tracked biofilm biomass and mean AMR burden, with colonization occurring at a probability of 0.05 and biofilm growth at 2% per time step. Shedding occurred with a probability of 0.20 and removed 10% of biofilm biomass, while cleaning was applied every 7 time steps with 95% biomass reduction. The model was evaluated using cleaning intervals of 1, 3, 7, 14, and 30 time steps and upstream AMR reduction experiments. It was validated by observing lagged AMR decay after upstream reduction (memory), identifying cleaning-dependent persistence thresholds, and confirming loss of persistence when colonization, growth or surface availability were removed. The complete model code is available in the associated software repository (Fiaz and Awan, 2026b).

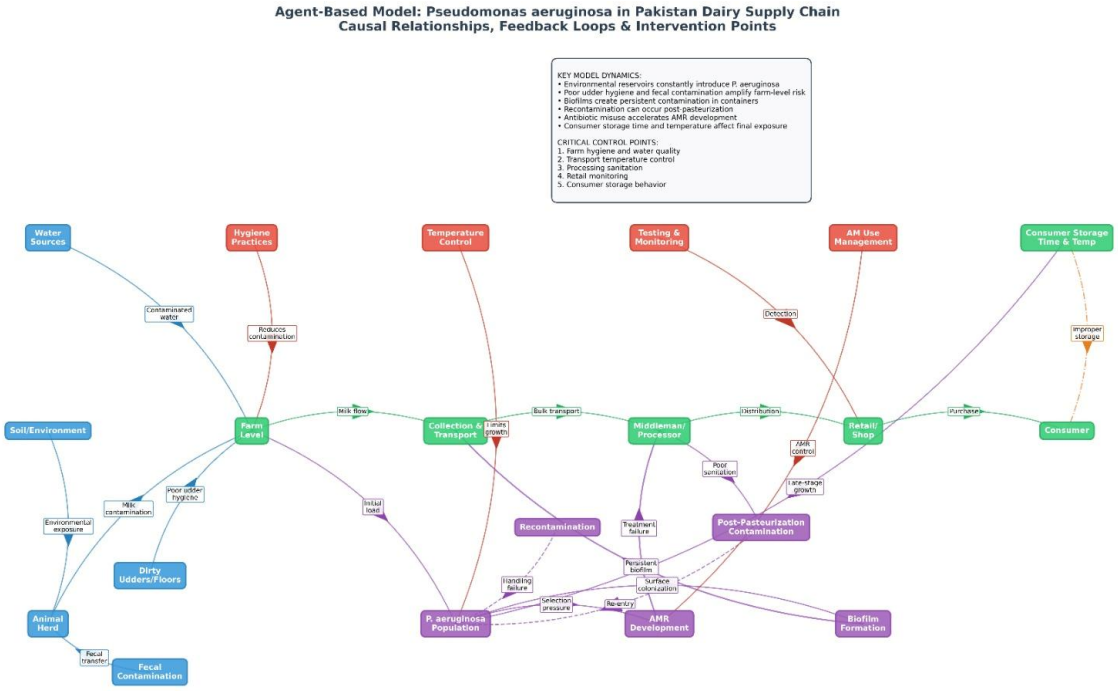


Fig. 2 Agent-based model schematic of the dairy supply chain illustrating key causal relationships, contamination pathways, and intervention points affecting *Pseudomonas aeruginosa* transmission from farm to consumer

Statistical Analysis Data analysis was performed using R statistical software (version 4.4.1). Because of the small number of isolates with available MDR classification and the sparse frequencies across several categorical risk factor levels, associations between assessed risk factors at retail shops and dairy farms and multidrug resistance in *Pseudomonas* strains were evaluated using Fisher's exact test. A p-value ≤ 0.05 was considered statistically significant.

RESULTS

Identification Out of 100 milk samples cultured on Cetrimide agar, 96 (96%) showed colonies (Fig. 3A). Colonies showing characteristic greenish pigmentation, occasionally creamy-white pigmentation, were presumptively identified as *Pseudomonas* spp. These isolates were further examined and all displayed Gram-negative rods on Gram staining (Fig. 3B). All 96 isolates were subjected to biochemical testing. Catalase positivity was indicated by immediate bubble formation after the addition of hydrogen peroxide (Fig. 3C). A rapid color change following oxidase reagent indicated oxidase positivity (Fig. 3D). On TSI agar, red slant and red butt without gas production indicated a negative reaction (Fig. 3E). Citrate utilization was demonstrated by a green-to-blue color change of the medium (Fig. 3F). Under UV light, all identified isolates exhibited blue fluorescence (Fig. 3G).

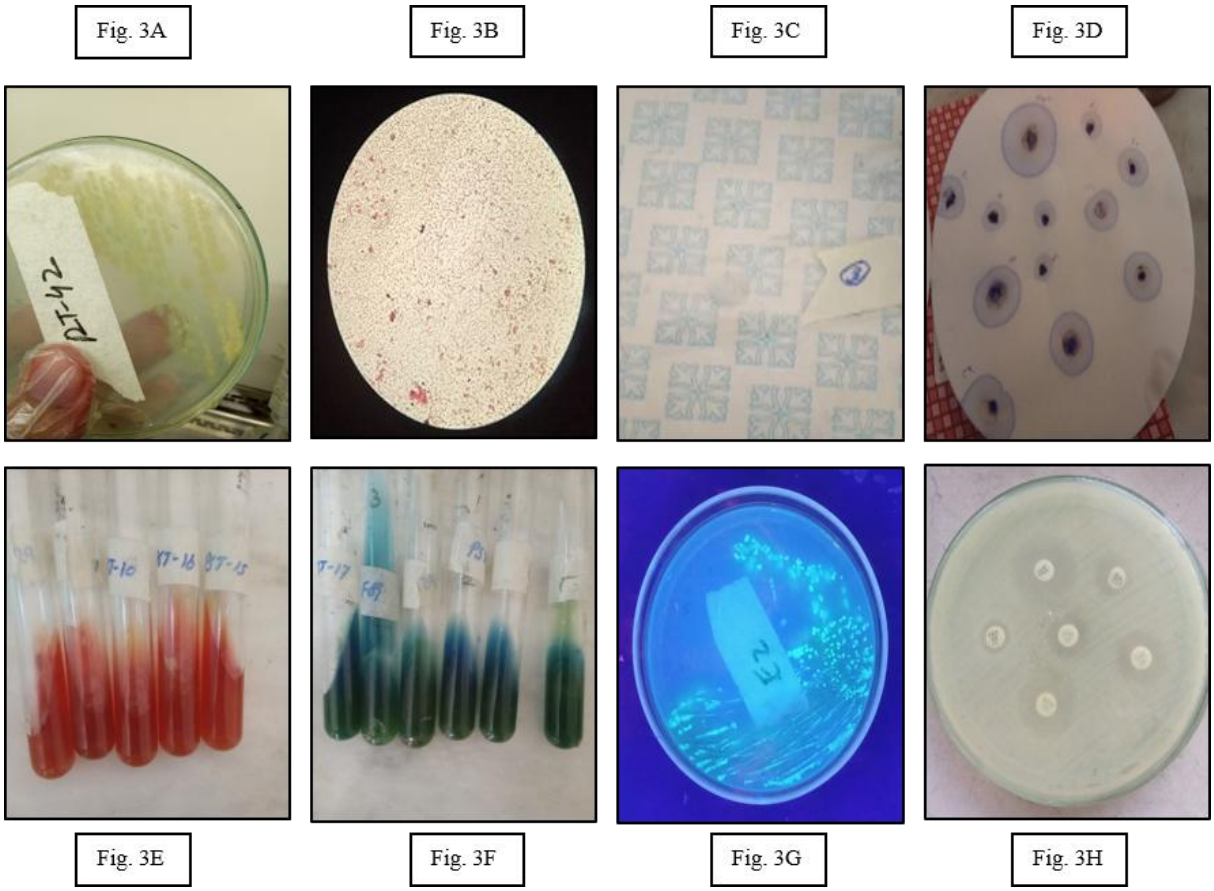


Fig. 3 (A) Characteristic Pseudomonas colonies on Cetrimide agar (Pure Culture) (B) Phenotypically identified Pseudomonas colonies exhibited Gram-negative rod- shaped bacteria on Gram staining (C) Phenotypically identified Pseudomonas spp. colonies showing positive catalase test (D) It shows a positive oxidase test, indicated by the appearance of a purple coloration on the filter paper in the petri plate (E) It shows red slant and red butt indicating sample negative (F) It shows citrate positive sample indicated by blue color of agar (G) It shows blue fluorescence of bacterial colonies on Cetrimide agar under ultraviolet light (H) Results of antimicrobial susceptibility test (Kirby Bauer Disc diffusion method)

Molecular Analysis by PCR PCR targeting the *toxA* and *algD* genes was carried out on all 96 biochemically tested isolates. No amplification of *toxA* (396 bp) or *algD* (520 bp) was detected among all 96 tested isolates. Only the positive controls produced bands at the expected sizes, while the nuclease-free water negative control showed no amplification, confirming valid PCR results (Fig. 4).

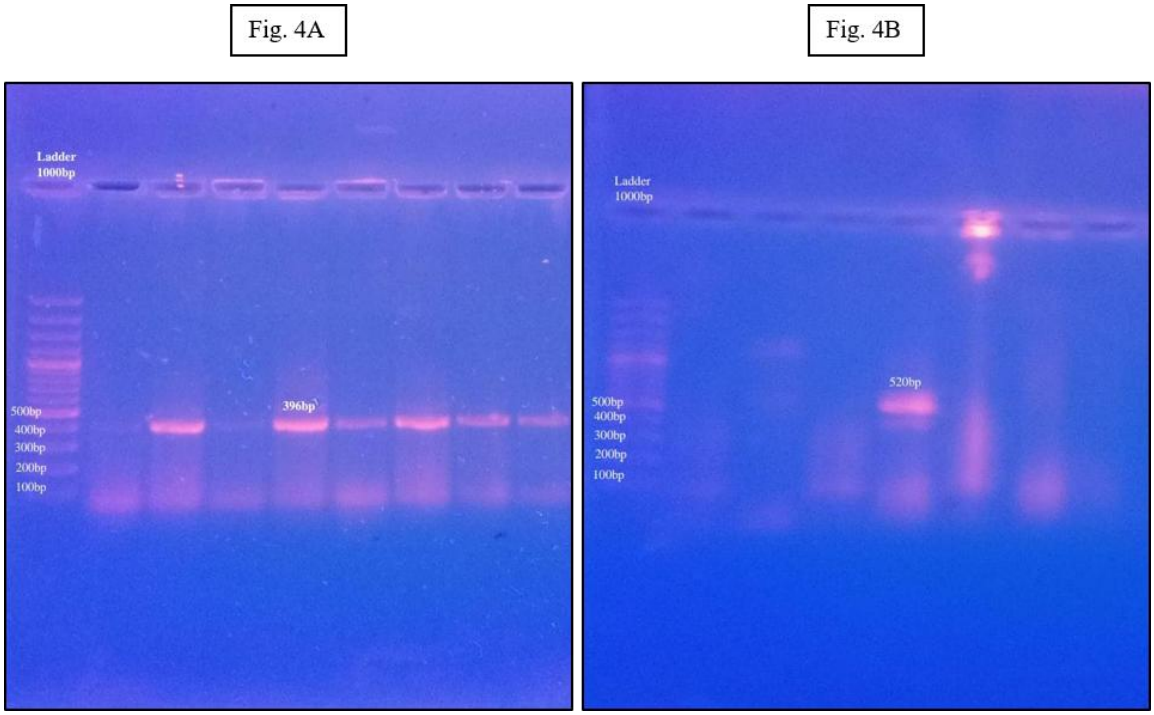


Fig. 4 Gel electrophoresis showing Pseudomonas aeruginosa control PCR products (A) positive control for toxA with a 396 bp band (B) positive control for algD with a 520 bp band

Antimicrobial Susceptibility All 96 presumptive *Pseudomonas* isolates were first screened by PCR for the targeted *toxA* and *algD* genes, and none showed amplification. Following PCR screening, 20 isolates were selected to include the different milk source categories comprising 10 isolates from retail milk, 5 from dairy farms, and 5 from packaged milk. Antimicrobial susceptibility testing of all 96 isolates was not feasible due to limited laboratory resources. Resistance patterns were interpreted according to CLSI guidelines (Fig. 3H). Out of the 20 isolates, all showed resistance to at least one antibiotic. Multidrug resistance was observed in 5 retail milk isolates (5/10, 50%) and 2 farm milk isolates (2/5, 40%). None of the packaged milk isolates were multidrug-resistant. Graphs (Fig. 5A, 5B and 5C) show antibiogram data with the percentages of resistant, intermediate and susceptible isolates across retail, packaged and farm milk. The highest resistance was observed for aztreonam (6/10, 60% in retail; 2/5, 40% in packaged and 4/5, 80% in farm), meropenem (5/10, 50%; 0/5, 0% and 3/5, 60%), piperacillin (5/10, 50%; 2/5, 40% and 2/5, 40%) and gentamicin (4/10, 40%; 4/5, 80% and 2/5, 40%). Moderate resistance was also noted for amikacin (3/10, 30%; 0/5, 0% and 1/5, 20%), piperacillin–tazobactam (1/10, 10%; 1/5, 20% and 0/5, 0%), imipenem (2/10, 20%; 0/5, 0% and 0/5, 0%) and ceftazidime (0/10, 0%; 0/5, 0% and 1/5, 20%). Highest susceptibility was observed for ceftazidime (7/10, 70%: 5/5, 100% and 3/5, 60%), amikacin (6/10, 60%; 1/5, 20% and 4/5, 80%), imipenem (6/10, 60%; 5/5, 100% and 2/5, 40%), piperacillin–tazobactam (6/10, 60%; 2/5, 40% and 3/5, 60%), piperacillin (2/10, 20%; 1/5, 20% and 0/5, 0%), levofloxacin (3/10, 30%; 1/5, 20% and 1/5, 20%), aztreonam (1/10, 10%; 2/5, 40% and 1/5, 20%) and meropenem (3/10, 30%; 3/5, 60% and 2/5, 40%). Gentamicin showed no susceptible isolates in any group. These resistance and susceptibility percentages should be interpreted with caution because antimicrobial susceptibility testing was performed on a limited number of isolates. Therefore, the findings are not representative of all 96 isolates.

Fig. 5A

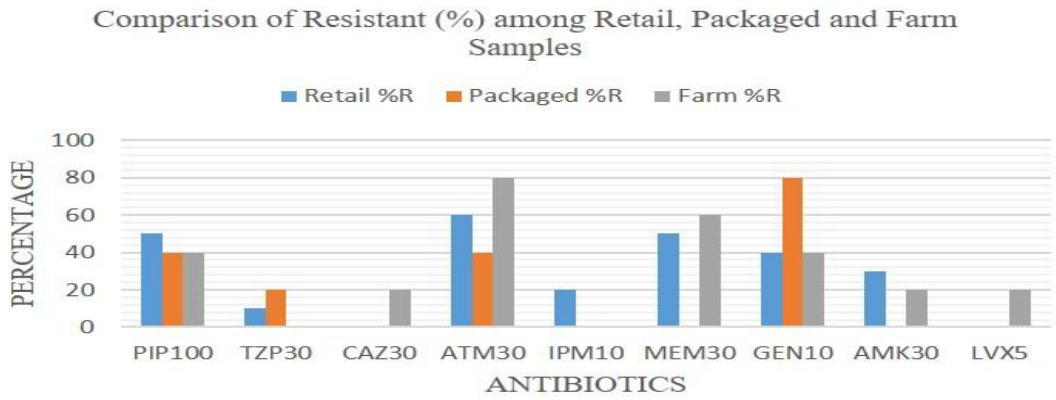


Fig. 5B

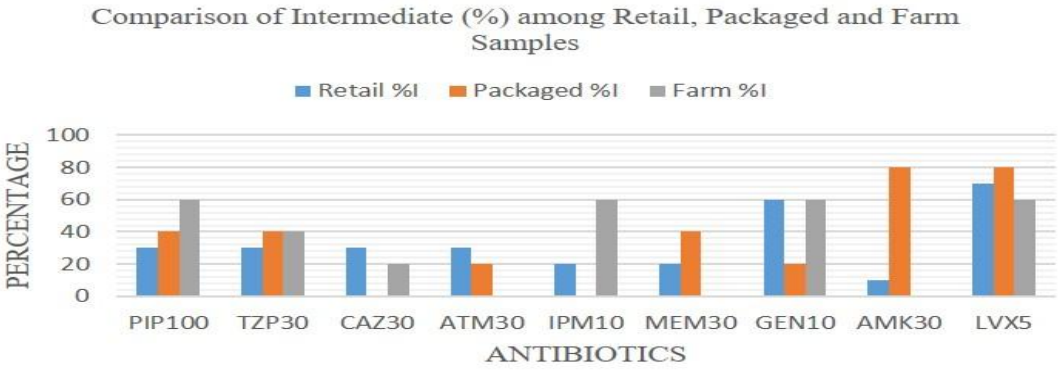


Fig. 5C

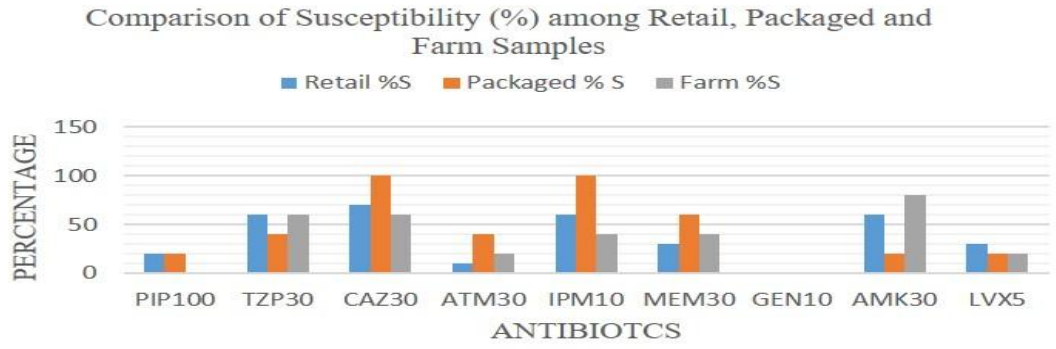
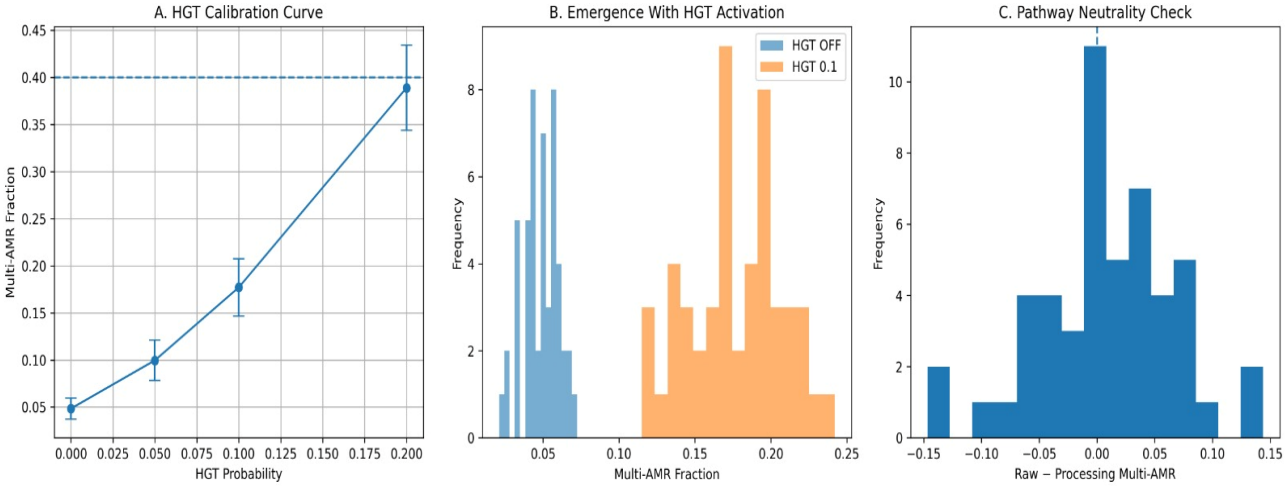


Fig. 5 (A) Graph shows percentages of resistant isolates of *Pseudomonas* spp. to all nine antibiotics among retail, packaged, and farm milk samples (B) Graph shows percentages of intermediate isolates of *Pseudomonas* spp. to all nine antibiotics among retail, packaged and farm milk samples (C) Graph shows percentages of susceptible isolates of *Pseudomonas* spp. to all nine antibiotics among retail, packaged, and farm milk samples

ABM 1: Emergence of Multidrug Resistance Simulation results demonstrated a monotonic increase in MDR prevalence with increasing HGT probability (Fig. 6A). In the absence of HGT, MDR levels remained low and reflected baseline contamination. As HGT probability increased, a rightward shift in resistance distribution was observed across simulation runs indicates emergent resistance during milk aggregation and storage (Fig. 6B). No consistent difference



was detected between raw and processing pathways, suggesting pathway neutrality in resistance dynamics (Fig. 6C).

Fig. 6 Emergence of multi-antimicrobial resistance (A) HGT calibration curve showing increasing multi-AMR fraction with rising horizontal gene transfer probability (B) Distribution of multi-AMR fractions with HGT off (blue) and on (orange) (C) Raw vs Processing pathway differences centered around zero, indicating pathway independence

ABM 2: Persistence via Biofilm Formation Biofilm-associated antimicrobial resistance exhibited delayed decline following simulated reductions in upstream contamination indicating persistence and memory effects (Fig. 7A). Variation in cleaning frequency revealed threshold behaviour, where frequent cleaning suppressed steady-state resistance while infrequent cleaning allowed sustained AMR burden (Fig. 7B). Removal of colonization, growth, or surface availability eliminated persistent resistance behaviour, confirming that persistence emerged from interacting biofilm processes (Fig. 7C).

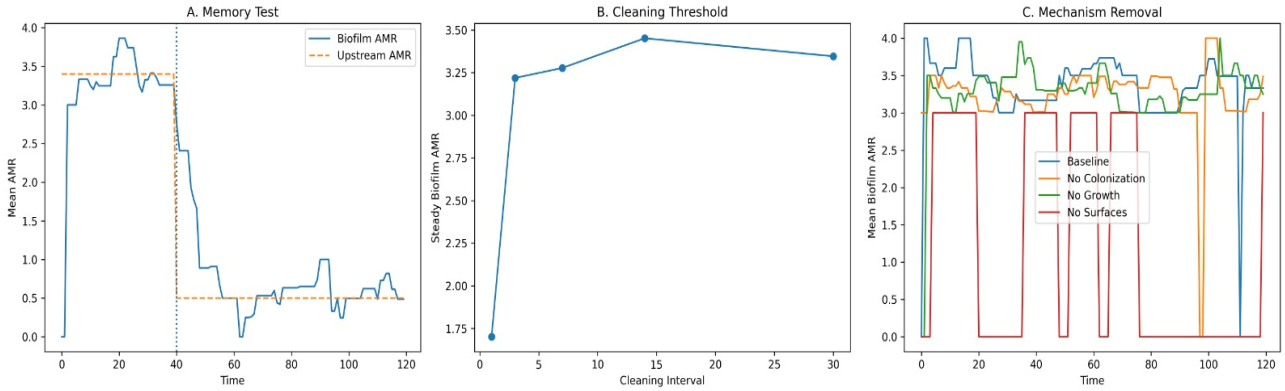


Fig. 7 Biofilm-mediated AMR persistence (A) Memory test showing lagged decline of biofilm AMR after upstream reduction (B) Steady-state biofilm AMR versus cleaning intervals highlighting persistence threshold (C) Mechanism removal over time under baseline (blue), no colonization (orange), no growth (green) and no surfaces (red) showing critical roles of surfaces, colonization and growth

Although upstream AMR decreased abruptly, biofilm-associated AMR declined gradually over time, indicating persistence and memory effects within infrastructure surfaces (Fig. 8).

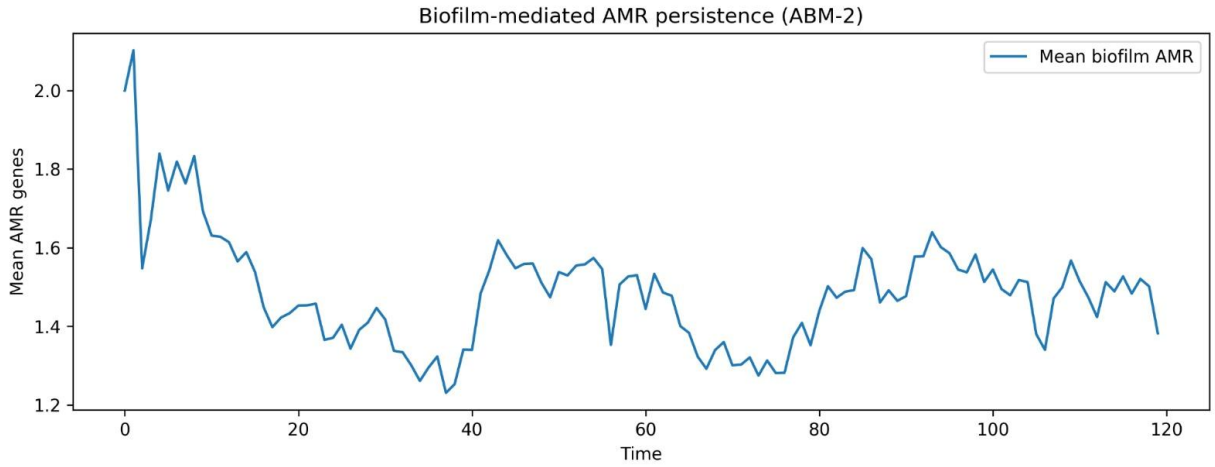


Fig. 8 Biofilm-mediated AMR persistence over time

Analysis of Questionnaire Data Questionnaire data for retail and farm samples and statistical analysis linking MDR *Pseudomonas* to handling practices are presented in Tables 1 and 2, respectively. In retail samples, cleaning frequency and use of gloves during milk handling were significantly associated with MDR, while no significant associations were found in farm samples. Several retail and farm practices, including milk pooling, use of steel containers, wearing clean clothing, and absence of hand wounds, were uniform. Therefore, these practices could not be statistically evaluated for association with multidrug resistance.

Table 1. **Analysis of Risk Assessment Associated with the Presence of MDR *Pseudomonas strains* in retail shop**

Independent Variable		**MDR**	**Non-MDR**	**p-value**
Is milk pooled before testing	Yes	5	5	
Type of container used for milk storage	Steel	5	5	
Bulk milk container/tank is covered	No	0	1	1
	Yes	5	4	
Cooling system present (Refrigeration or Ice)	No	1	2	1
	Yes	4	3	
Time between milk arrival and cooling	≤1 hr	5	4	1
	1-hrs	0	1	
Availability of hot water for cleaning tanks/utensils	No	3	4	1
	Yes	2	1	
Cleaning done with cold water only	No	2	1	1
	Yes	3	4	
Cleaning frequency	Alternate days	4	0	*0.0476
	Daily	1	5	
Use of detergent/disinfectant	No	2	0	0.4444
	Yes	3	5	
Gloves used while handling milk	No	5	1	*0.0476
	Yes	0	4	
Clean clothing/uniform worn	Yes	5	5	
Open wounds/cuts on hands	No	5	5	
Hairnet/head cover used	No	4	5	1
	Yes	1	0	

Hand washing facility available	No	1	0	1
	Yes	4	5	
Education Level of Milk Handler / Supervisor	High School(Grade 9-12)	0	1	0.5367
	Illiterate	4	2	
	Primary (Grade 1-5)	1	2	

* $P \leq 0.05$ was considered statistically significant.
Blank cells indicate that no P value was calculated because all respondents gave the same answer.

Table 2. **Analysis of Risk Assessment Associated with the Presence of MDR *Pseudomonas strains* in farm samples**

Independent Variable		**MDR**	**Non-MDR**	**P-value**
Is milk pooled before testing from multiple animals?	Yes	2	3	
Type of container used for milk collection	Steel	2	3	
Availability of hot water for cleaning	No	1	3	0.4
	Yes	1	0	
Cleaning done with cold water only	No	1	0	0.4
	Yes	1	3	
Cleaning frequency of utensils/bulk tanks	Daily	2	3	
Gloves used during milking/handling	No	1	3	0.4
	Yes	1	0	
Clean clothing/uniform worn	Yes	2	3	
Open wounds/cuts on hands	No	2	3	
Hairnet/head cover used	Yes	2	3	
Hand washing facility available	Yes	2	3	
Teat Preparation done	Yes	2	3	
Milking Method	Both	0	2	0.4
	Machine	2	1	
Pipeline cleaning frequency	Daily	2	3	
Bulk tank cleaning after every milk pickup	Yes	2	3	
Bulk tank cleaning methos after milk pickup	Manual	2	3	
Bulk tank and pipeline inlet clean & shiny	Yes	2	3	
Milk storage container/bulk tank is covered	Yes	2	3	
Cooling system available on farm	Yes	2	3	
Education Level of Milk Handler / Supervisor	High School (Grade 9-12)	1	0	0.6017
	Illiterate	0	2	
	Primary (Grade 1-5)	1	1	

No statistically significant associations were observed ($P > 0.05$).
Blank cells indicate that no P value was calculated because all respondents gave the same answer.

DISCUSSION

This study aimed to detect virulence-associated markers *toxA* and *algD* in presumptively identified *Pseudomonas* spp. from farm, retail and packaged milk. Neither *toxA* nor *algD* was detected among the tested isolates; however, their absence does not exclude the presence of other virulence determinants. In contrast, *algD* was detected in 80% of *P. aeruginosa* isolates from poultry meat and meat products along with other virulence-associated genes, including *pslD*, *lasR*, *rhlR*, *exoS* and *exoU* (Abdullah et al., 2024). Antimicrobial susceptibility testing was performed using culture-based methods (Swetha *et al.*, 2017). Molecular confirmation was negative, so the isolates were reported as

Pseudomonas spp. Cetrimide agar is widely used for presumptive isolation of *P. aeruginosa*. It is selective rather than species-specific and supports the growth of multiple *Pseudomonas* species and other Gram-negative bacteria (Lambe and Stewart, 1972, Akoglu *et al.*, 2012, Gamal *et al.*, 2022).

Isolates showed marked resistance to aztreonam, meropenem, piperacillin, and gentamicin. Similar resistance to aztreonam (67%) and gentamicin (58%) has been reported among *P. aeruginosa* isolates from poultry meat and meat products in Pakistan, although meropenem showed high susceptibility (84%) in that study (Abdullah *et al.*, 2024). Similarly, high resistance to meropenem (100%), imipenem (100%), piperacillin (71.42%), and gentamicin (64.28%) was reported among *P. aeruginosa* isolates from burn wound infections (Ahsan *et al.*, 2024). In dairy products, all biochemically identified *P. aeruginosa* isolates were resistant to the tested antibiotics except meropenem, while the *blaIMP* gene was detected in all molecularly confirmed isolates (Tanaka *et al.*, 2024). Highest susceptibility was observed for ceftazidime, followed by amikacin and imipenem, with moderate susceptibility for piperacillin–tazobactam, piperacillin, and levofloxacin. The observed resistance may reflect antimicrobial selection pressure or contamination occurring at different stages of the dairy production and handling chain. The present study cannot determine the stage at which resistance originated. Most isolates remained susceptible to ceftazidime and amikacin. This may be due to low selective pressure and the absence of specific resistance genes.(Arslan *et al.*, 2011, Meng *et al.*, 2020, Briega *et al.*, 2025).

All *Pseudomonas* isolates from retail, packaged, and farm milk were resistant to at least one antibiotic. Multidrug resistance (MDR) was detected in 50% of retail milk isolates (5/10) and 40% of farm milk isolates (2/5), with no MDR in packaged milk isolates. Comparable MDR rates (30–85%) have been reported in raw milk and dairy products (Meng *et al.*, 2020, Briega *et al.*, 2025). Cheese showed no MDR (Arslan *et al.*, 2011), reflecting differences in processing, hygiene, and antimicrobial exposure.

In agent-based model 1, the first graph (HGT Calibration Curve) shows that the multi-drug-resistant fraction increases steadily with horizontal gene transfer (HGT) probability, from about 5% at zero HGT to nearly 39% at 0.2 HGT, confirming a clear upward trend. The horizontal dotted line indicates the observed laboratory MDR prevalence of 40%. The simulated prevalence at HGT = 0.20 (~0.39) closely approximated the empirical value (Fig. 5A). Previous studies have reported that Pseudomonas can acquire AMR genes through plasmid-mediated conjugation and extracellular DNA. Therefore, heterogeneity in resistance across milk batches may occur (Thomas and Nielsen, 2005, Couce and Blazquez, 2009). The second graph compares resistance distributions with HGT off and at an HGT probability of 0.1. Without HGT, values cluster around 2–7%, peaking near 4–5%. With HGT, the distribution shifts to the right, with values mostly between12–24%, peaking near 18–20%, showing increased resistance under the simulated gene transfer conditions (Fig. 5B). The third graph tests pathway neutrality by plotting differences between raw and processing pathways, which center tightly around zero (−0.15 to +0.15). This indicates no substantial difference in simulated resistance between the pathways. The HGT model provides a theoretical mechanism for the emergence of antimicrobial resistance (AMR) under the specified simulation assumptions; however, the laboratory component of this study did not directly demonstrate horizontal gene transfer. This suggests that, under the model assumptions, HGT had a greater influence on resistance emergence than the simulated supply chain pathway (Fig. 5C).

In agent-based model 2, the graphs show biofilm-associated AMR dynamics under different conditions. The first graph (Memory Test) shows biofilm AMR rising to about 3.8 by time 25 and remaining above upstream AMR (~3.4). After upstream AMR drops sharply to ~0.5 at time 40, biofilm AMR declines gradually with a lag, fluctuating between 0 and 1 until time 120, demonstrating biofilm-associated resistance memory (Fig. 6A). The second graph (Cleaning Threshold) shows biofilm AMR is lowest (~1.7) at the shortest cleaning interval (1), then rises sharply to ~3.45 at interval 15 and remains stable, indicating frequent cleaning is needed to reduce AMR (Fig. 6B). The third graph (Mechanism Removal) tracks biofilm AMR over time under four conditions: which are baseline (~3.5–4.0), no colonization (~3.0), no growth (~3.0 with more variation) and no surfaces, where AMR repeatedly drops to zero. It highlights that surface availability, colonization, and growth are critical for maintaining biofilm AMR (Fig. 6C). Bacteria in biofilms can resist antibiotics and chemical disinfectants, with persister cells contributing to this resistance through regulatory and protective mechanisms. Biofilm formation is an important factor in bacterial persistence and resistance, and antibiofilm activity against MRSA has also been demonstrated using pyocyanin derived from *P. aeruginosa* (Kara *et al.*, 2025). This study supports ABM-2 findings, where biofilm reservoirs sustained AMR levels over time despite reduced upstream contamination and cleaning, consistent with a memory effect (Stewart, 2002). Biofilm AMR rose to ~3.1 genes under sustained contamination and declined only gradually after upstream reduction at time 40, reaching ~0.5 genes after ~40–50 additional steps. This delayed decay demonstrates biofilm-mediated persistence and memory of AMR (Fig. 7).

MDR occurrence in retail milk was associated with infrequent cleaning and lack of glove use. These practices may facilitate *Pseudomonas* adherence and biofilm formation on stainless steel surfaces (Carrascosa *et al.*, 2021, Aziz *et al.*, 2022). Effective disinfection is also important for controlling environmental *Pseudomonas* contamination, as several

disinfectants achieved complete microbial reduction against *P. aeruginosa* when used at sufficient concentrations and exposure times (Ibrahim *et al.*, 2024). Use of slightly acidic electrolyzed water effectively controlled persistent *Pseudomonas* in dairy environments (Kawai *et al.*, 2017). Packaged milk showed lower resistance and higher susceptibility. Retail milk had slightly higher resistance than farm milk, highlighting the possible influence of hygiene and pasteurization (Gamal *et al.*, 2022).

In conclusion, AMR was detected among *Pseudomonas* spp. Isolates, mainly from retail and farm milk. MDR was absent among the five packaged milk isolates tested for antimicrobial susceptibility. High resistance to aztreonam was observed, but the present laboratory data do not establish horizontal gene transfer as its source. Poor cleaning and lack of glove use were associated with MDR among the tested retail isolates. Agent-based modeling provided theoretical support for resistance emergence through horizontal gene transfer and persistence through biofilm-associated processes under the specified model assumptions.

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Author Contributions MTF was the principal contributor and conducted sample collection, sample processing, data collection, data processing, analysis, manuscript drafting, summarization and organization. MHM provided study design, academic guidance and supervised the research. FA conceived the study, provided the study design, supervised the research and contributed to statistical and modeling analysis. AR supervised the research and provided academic guidance. All authors reviewed and approved the final manuscript.

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