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Biochemical Characterization and Antibiotic Profile against *Salmonella* Species Isolated from Milk and Milk Products

Original Article

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Abstract

Salmonella species are among the most common foodborne pathogens associated with milk and dairy products. This study investigated the microbiological characteristics and antibiotic resistance patterns of *Salmonella* spp. in milk, ice cream, and cheese samples collected from August to December 2024 in Layyah District, Punjab, Pakistan. A total of 150 randomly selected samples (50 each of milk, ice cream, and cheese) were analyzed. Isolation and identification were performed using standard biochemical tests and further confirmed using the API 10S identification kit. *Salmonella* contamination was detected in 20% of milk samples, 10% of ice cream samples, and 6% of cheese samples. Among the isolates, *Salmonella Typhi* was the predominant serotype, found in milk (6 samples), ice cream (3), and cheese (2), followed by *S. Paratyphi A* in milk (4), ice cream (2), and cheese (1). Biochemical profiling revealed typical characteristics of *Salmonella*, including glucose fermentation with acid and gas production, hydrogen sulfide (H_2S) production, and citrate utilization. Antibiotic susceptibility testing using the disc diffusion method indicated significant resistance, with notably low zones of inhibition for oxacillin (0 mm), polymyxin B (0.9 mm), trimethoprim (0.8 mm), and amoxicillin (0.5 mm), while imipenem (10.7 mm), ciprofloxacin (10.8 mm), and clindamycin (10.3 mm) showed moderate effects. The findings highlight the public health risk posed by contaminated dairy products and the emergence of multidrug-resistant (MDR) *Salmonella* strains. Strengthening food hygiene practices, ensuring responsible antibiotic use in dairy production, and implementing robust surveillance measures are essential to safeguard public health and ensure compliance with food safety standards.

Keywords: Salmonella, Biochemical Characterization, Antibiotic Resistance, Food Safety, Milk & Milk Products.

Introduction

Salmonella species belong to the *Enterobacteriaceae* family, comprising aerobic, non-sporulating, Gram-negative bacilli typically measuring 1–3 × 0.5 µm in diameter. *Salmonella* was first described as a distinct bacillus isolated from the mesenteric lymph nodes and spleen of a patient suffering from typhoid fever, and it was successfully cultured in tissue in the late 19th century (Eberth, 1880; Khan, 2010). Globally, salmonellosis remains one of the major zoonotic diseases responsible for foodborne illnesses, particularly in developing countries (Luo, Yi, Yao, Zhu, & Qin, 2018). Milk and its derivatives, especially those produced from raw or partially pasteurized milk, are frequently implicated in zoonotic disease transmission to humans (Omar, Al-Ashmawy, Ramadan, & El-Sherbiny, 2018). While milk is valued as a nutrient-rich food essential for human nutrition, it is also highly susceptible to contamination by pathogens. Contamination may originate internally or externally from the udder, as well as from equipment and utensils used during processing. Contributing environmental factors include water quality, personnel hygiene, animal health, milking frequency and method, and equipment cleanliness (Adzitey, Asiamah, & Boateng, 2020).

Pakistan ranks fourth globally in milk production, with 97% of its milk consumed in raw form and only 3% as pasteurized (Dairy Sciences - Current Technological Trends and Future Prospects, Knowledge Scientific Publisher, USA). The dairy sector plays a vital economic role, especially for the approximately 8 million smallholder farmers in rural regions (Khan, Khan, Avais, Maqbool, Salman, & Rehman, 2009). Government initiatives increasingly aim to improve dairy farming by promoting high-yielding breeds to meet the rising demand for animal protein (Nadeem & Ahmad, 2024). In Punjab, tropical and subtropical climates support specialized breeds of cattle and buffalo. Annually, Pakistan produces roughly 2 billion liters of milk, 75% of which comes from rural cows. Milk contributes to 30% of commercial food products—including cheese, ice cream, chocolate, and butter—and provides 5–10% of daily caloric intake in many countries. However, typhoid fever outbreaks in Pakistan are often traced to the consumption of milk contaminated with pathogens such as *Salmonella* spp. and *Staphylococcus aureus* (Bano *et al*., 2020).

Milk's favorable nutrient composition, water content, and near-neutral pH (6.4 to 6.8) create an ideal environment for microbial growth. Factors such as farm management, animal density, and location significantly affect pathogen presence. Additionally, sampling methods, seasonal variability, and product types of influence contamination levels. Frequently isolated milk pathogens include *Salmonella spp.*, *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, *Campylobacter*, and *E. coli* O157: H7 (Neven, Rashad, Medhat, Refaat, Elsayed, Ismail, & Soudy, 2023). Consuming raw milk significantly increases the risk of infection by these pathogens. Cheese, another widely consumed dairy product, is considered nutritionally rich, easy to digest, and filling. Common Egyptian cheeses include Soft White Cheese, Domiati, Tallaga, and Kariesh.

Despite their nutritional value, cheese can harbor pathogens such as *Salmonella* and *E. coli*, especially when made from raw or contaminated milk. Several foodborne illness outbreaks in recent years have been attributed to such cheeses. Because *Salmonella* primarily affects the gastrointestinal tract, its presence in cheese is often due to fecal contamination from infected humans or animals (El-Bagoury, Shelaby, & Saied, 2019). Ice cream, a popular frozen dairy product in Egypt, is enjoyed by people of all ages, especially children. Although it is often made from pasteurized milk and considered nutrient-dense, ice cream remains vulnerable to microbial contamination during manufacturing, handling, transport, and packaging. This contamination may originate from water, raw ingredients, or poor hygiene practices. Additives, utensils, and processing equipment also contribute to microbial load. *Salmonella* and *E. coli* are frequently associated with foodborne illnesses linked to ice cream consumption (Sobeih, Al-Hawary, Khalifa, & Ebied, 2020).

Due to the widespread consumption of milk and milk products, contamination with pathogenic microorganisms such as *Salmonella spp.* poses a significant public health threat globally. Thus, the objectives of this study focus on detecting and characterizing *Salmonella* species in milk and related dairy products. First, the study aims to isolate *Salmonella spp.* from collected samples of milk, cheese, and ice cream. Second, it seeks to identify these isolates using biochemical characterization. Finally, the study evaluates their antimicrobial susceptibility profiles to determine resistance patterns against commonly used antibiotics.

Methods and Materials

Study Area

This study was conducted at the Department of Microbiology, Faculty of Veterinary and Animal Sciences (FVAS), Dera Ismail Khan, from August to December 2024. Milk and milk product samples were collected from the Layyah District in Punjab, Pakistan. Geographically, Layyah lies between latitudes 30°45' to 31°24' N and longitudes 70°44' to 71°50' E. It is bordered by Bhakkar District to the north, Jhang to the east and west, and Dera Ghazi Khan to the south, separated by the Indus River. This study site was selected due to the lack of existing data regarding the microbiological quality and antibiotic resistance profiles of dairy products in the region.

Sample Collection, Processing, and Analysis

A total of 150 samples were collected randomly from local milk, cheese, and ice cream vendors, comprising 50 samples each of raw milk, ice cream, and cheese. Ethical approval for the study was obtained from the Ethical Review Board (ERB) of Gomal University (Reference No: 25/ERB/GU). Each sample (25 mL) was collected aseptically in sterile screw-capped bottles, following the guidelines by Gumse *et al*. (2023), and labeled with the sample type and collection date. For microbial isolation, 1 mL of milk and 1 gram each of cheese and ice cream were transferred into Rappaport Vassiliadis R10 enrichment broth and incubated at 37°C for 24 hours. A loopful from the enriched broth was then streaked onto MacConkey agar plates and incubated at 37°C for 24–48 hours. MacConkey agar was employed to differentiate lactose fermenters (which form pink colonies) from non-lactose fermenters, such as *Salmonella*, which appear as colorless colonies. Following incubation, Gram staining was performed using the method described by Merchant and Packer (1967). Bacterial smears were prepared on slides, heat-fixed, and subjected to a sequential staining process using crystal violet, iodine, decolorizer, and safranin. Slides were examined under a 100× oil immersion lens. *Salmonella* species appeared as Gram-negative rods, showing a pink coloration. Further biochemical characterization was carried out to confirm the identity of the isolates. The catalase test was used to detect the presence of the catalase enzyme, with bubble formation indicating a positive result (Reiner, 2010). The oxidase test was performed using N-Tetramethyl-p-phenylenediamine; a purple color within 60–90 seconds indicated a positive outcome (Shields & Cathcart, 2010).

Biochemical Analysis

The Analytical Profile Index (API 10S E) kit was employed for detailed biochemical profiling of the isolates. Colonies were suspended in sterile water and used to inoculate API strips, which were incubated at 37°C for 24 hours. Color changes in the wells were recorded and matched with the API codebook to determine the organism's identity (Attiq *et al*., 2024). Additional biochemical tests included the ONPG test, to detect β-galactosidase activity; the Lysine Decarboxylase (LDC) test, which assesses lysine metabolism under anaerobic conditions; and the Indole test, which detects the ability of bacteria to produce indole from tryptophan using Kovac's reagent. Citrate utilization was assessed by the ability of the organism to use citrate as the sole carbon source, indicated by a color change due to alkaline byproducts. The Arginine Dihydrolase (ADH) and Ornithine Decarboxylase (ODC) tests determined the organism's ability to decarboxylate specific amino acids. Hydrogen sulfide (H_2S) production was identified by the formation of black precipitates in the medium. Urease activity was tested to distinguish urease-producing organisms, with *Salmonella* being urease-negative. Lastly, the TDA test assessed tryptophan deaminase activity, indicated by a green color change upon reagent application.

Antibiotic Susceptibility

Antimicrobial susceptibility testing was performed according to Clinical and Laboratory Standards Institute (CLSI) guidelines using the disc diffusion method on nutrient agar. Ten commercial antibiotic discs were tested: Imipenem (10 µg), Ciprofloxacin (5 µg), Clindamycin (10 µg), Oxacillin (1 µg), Polymyxin B (300 µg), Trimethoprim (5 µg), and Amoxicillin (50 µg). The antibiotic discs were placed on agar plates using sterile forceps, followed by incubation at 37°C for 24 hours. Zones of inhibition were measured in millimeters, and isolates were classified as resistant, intermediate, or susceptible based on CLSI interpretive criteria (Gwaza & Adie, 2024).

Results and Discussion

The current study aimed to isolate and identify *Salmonella* species from various milk and milk product samples, characterize their biochemical properties, and assess their antibiotic resistance profiles. A total of 150 samples were collected, equally divided among milk, cheese, and ice cream, representing commonly consumed dairy products with the potential risk of contamination. The use of conventional microbiological techniques for *Salmonella* isolation, including enrichment in Rappaport-Vassiliadis broth followed by selective culturing on MacConkey agar, is a well-established protocol that enhances the sensitivity and specificity of detecting *Salmonella* spp. in food matrices (Gwida *et al*., 2014; Umar *et al*., 2024). This methodological approach allowed the successful recovery and isolation of *Salmonella* strains from the tested samples (Figure 1, Figure 2).

Catalase Test

As part of the initial enzymatic characterization, the catalase test was performed to determine the presence of the catalase enzyme, which decomposes hydrogen peroxide (H_2O_2) into water and oxygen. The presence of catalase is usually indicated by the rapid formation of bubbles due to oxygen release. Interestingly, in this study, none of the tested samples exhibited visible bubbling during the catalase test. While this might initially suggest a negative reaction, it is important to note that *Salmonella typhi* is known to produce catalase weakly, and its enzymatic activity may be subtle or undetectable under certain test conditions (Reiner, 2010; Faisal, Alam, & Sajed, 2017). Factors such as the age and concentration of the bacterial inoculum, as well as the freshness and strength of the hydrogen peroxide used, can influence test outcomes. Therefore, the absence of visible bubbles should be interpreted cautiously, and further repeat tests with fresh reagents and adequate bacterial load are advisable to avoid false-negative results (Figure 3).

Biochemical Characterization

Comprehensive biochemical testing was conducted on all 18 isolates initially screened as *Salmonella* positive. Using the Analytical Profile Index (API) system allowed for a detailed and systematic assessment of metabolic and enzymatic characteristics to confirm the identity of *Salmonella* species and differentiate between *S. Typhi* and *S. Paratyphi A*. The biochemical profiles obtained were consistent with classical descriptions found in the literature. For example, *S. Typhi* isolates tested positive for glucose fermentation, lysine decarboxylase (LDC) activity, and hydrogen sulfide (H_2S) production, a signature trait of many *Salmonella* serovars (Lal & Cheeptham, 2015; McWilliams, 2012). Conversely, *S. Paratyphi A* displayed positivity for glucose, arabinose fermentation, and ornithine decarboxylase (ODC) activity, while testing negative for LDC and H_2S production (Figure 4). These differential biochemical markers are crucial in clinical and food microbiology laboratories for accurate serovar identification, which is essential for epidemiological tracking and infection control measures (Attiq *et al*., 2024).

Prevalence of Salmonella in Dairy Products

Among the 150 dairy product samples analyzed, *Salmonella* was detected in 18 samples, corresponding to an overall prevalence of 12%. Notably, milk samples exhibited the highest contamination rate at 20% (10 out of 50 samples), followed by ice cream at 10% and cheese at 6%. This gradient of contamination indicates that raw or inadequately processed milk may serve as a significant reservoir for *Salmonella* and pose a substantial risk for foodborne illnesses in the community. These findings are consistent with previous studies conducted in South Asia, which have reported similar prevalence rates of *Salmonella* in dairy products and emphasized the role of milk as a critical vehicle for pathogen transmission (Hasan, 2021; Gwida *et al*., 2014). The lower contamination rates in cheese and ice cream may be attributed to the manufacturing processes, which often involve heat treatment or the addition of preservatives, but contamination during handling and storage remains a concern. The detection of *Salmonella* in these products underscores the urgent need for improved hygiene, sanitation, and monitoring practices at every stage of dairy production, from farm to consumer.

Antibiotic Resistance Profiles

The study's antibiotic susceptibility testing revealed alarming resistance patterns among the isolated *Salmonella* strains, reflecting a growing public health challenge. Imipenem, a carbapenem antibiotic regarded as a last-resort treatment for multidrug-resistant infections, demonstrated the highest activity against both *S. Typhi* and *S. Paratyphi*

A, with inhibition zones indicating moderate susceptibility (Gwaza & Adie, 2024). Ciprofloxacin, a commonly prescribed fluoroquinolone for enteric fever, also showed considerable efficacy, albeit lower than imipenem. However, resistance to several other antibiotics was prominent. Oxacillin exhibited no inhibitory effect on any isolates, and minimal zones of inhibition were observed for polymyxin B, trimethoprim, and amoxicillin, indicating extensive resistance. Clindamycin showed moderate resistance levels, further complicating treatment options Figure 5. These resistance trends are consistent with global reports highlighting the emergence of multidrug-resistant *Salmonella* strains, especially in regions where antibiotic misuse and overuse are widespread (Gwaza & Adie, 2024; Attiq *et al*., 2024). The higher resistance noted in *S. Paratyphi A* compared to *S. Typhi* may suggest serovar-specific differences in resistance mechanisms or antibiotic exposure histories, which warrant further molecular investigation. Such antimicrobial resistance not only limits therapeutic options but also poses risks for treatment failure, prolonged illness, and increased healthcare costs.

Figure 1

Growth of bacterial colonies from a milk sample on MacConkey agar

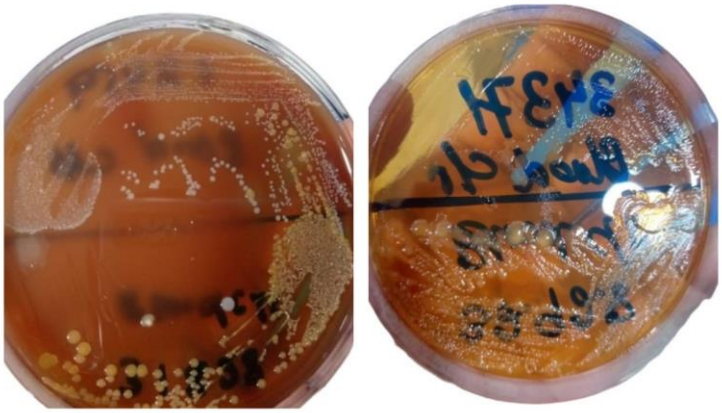

Figure 2

Bacterial colonies were isolated from cheese and ice cream samples, were cultured on MacConkey agar. The results showed variation in colony morphology, with some colonies appearing large while others were smaller in size.

Figure 3
Catalase Test

Figure 4
Biochemical Characterization of Salmonella Paratyphi a Isolates

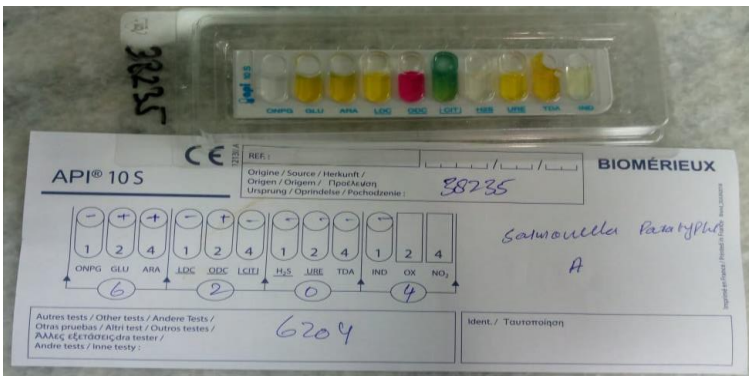

Table 1
Biochemical Characterization of Salmonella spp. Isolates

Test	*S. Typhi*	*S. Paratyphi A*
ONPG	–	–
GLU	+	+
ARA	–	+
LDC	+	–
ODC	–	+
CIT	–	–
H_2S	+	–
URE	–	–
TDA	–	–
IND	–	–

Table 2
Isolation Rate of Salmonella and Its Association with Different Samples

Sample Type	No. examined	No. positive	Prevalence (%)
Milk	50	10	20.0
Cheese	50	3	6.0
Ice-Cream	50	5	10.0
Total	**150**	**18**	**12.0**

Table 3
Antimicrobial Resistance of Salmonella spp. Isolates

Antibiotic	S. Typhi (mm)	S. Paratyphi (mm)	Disc content (ug)
Imipenem (IPM)	14.2	10.7	10
Ciprofloxacin (CIP)	16.5	10.8	5
Clindamycin (DA)	12	10.3	10
Oxacillin (OX)	0	0	1
Polymyxin B (PB)	1.5	0.9	300
Trimethoprim (TMP)	1.2	0.8	5
Amoxicillin (AMC)	0.8	0.5	50

Figure 5
Antimicrobial Resistance of Salmonella spp. Isolates

Conclusion

In conclusion, this study successfully isolated *Salmonella* species from milk, cheese, and ice cream samples, with milk showing the highest contamination rate. Biochemical characterization effectively differentiated *Salmonella Typhi* and *Salmonella Paratyphi A*, confirming their distinct metabolic profiles. Antibiotic susceptibility testing revealed moderate sensitivity to Imipenem and Ciprofloxacin, while high resistance was observed against several commonly used antibiotics, including Oxacillin, Polymyxin B, Trimethoprim, and Amoxicillin. These findings underscore the significant risk of *Salmonella* contamination in dairy products and highlight the urgent need for improved hygiene practices and careful antibiotic stewardship to manage antimicrobial resistance.

Implications and Recommendations

The study findings have significant implications for public health, food safety, and clinical management of *Salmonella* infections. The identification of *Salmonella* in commonly consumed dairy products, particularly milk, highlights the critical need for stringent hygiene practices during milking, processing, and distribution. Regular surveillance of *Salmonella* prevalence and resistance patterns is essential to detect emerging threats and guide effective interventions. Furthermore, the antibiotic resistance patterns observed emphasize the urgency of implementing rational antibiotic stewardship programs. Educating healthcare providers and the public about the prudent use of antibiotics and

restricting over-the-counter access to antibiotics can help slow the spread of resistant strains. Policy frameworks supporting these measures should be strengthened alongside continuous laboratory-based monitoring of resistance profiles (Hasan, 2021; Gwida *et al*., 2014; Lal & Cheeptham, 2015).

Declarations

Ethical Approval and Consent to Participate: This study strictly adhered to the Declaration of Helsinki and relevant national and institutional ethical guidelines. Informed consent was not required, as secondary data available on websites was obtained for analysis. All procedures performed in this study were by the ethical standards of the Helsinki Declaration.

Consent for Publication: Not Applicable.

Availability of Data and Materials: Data for this study will be made available upon request from the corresponding author.

Competing Interest: The authors declare that they have no competing interests.

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Authors' Contribution: All three have actively participated in the conduct, writing, and submission to the journal.

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