COMPREHENSIVE EXPLORATION OF ANTIBIOTIC USE IN DAIRY COWS: INVESTIGATING MILK AND CURD MICROBIOTA, ANTIMICROBIAL RESISTANCE GENE IN NATIVE VERSUS CROSSBREEDS

A THESIS TO BE SUBMITTED TO

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

The University Of Trans-Disciplinary Health Sciences and Technology

FOR THE AWARD OF THE DEGREE OF
M.SC IN BIOLOGICAL SCIENCES (BIOINFORMATICS AND FUNCTIONAL GENOMICS BY RESEARCH)

BY

SUNAINA N

UNDER THE GUIDANCE OF

DR PAVITHRA N

ASSOCIATE PROFESSOR, TDU

MARCH 2026

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY
Private University Established in Karnataka by ACT 35 of 2013

BENGALURU - 560064

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

Private University Established in Karnataka by ACT 35 of 2013

BENGALURU - 560064

DECLARATION BY THE CANDIDATE

I declare that this thesis entitled "**Comprehensive exploration of antibiotic use in dairy cows: Investigating milk and curd microbiota, antimicrobial resistance gene in native versus crossbreeds**" submitted for the award of Master of Science to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of guide Dr. Pavithra N, Associate professor, Functional genomics and Bioinformatics, TDU. I also wish to inform that no part of the research has been submitted for a degree or examination at any university. References, help and material obtained from other sources have been duly acknowledged.

I hereby confirm the originality of the work and that there is no plagiarism in any part of the dissertation.

Place: Bengaluru **Signature of the Candidate**

Date: 23/03/2026

Name of candidate: Sunaina N

Reg. No.:2023MSCR04

March 2026

THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY

Private University Established in Karnataka by ACT 35 of 2013

BENGALURU - 560064

CERTIFICATE

This is to certify that the work incorporated in this thesis **"Comprehensive exploration of antibiotic use in dairy cows: Investigating milk and curd microbiota, antimicrobial resistance gene in native versus crossbreeds"** submitted by Ms. Sunaina N was carried out under my supervision. No part of this thesis has been submitted for a degree or examination at any university. References, help and material obtained from other sources have been duly acknowledged. I hereby confirm the originality of the work and that there is no plagiarism in any part of the dissertation.

Research Supervisor / Guide

Dr Pavithra N,
Associate professor,
Functional Genomics and Bioinformatics
The University of Trans-Disciplinary Health Sciences and Technology,
Yelahanka, Bangalore – 560064

March 2026

Acknowledgement

I would like to express my gratitude to all those who have supported and constantly encouraged me during the master's research and project work.

I want to thank Functional genomics and Bioinformatics team (FGB) from The University of Trans-Disciplinary Health Sciences and Technology, Bangalore for assigning me this research work to carry out as a project of my PG program.

I have furthermore to thank my guide Dr. Pavithra N, Associate Professor, Functional Genomics and Bioinformatics group, who gave me the permission and encouraged me to carry out my research project.

I would like to thank my Master's Advisory Committee members (MAC) Dr Kumar S K Mohan, IVC Road, Devanahalli Bengaluru for his constant encouragement and regular inputs given and guiding me for identification of the different cow breeds, for the milk samples collection and for procuring commercial starter culture from Hoskote Dairy and Dr. Abdul Kareem, Associate Professor, Conservation of Natural Resources group, TDU for constant support, guidance and inputs given during the MAC meetings.

I would like to thank Dr Lavanya Devi, Associate professor, Functional genomics and Bioinformatics, TDU and Mr. Ravikumar, Assistant Registrar-Academics, for their support, and constant guidance.

I would like to express my gratitude to Dr. Santhosh, Assistant professor, TDU for supporting me to carry out the project in terms of all the required computational and bioinformatics support for the project progress.

I extend my thanks to intern students Santhoshi, Jeevitha and Radha from Bishop Heber college for their aid in the milk sample collection, curd preparation and other processes involved during the experiments.

I would extend my gratitude to Ms. Ifra Masum, Ms. Rajeswari B, Ms. Varalaxmi and Ms. Namitha for constantly aiding me in the Molecular biology techniques and helping throughout to carry out my research work successfully.

I would express my sincere gratitude to RIST (Rural India Supporting Trust) for the funding.

I would like to express my sincere gratitude and special heartfelt thanks to my parents and my dear friends, without their selfless love, constant support and encouragement, could never got through this journey.

DrillBit

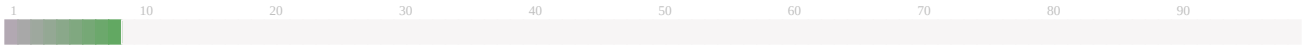
The Report is Generated by DrillBit Plagiarism Detection Software

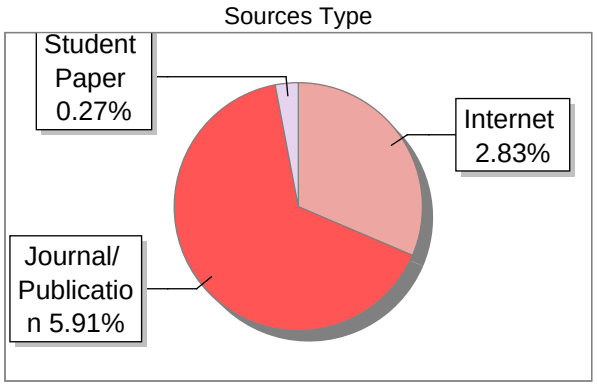
Submission Information

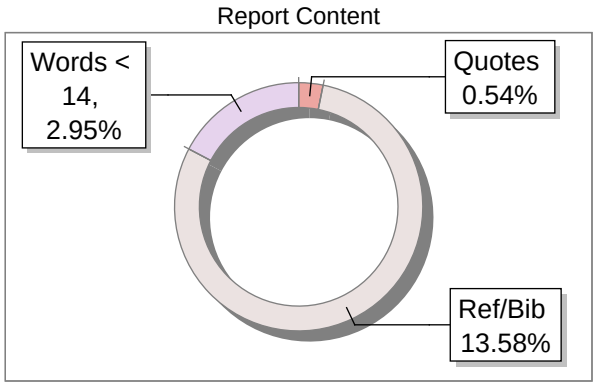
Author Name	Sunaina N
Title	“Comprehensive exploration of antibiotic use in dairy cows: Investigating milk and curd microbiota, antimicrobial resistance genes in native versus crossbreeds”
Paper/Submission ID	5267305
Submitted by	ravi.g@tdu.edu.in
Submission Date	2026-02-13 14:28:47
Total Pages, Total Words	64, 15677
Document type	Thesis

Result Information

Similarity **9 %**







Exclude Information

Quotes	Not Excluded
References/Bibliography	Not Excluded
Source: Excluded < 14 Words	Not Excluded
Excluded Source	**0 %**
Excluded Phrases	Not Excluded

Database Selection

Language	English
Student Papers	Yes
Journals & publishers	Yes
Internet or Web	Yes
Institution Repository	Yes

A Unique QR Code use to View/Download/Share Pdf File

Table of Contents

List of Tables

List of Figures

Synopsis

Title

"Comprehensive exploration of antibiotic use in dairy cows: Investigating milk and curd microbiota, antimicrobial resistance genes in native versus crossbreeds"

Background

The extensive use of antibiotics in dairy farming has significantly contributed to the emergence and spread of antimicrobial resistance (AMR), posing risks to animal health, public health, and environmental sustainability. In India, the dairy sector includes both native and crossbred cattle, where antibiotic usage practices, awareness levels, and stewardship measures vary widely.

Simultaneously, milk microbiota plays a crucial role in dairy product quality, fermentation characteristics, and potential transmission of antimicrobial resistance genes (AMR). Variations in microbial diversity between native and crossbred breeds may influence both product quality and resistance development patterns. Therefore, integrating survey-based antibiotic usage data with microbiome profiling and AMR gene detection provides a comprehensive One Health perspective.

Objectives

- To analyze and compare antibiotic use in focusing on the frequency, type, and dosage of antibiotics administered.
- To investigate the composition and diversity of the milk and curd microbiota in native versus crossbred dairy cows, also examining the pH, moisture content and texture analysis of milk and curd.
- To detect and compare the prevalence of antimicrobial resistance genes in the milk of native and crossbred dairy cows, assessing the impact of antibiotic practices on the spread of resistance.

Methodology

A structured questionnaire survey was conducted among veterinary stakeholders to evaluate antibiotic prescription practices, awareness of AMR, and adherence to guidelines. Data were analyzed in percentage format.

Milk samples were collected from five cattle breeds: Malnad gidda, Sahiwal, Gir (native breeds), and Jersey and Holstein Friesian (crossbreeds). Samples were processed for microbiome analysis, starter culture preparation, and curd production. Physicochemical parameters including pH and moisture content were measured. Texture Profile Analysis (TPA) of curd samples was performed to assess hardness, cohesiveness, springiness, gumminess, chewiness, and resilience.

Metagenomic DNA was extracted from milk, curd, and starter culture samples. DNA quality and concentration were assessed using Nanodrop and Qubit. PCR amplification targeted the 16S rRNA gene (1500 bp) for microbiome analysis and the mecA gene (1900 bp) for AMR detection. Amplicons were confirmed through gel electrophoresis. Libraries were prepared and sequenced using the Oxford Nanopore platform. Bioinformatic analysis generated taxonomic classification from domain to species level. Diversity analysis was done.

Results

Survey findings revealed that 69.6% of stakeholders expressed concern about antibiotic use in dairy cows. However, only 35.7% were aware of specific prescription guidelines or alignment with WHO standards. A high proportion (89.3%) agreed that antibiotics should only be prescribed by veterinarians, and 76.8% emphasized minimizing antibiotic use to control AMR. While 86.2% recognized the link between antibiotic overuse and AMR, only 16.1% believed cattle owners were aware of antibiotic resistance issues. Mastitis (86.79%) was the predominant condition treated with antibiotics, followed by metritis (45.94%). Tetracycline (47.6%), and penicillin (30.9%) were the most frequently prescribed antibiotics, indicating reliance on broad-spectrum agents.

Milk pH ranged from 6.73 to 6.83 across breeds, while moisture content varied between 82.55% and 90.78%. After fermentation, curd pH decreased to 4.59–4.72, confirming successful lactic acid fermentation. Texture analysis showed significant breed-based variation, with Malnad gidda curd exhibiting higher hardness and gumminess compared to other breeds. PCR amplification of the 16S rRNA gene was successful in all samples (1500 bp). The mecA gene was detected in milk samples, confirming the presence of methicillin resistance determinants. Sequencing analysis was done with obtained raw reads and downstream analysis was carried out by processing the reads further.

Taxonomic analysis revealed substantial microbial diversity across breeds and sample types. At the phylum level, Firmicutes dominated starter culture and curd samples, reflecting selective enrichment during fermentation. Genus-level analysis showed Lactococcus dominance in several milk samples, while Streptococcus was predominant in curd. Species-level variation indicated breed-specific microbial patterns, including the presence of Lactococcus lactis and Bacillus cereus group. Upset plot analysis demonstrated 190 shared species in starter cultures, 114 in curd, and 101 in milk, indicating both shared core microbiota and breed-specific variations.

Diversity indices (Shannon and Simpson) were higher in milk samples of native breeds (Gir and Sahiwal). Fermented products showed reduced richness, suggesting selective microbial growth during fermentation. PCoA analysis indicated no significant clustering differences among breeds, suggesting broadly similar microbial communities

Conclusion

The study highlights substantial concerns regarding antibiotic usage in the Indian dairy sector, with widespread recognition of AMR risks but limited awareness of administration guidelines. Mastitis remains the primary trigger of antibiotic administration, with broad-spectrum antibiotics frequently prescribed.

Microbiome analysis demonstrated breed-specific variations in milk microbial diversity, with native breeds exhibiting higher richness. Fermentation resulted in selective enrichment of Firmicutes, particularly Streptococcus and Lactococcus species. Detection of the mecA gene confirms the presence of antimicrobial resistance determinants in milk, underscoring the potential public health implications.

Overall, the findings emphasize the need for strengthened antibiotic stewardship, enhanced veterinary education, and responsible prescribing practices. Integrating microbiome profiling with AMR surveillance provides valuable insights for sustainable dairy management under a One Health approach.

"Comprehensive exploration of antibiotic use in dairy cows: Investigating milk and curd microbiota, antimicrobial resistance genes in native versus crossbreeds"

1.1 Abstract

The indiscriminate use of antibiotics in dairy farming has raised serious concerns regarding antimicrobial resistance (AMR), food safety, and variations in dairy microbiota. This study aimed to analyze antibiotic usage patterns in dairy cows, investigate the microbiota composition of milk and curd from native and crossbred cows and assess the occurrence of antimicrobial resistance gene in milk samples. A structured questionnaire survey revealed that 69.6% of stakeholders expressed concerns regarding antibiotic use in dairy cows. Significantly, 87.5% acknowledged the devastating consequences of antibiotic overuse, and 76.8% agreed that minimizing antibiotic use is vital for controlling AMR. Mastitis was identified as the most common condition treated with antibiotics (86.79%), with tetracycline (47.6%), gentamycin (38%) and penicillin (30.9%) being the most frequently prescribed antibiotics. Physicochemical analysis revealed relatively stable values across different samples of milk, starter culture and curd. Nanopore sequencing of milk, curd, and starter culture samples generated good-quality reads, enabling comprehensive microbiome profiling. Taxonomic analysis showed dominance of the phylum *Firmicutes* across all fermented samples, while genus level varied among breeds, with *Lactococcus*, *Streptococcus*, and *Bacillus* being predominant. Native breeds, particularly Gir and Malnad gidda, exhibited higher microbial richness and diversity in milk samples. The AMR gene (*mecA*) analysis in milk samples confirms the presence of AMR resistance, indicating a potential link between antibiotic usage practices and resistance. Broadly, the findings highlight the very essential need for improved antibiotic supervision and promotion of ethno-veterinary practices in disease management protocols to mitigate AMR in the Indian dairy sector.

Keywords: Antimicrobial resistance, Native cows, Mastitis, Microbiome, Ethno-veterinary practices.

1.2 Introduction

The human nutritional needs can be fulfilled at an affordable expense; in particular, milk, which is one of the least expensive sources of nutrients when compared to other food products, provides 30% of the daily requirements of eight nutrients (protein, calcium, iron, fibre, vitamins A, C, D, and E). Whole milk can provide 30% of a healthy adult's calcium needs, and most dairy products are cost-effective. Dairy products gained first position in the ranking of the cost of vitamin D and obtained a good position in the ranking of protein and vitamin A. Due to the diverse range of nutrients found in milk and dairy products, this allows the consumers to intake the required amounts of nutrients and ingest foods of dietary quality. Global health authorities acknowledge the health advantages of dairy products and advise consumption of them as part of a balanced diet and a healthy diet. Milk and dairy products furnish important micro-nutrients like phosphorus, magnesium, calcium etc, thus benefits consumers to take in appropriate amounts of nutrients and ingest dietary quality food [1].

Crossbreeding of dairy cattle has played a major role in India to become the world's top milk producer.

India has increased milk production from 17 million tonnes in 1950-51 to approximately 137.69 million tonnes in 2013-14, making it the highest producer globally. In 2013-14, India had a per capita milk availability of approximately 307 grams, more than the global average of 294 grams per day (Economic Survey- 2014-15). This was accomplished by crossing Indian descript and non-descript cattle with exotic dairy breeds such as Holstein Friesian, Jersey, and Brown Swiss.

Crossbreeding programs involve high initial investment and maintenance costs. Moreover, crossbred animals are more susceptible to infectious diseases like foot and mouth disease, babesiosis, theileriosis, mastitis, milk fever, and ketosis due to limited access to high-quality feed and fodder. They also exhibit increased propensity to heat stress and shock. India has been increased the milk yield by 4 to 6% per year over the past 20 years. Crossbreeding programmes initiated during the year 1950 in India between native and crossbreed cattle majorly with Holstein Friesian (HF) and Jersey for increasing the milk production [2].

According to estimation, by 2030 the global consumption of antibiotics will increase by up to 200%, in comparison with the 42 billion daily doses administered in 2015. Of these, 73% of antibiotics are applied to animals to prevent and treat infections like mastitis, foot and mouth disease, etc., but also to improve weight gain and productivity [3].

Disease resistance is an important trait of economic importance in livestock. Native breeds cattle exhibited high resistance to parasitic and tick-borne protozoan diseases.

Studies examining the role of breed in the occurrence of haemoprotozoan diseases found that Holstein Friesian (HF) and Jersey crossbred cattle have considerably prevalent to these diseases than native breeds. *Staphylococcus aureus* is a common commensal microorganism with ability to become an opportunistic pathogen, which is leading to superficial and invasive clinical conditions in cattle as well as humans.

Heat tolerance is one of the important traits of economic importance in dairy cattle. Zebu cattle are more heat tolerant than crossbred cattle, as revealed by the smaller

magnitude of their physiological reactions to heat stress when compared to crossbred cattle [4].

Milk, a fundamental of human nutrition, has been revered for its nutritional value and versatility for centuries. Beyond its rich content of proteins, vitamins, and minerals, milk is also a complex microbial ecosystem teeming with an array of microorganisms that play a crucial role in its composition, flavour, and safety.

Milk has been widely renowned for its nutritional content and versatility, making it a crucial part of human diets.

In addition to being high in proteins, vitamins, and minerals, milk is also a complex microbial ecosystem that is niche to a wide variety of microorganisms that are essential to its safety, flavour, and composition.

A diverse community of microorganisms, mostly bacteria, yeasts, and molds, find a unique residence in milk.

As the basic components of the starter cultures used in fermented food products, the LAB group of bacteria plays a vital role in the fermentation as well as an industrial significance.

Bacteria are the most important members of the milk microbiota. Lactic Acid Bacteria (LAB) are of particular importance, as they play an essential role in transforming lactose (milk sugar) into lactic acid through fermentation. This process not only contributes to the characteristic tangy flavour of dairy products but also acts as a natural preservation mechanism by lowering the pH and suppressing the growth of harmful pathogens. *Streptococcus, Lactobacillus*, and *Lactococcus* are common genera of LAB found in milk.

The microbes such as *Lactococcus lactis* plays an important role in the food industry, specifically in the production of dairy products, and in the health zone due to its exceptional fermentation process. The *L. lactis* bacteria potentially contributes to the characteristics such as lactose fermentation capabilities, proteolytic activity, exopolysaccharide (EPS) production, and flavour production; they also play an essential role in the formation of aroma, texture and acidity in the final fermented products [5, 6]

Indian zebu cattle can be identified by their capacity to adapt to harsh weather, survive on limited feed and nourishment, and have better immunity against tropical diseases and environmental stress.

There are a wide variety of cattle breeds that are primarily reared for milk and draught in India. A few of Indian native cattle breeds with superior traits like adaptability were previously introduced to other nations with the objective to improve and advance their own native breeds [7].

Even though livestock are the most important animal species in India that contribute significantly to the country's agricultural economy, some of the prominent cow breeds have witnessed population declines or breed characteristics becoming diluted due to the present way of production.

Gir cow

The Gir breed of zebu cattle is purely dairy-bred, and its name originates from the Gir forest and hills in the southern part of Gujarat's Saurashtra area, namely in the districts of Junagadh, Gir-Somnath, Amreli, Bhavnagar, Porbandar, Rajkot, and Jamnagar [8].

Gir cattle are also found in other parts of Gujarat, Rajasthan and Maharashtra states. Due to its specific fundamental physical traits, this species is better able to withstand heat and withstand tropical diseases [9].

Pure Gir cattle have wide range of body coat colour. Red colour is the most predominant colour in 80% animals. In some animals red colour is speckled with white colour or vice-versa. Some animals also have yellowish light to dark red coat colour.

Sahiwal cow

Sahiwal cow is a milk breed of zebu cattle that got its name from its original habitat the Sahiwal area in the Montgomery district of Punjab in Pakistan [10].

The Indian states of Punjab, Haryana, Uttar Pradesh, and Madhya Pradesh were home to the majority of milch breeds Sahiwal cattle. They can thrive easily in high humid areas [11].

Malnad Gidda cow

Malnad Gidda cows are a small, hardy, dwarf breed native to Karnataka's Malnad region. They are predominantly black with occasional fawn or brown shades. Their size makes them ideal for grazing in hilly terrains, with an average height of ~90 cm and body length of ~87 cm.

Milk yield ranges from 0.5 to 4 litres per day, with a high-fat content of 5.5–8%. Though low in quantity compared to crossbred cows, their milk is valued for its quality, making it suitable for traditional dairy products.

The breed is renowned for its resistance to local diseases and its ability to thrive in challenging environments with minimal care. This resilience makes it a low-cost option for smallholder farmers in the Malnad region.

Malnad Gidda cows are integral to sustainable farming practices in Karnataka. Their dung and urine are used for compost and pest control. However, the breed faces threats of genetic dilution due to crossbreeding [12].

Jersey cow

Jersey cows are smaller compared to many other dairy breeds, such as Holsteins. Adult cows weigh around 350–400 kg and stand about 115–120 cm at the withers. Bulls are larger, weighing 600–700 kg.

Their coat varies from light fawn to deep brown, often with white markings. Purebred Jerseys typically have a lighter band around their muzzles, dark tails, and black hooves .

They thrive in a variety of climates due to their small frame, efficient grazing, and robust health.

Jersey milk is renowned for its richness, containing higher levels of butterfat (around 4.84%) and protein (3.95%) compared to other breeds. This makes their milk ideal for butter and cheese production.

Jerseys convert feed into milk solids more efficiently than most dairy breeds, producing high-quality milk relative to their smaller body size and feed intake.

Jersey cows are generally docile and easy to handle, although bulls may be aggressive. This trait, combined with their size, makes them suitable for smaller farms.

Jersey calves are smaller and more prone to heat loss, requiring attentive care in colder climates.

These traits make Jersey cows a popular choice worldwide for both small-scale and commercial dairy operations. Their adaptability and efficient production are key to their global success.

Holstein Friesian cow

Holstein Friesians are large-framed cows, with noticeable height, broad chest width, and deep body structure. They have a typical black-and-white or red-and-white coat pattern, depending on the genetic variant. Their skeletal structure is designed to support high milk production.

Their udders are well-formed and strongly attached, optimized for high-yield milking systems. Udder traits are a primary focus in breeding programs.

They have been selectively bred for long productive lifespans, focusing on efficient reproduction and resistance to environmental stressors.

Known for exceptional milk yields, Holstein Friesians dominate global dairy production, with lactation periods yielding significantly higher milk quantities than most other breeds.

Although originally from temperate regions, they have been successfully crossbred to thrive in tropical and subtropical climates, especially with improved nutrition and management practices.

This breed is a key contributor to genetic programs worldwide, aimed at improving dairy traits in crossbred populations [13].

Massive parallel DNA sequencing is made possible by next-generation sequencing (NGS) technology, which generates enormous amounts of precise information. NGS platforms are widely used for clinical research.

The bacterial 16S rRNA gene consists of nine variable regions (V1-V9) separated by highly conserved sequences across various taxa.

The standard parallel-type short-read sequencer cannot produce reads covering the whole 16S rRNA gene, several regions of it have been targeted for sequencing, which often causes ambiguity in taxonomic classification.

New sequencing platforms have overcome these technical restrictions, particularly those affecting read length. A prime example is the MinION sequencer from Oxford Nanopore Technologies, which is capable of producing long sequences with no theoretical read length limit. Real-time sequencing data generation from MinION speeds up data processing turnaround times.

Previous investigations on the intramammary flora using second-generation sequencing have amplified partial sequences of the 16S rRNA gene, such as V1-V3 or V2-V3, and analysed them at the genus level [14].

Antimicrobial resistance (AMR) has emerged as a significant public health issue, particularly in nations with low and middle-income nations. Although there is little evidence linking the formation of AMR in humans to food intake, resistant strains may be spread from animals to humans through animal-source foods. Antibiotics used in farm animals often belong to the same classes of antibiotics used in humans, therefore posing a risk of resistance transmission between animals and humans [15].
Primers for antimicrobial resistance gene detection were designed based on conserved gene regions, ensuring specificity and efficient simultaneous amplification in multiplex PCR, as demonstrated by Strommenger et al.
Staphylococcus aureus strains resistant to methicillin and many other antibiotics are the main causes of nosocomial infections across the world. The mecA gene is a part of 21 to 60 kb staphylococcal chromosome cassette mec, a mobile genetic element that may also contain genetic structures such as pUB110 which encode resistance to non-beta lactam antibiotics.
India being a largest producer of milk worldwide, the issues of MRSA in dairy farms remain a major challenge. Pathogenic bacteria found in livestock are zoonotic and develops antibiotic resistance in these pathogenic bacteria which is susceptible to spread to humans through food chain.
Staphylococcal cassette chromosome *mec* (SCC*mec*) typing is crucial for identifying clones of methicillin-resistant staphylococci. SCC*mec* are the mobile genetic element genes encoding for PBP2a, which could be transmitted from one bacterial species to another.
Infections caused by the resistant bacterial strains in humans are rising, which includes infection caused by *Staphylococcus spp., Escherichia coli, Salmonella spp.*, and *Campylobacter spp*.
Staphylococci cause sickness in humans, more specifically in those whose immune system is weak. This also is the major diseases in dairy animals, like mastitis, wounds and other infections [16, 17].
By comparing native and crossbred dairy cows, this study seeks to evaluate antibiotic usage patterns and their effects on antimicrobial resistance and milk microbiome diversity. Also, an overall focus on antibiotic frequency and dosage, the composition of the milk and curd microbiota, physicochemical characteristics, and the occurrence of resistance genes.

2.1 Literature Review

The use of antibiotics in dairy farming is widely documented due to growing concerns over antimicrobial resistance (AMR) and its implications for both animal and human health.

The use of antibiotics in food-producing animals has been a public health issue for decades. In 1977, a WHO committee stated that use of antimicrobials in food-producing animals chooses for resistant bacteria, and such bacteria are transmitted to humans in food and by means of direct contact with animals. WHO guidelines recommend overall reduced use of medically significant antimicrobials in food-producing animals [18].

Pol and Ruegg (2007) and Ruegg (2017) report that antibiotics are commonly administered to dairy cows, primarily to treat infections and prevent diseases like mastitis. This preventive and therapeutic approach highlights the reliance on antibiotics in managing dairy cattle health, which, while beneficial for productivity, poses risks for AMR development [19, 20].

The indiscriminate administration of antibiotics without determining the in-vitro sensitivity of the causative organisms is considered to be a major contributing factor to treatment failure [21].

Veterinarians play a critical role in educating prospects about the responsible use of antibiotics and promoting for measures to mitigate the threat of antibiotic resistance developing on dairy farms. Actually, the effectiveness of measures to reduce antibiotic resistance has primarily reliant on evolving viewpoints on the prescription and use of antibiotics among veterinarians and their clients [22].

Critically, the use of broad-spectrum antibiotics has been associated with higher AMR risks. Schar et al. (2018) and Chantziaras et al. (2014) emphasize that broad-spectrum antibiotics, although effective against a range of pathogens, contribute significantly to resistance development. These studies call for a more targeted approach in antibiotic administration, advocating for restricted use and closer monitoring of antibiotic practices to minimize resistance spread [23, 24].

The microbiota composition in milk is vital to dairy cow health, influencing milk quality and disease resistance. Studies have demonstrated that breed differences between native and crossbred cows affect microbiota diversity, with native breeds generally showing higher diversity and resilience against pathogens. Zhang et al. (2019) and Addis et al. (2016) report that native breeds exhibit a broader range of beneficial bacteria, enhancing immune function and providing protection against mastitis-causing pathogens [25, 26].

Seasonal variations and farming practices have been shown to significantly influence the microbial composition of raw milk. *Van De Grift et al.* (2025) reported distinct differences in the milk microbiome between conventional and organic farming systems, with seasonal shifts further contributing to variations in microbial diversity and community structure.

Recent integrative studies combining ethnobiological knowledge with multi-omics approaches have provided deeper insights into the microbial diversity and functional

potential of Indian fermented foods. Samantaray et al. (2025) highlighted that traditional fermentation practices harbour complex microbial consortia with significant roles in nutrition, food safety, and health-promoting functionalities.

Moreover, the crossbreeding of cows has been associated with reduced microbial diversity, which may compromise immune response and increase susceptibility to infections (Derakhshani et al., 2018; Barkema et al., 2009). Metcalf et al. (2019) support these findings, noting that crossbreeding can shift microbiota composition, potentially weakening pathogen defences. These studies highlight the significance of understanding breed-specific microbiota to guide breeding practices and inform disease management strategies in dairy herds [27].

A systematic evaluation of antimicrobial resistance in milk through the decade long systematic review has highlighted the widespread occurrence and persistence of resistance genes across dairy systems. *Sievers et al.* (2025) emphasized that milk serves as a potential reservoir for antimicrobial resistance genes, with variations influenced by antibiotic usage, farm management practices, and processing conditions.

The presence of antimicrobial resistance genes (ARGs) in milk raises significant concerns regarding the transmission of resistance from animals to humans via the food chain. Research indicates that antibiotic use in dairy farming plays a pivotal role in ARG occurrence within milk microbiota, with intensive antibiotic practices correlating with higher ARG levels in these environments. Locatelli et al. (2017) and Tian et al. (2016) found that herds with frequent antibiotic exposure showed elevated ARG presence, suggesting a direct link between antibiotic administration and resistance gene amplification [28].

Furthermore, native breeds appear to have lower ARG levels in their milk microbiota compared to crossbred cows. Klein-Jöbstl et al. (2014) attribute this difference to variations in antibiotic exposure and potentially distinct immune responses among breeds. This relationship between breed and ARG occurrence underscores the role of genetic factors in influencing resistance.

The study focused on the *mecA* AMR gene due to β-lactam antibiotics are widely used as broad-spectrum agents in veterinary settings, leading to strong selective pressure for resistance. The *mecA* gene encodes an altered penicillin-binding protein (PBP2a), which reduces the efficacy of these antibiotics and is a key determinant of methicillin resistance in staphylococci. The relevance of staphylococcal infections such as mastitis, serious infection in dairy cattle, *mecA* serves as a clinically significant and widely recognized marker for AMR surveillance.

Additionally, the study employed an in-house designed primer specifically targeting the *mecA* gene, which was optimized and validated for sensitivity and specificity within the scope of this work. Due to the targeted objective of assessing β-lactam resistance, the analysis was limited to this marker.

Although more research is required to increase accuracy, the MinION sequencer has shown successful long-read outputs, portability, and real-time analysis in contrast to short-read sequencing [29].

From full-length 16S rRNA gene sequences that comprise nine hypervariable regions (V1-V9) as phylogenetically relevant markers, researchers must choose the most useful target regions for taxonomic identification. Oxford Nanopore Technologies (Oxford, UK) has released MinION, the first commercial sequencer utilizing nanopore technology. It has significant benefits over other DNA sequencing methods, including a long-read output, affordability, and the capacity to analyze data quickly in real time [30].

The application of Ayurvedic formulations in treating mastitis presents an opportunity to reduce dependency on antibiotics and address antimicrobial resistance (AMR) concerns. Network pharmacology provides a valuable framework to investigate the multi-target and synergistic effects of Ayurvedic compounds, allowing a deeper understanding of how these formulations modulate immune responses and inhibit bacterial growth. Huang et al. (2014) and Hopkins (2008) outline network pharmacology's potential to explore these complex molecular interactions, emphasizing its ability to elucidate the multi-pathway impact of natural compounds [31].

Herbal formulations have shown promise in modulating immune responses and providing anti-inflammatory and antibacterial effects, particularly relevant in treating bovine mastitis. Patil et al. (2019) and Zhang et al. (2017) highlight the efficacy of such herbal treatments, demonstrating their role as complementary therapies to antibiotics in dairy cattle. These studies observe that specific Ayurvedic compounds can activate immune-modulating pathways, leading to enhanced resilience against bacterial infections, a critical advantage in mastitis management.

2.2 Research Gap

1. **Limited Comparative Data on Native vs. Crossbred Dairy Cows:** Existing studies broadly examine antibiotic use in dairy farming but lack direct comparisons of microbiota and resistance patterns between native and crossbred cows.

2. **Insufficient Understanding of Resistance Gene Occurrence:** Studies emphasize the role of broad-spectrum antibiotics in resistance development but provide limited data on the specific antimicrobial resistance genes present in milk and their variation between native and crossbred cows.

3. **Impact of Crossbreeding on Microbial Diversity:** Crossbreeding is linked to reduced microbiota diversity, but we need more research on how this affects the cows' health and their ability to resist infections.

4. **Understanding How Breed Affects Microbiota:** While it's known that native and crossbred cows have different microbiota, more research is needed to understand how these differences affect their immune system and ability to fight diseases like mastitis.

2.3 Objectives

- To analyze and compare antibiotic use in focusing on the frequency, type, and dosage of antibiotics administered.

- To investigate the composition and diversity of the milk and curd microbiota in native versus crossbred dairy cows, also examining the pH, moisture content and texture analysis of milk and curd.

- To detect and compare the occurrence of antimicrobial resistance genes in the milk of native and crossbred dairy cows, assessing the impact of antibiotic practices on the spread of resistance.

3. Materials and method

3.1 Assessment of antibiotics use in dairy cows and its impact

Survey was conducted among the veterinarians to analyze the antibiotics use in focusing on the frequency, type, and dosage of antibiotics administered.

- Google forms were created with a set of questionnaires with respect to antibiotics use for the cattle through veterinarians majorly. (Annexure 1)
- Questionnaires were framed by referring the literature, through the guidance of Ayurvedic doctor suggestions.
- Google forms were circulated among veterinarians in two modes: 1. Online 2. Offline

3.2 Milk sample collection

Milk samples were collected from both native (3 native breeds such as Malnad gidda, Sahiwal and Gir) and crossbred (2 crossbreeds such as Jersey and Holstein Friesian) dairy cows under aseptic conditions to ensure sample integrity and prevent contamination.

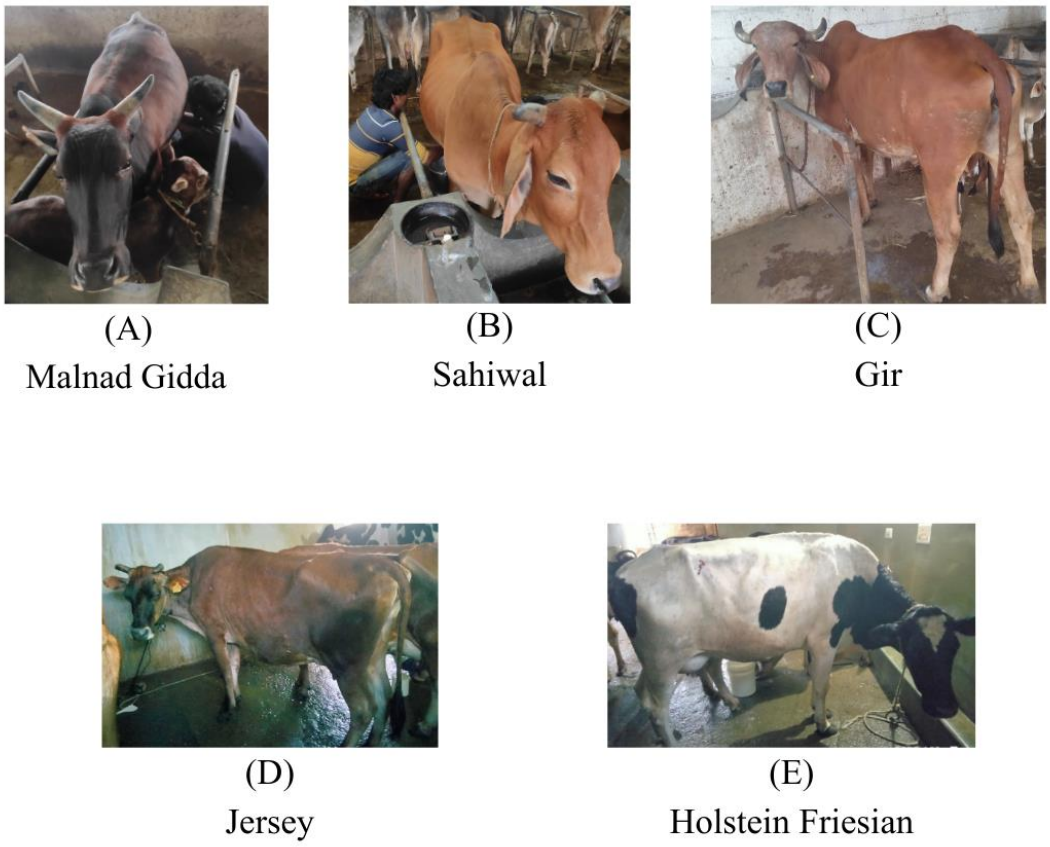


Figure 1. Images of five different breeds of cows in the dairy farm (3 native breeds and 2 crossbreeds)

3.3 Milk samples storage and processing

Milk samples were stored in the ice packs after collected, till they are brought to the laboratory. Then the samples were shifted to -20° C freezer. Milk samples were segregated for microbiome analysis, for starter culture and curd preparations, also preserved some amount of sample for physicochemical analysis (pH, moisture, etc).

Formation of Starter culture for curd preparation: Starter culture was prepared using red chilli.

- Milk was boiled at 80°C and cooled down to 40°C, and red chilli along with the stalk was dipped in the warm milk and incubated at 37°C for 12 hours. Later this culture was used to prepare curd.

Preparation of Curd using starter culture: Curd was prepared by boiling milk at 80°C

- Then, cooled down at 40°C, around 10 grams of freshly prepared starter culture was added.
- After adding starter culture kept in the incubator at 37°C for 4-6 hours for fermentation to take place to convert it to curd.
- This curd is used for microbiome studies and other analysis like moisture, pH and texture.

3.4 Physicochemical analysis method

pH: 10ml of sample was used to measure the pH of each sample with the help of pH meter.

Moisture content: 1 gram of the sample was taken and added into the moisture analyzer, the moisture content will be absorbed by the instrument at the temperature of 120ºC, and the moisture content percentage will be displayed on the moisture analyzer.

Texture analysis: A texture analyzer, with a 5 kg load cell and a cylindrical probe (25.4 mm in diameter) provided with texture exponent programs was used to examine the texture profiles of curd samples. Initially, the samples were stored at 25ºC until TPA analysis. TPA was carried out by compressing twice with a probe to penetrate 10 mm at a speed of 5 mm/s. Using software, TPA's hardness, springiness, adhesiveness, cohesiveness, chewiness, gumminess, and resilience were assessed. Every measurement was performed three times for every sample.

Figure 2. Determination of physicochemical properties of milk, starter culture and curd: (a) pH measurement using a digital pH meter, (b) moisture content estimation using a moisture analyser, and (c) texture profile analysis using a texture analyser

3.5 Metagenomic DNA isolation and QC (Quality check of isolated DNA)

DNA isolation was done for all the 5 breeds samples (milk, starter culture and curd samples) using Dneasy Powerfood Qiagen microbial kit method as per the manufacturer's protocol.

The detailed procedure for DNA extraction is as follows mentioned below:

Take 2 ml of sample in the Eppendorf tube

↓

Centrifuge at 14000 rpm, for 15 minutes at 4°C

↓

Keep the pellet and discard the supernatant

↓

Add preheated (70°C) 500µl MBL solution to the pellet

↓

Vortex the tube for resuspension

↓

Incubate the tube at 70°C for 5 minutes to dissolve the pellet

↓

Transfer the contents of the tube into the powerbead tube provided in the kit

↓

Incubate the powerbead tube for 10 minutes at 70°C

↓

Vortex the powerbead tube horizontally for 10 minutes at maximum speed

↓

Centrifuge at 14000 rpm for 2 minutes at 4°C (Note: Supernatant has DNA)

↓

Transfer the supernatant to another Eppendorf tube and discard the pellet

↓

Add 100µl of IRS solution for DNA precipitation

↓

Incubate the tube at 4°C for 30 minutes

↓

Centrifuge at 14000 rpm at 4°C for 2 minutes

↓

Keep the supernatant and discard the pellet

↓

Add 900µl of MR solution and vortex to mix for 1 second

↓

Transfer to the spin column given in the kit and centrifuge at 14000rpm at 4°C for 2 minutes

↓

Remove the flow through and add 650µl of PW solution

↓

Centrifuge 14000rpm at 4°C for 2 minutes and remove the flow through

↓

Add 650µl ethanol and centrifuge 14000rpm at 4°C for 2 minutes

↓

Remove the flow through and dry spin at 14000 rpm at 4°C for 2 minutes

↓

Add 20µl of EB in the collection tube and incubate at room temperature for 5 minutes

↓

Centrifuge at 4°C, 14000rpm for 1 minute

↓

Add 10µl of EB in the collection tube and incubate at room temperature for 5 minutes

↓

Centrifuge at 4°C 14000rpm for 1 minute

↓

DNA is obtained. (in the collection tube)

The purity and quantity of the isolated DNA were determined using NanoDrop by measuring both the 260/280 and 260/230 absorbance ratios.

3.6 Bacterial identification of 16S rRNA gene amplification (V1-V9 region of the genomic DNA) and QC (Quality check of amplified product)

PCR amplification of the 16S rRNA gene was conducted using KAPA HiFi HotStart Ready Mix. The conditions for PCR amplification are as follows for 16S rRNA gene: 3 min at 95°C initial denaturation, 34 cycles of 20 seconds at 98°C denaturation, 30 seconds at 61.5°C and 1 min 30 seconds annealing temperature, at 72°C for elongation followed by final extension at 72°C for 5 min.

Primer no.	Code	Sequence (5'-3')
1	16S_rRNA_27F	AGAGTTTGATCATGGCTCAG
2	16S_rRNA_1392R	GGTTACCTTGTTACGACTT

Table 1. 16S rRNA bacterial gene identification and amplification primer details

Bacterial identification of AMR gene amplification [Methicillin resistance gene (mecA)]

The PCR amplification of the AMR gene *mecA* was performed using KAPA HiFi HotStart Ready Mix in a total reaction volume of 10 µL, containing 5 µL master mix, 1 µL of each primer (final concentration 1 µM, stock concentration: 10 µM) and 3 µL template DNA. An initial denaturation step at 95°C for 3 min was used to ensure complete template denaturation and activation of the HotStart polymerase, followed by 34 cycles of denaturation at 98°C for 20 seconds, annealing at 57.5°C for 30 seconds, and extension at 72°C for 1 min 30 seconds. A final extension was carried out at 72°C for 5 min.

The 98°C temperature was used for the cycling denaturation is mainly to activate the HotStart polymerase which ensures efficient denaturation for high fidelity enzymes (could be based on enzyme optimization) in the master mix like KAPA HotStart Master Mix.

The extension time was optimized by increasing the longer duration based on the expected amplicon size which was between 1500 to 2000 base pairs depending on the different amplicon sizes with regard to the respective primers.

G2P (GeneToProtein) 1 kb DNA ladder was used is a ready-to-use molecular weight standard designed for sizing linear double-stranded DNA fragments in agarose gels, ranging from 250 bp to 10,000 bp. This molecular size marker estimated the PCR amplicon size of 1500bp.

Most commercial ladders (including G2P-type preparations) use recombinant Taq DNA polymerase-amplified products or restriction-digested plasmid DNA.

The reactions were performed in a BIO-RAD T100 gradient 96-well thermal cycler.

Primer no.	Code	Sequence (5'-3')
1	mecA_new_F	GTTGTAGTTGTCGGGTTTG
2	mecA_new_R	TGAGCTATATGAGAACGGT

Table 2. mecA AMR gene identification and amplification primer details

All the PCR products were quantified using Nanodrop and Invitrogen Qubit 4 fluorometer. The DNA integrity was checked using a 1% Agarose TAE gel. 1 kb DNA ladder was also loaded as a molecular size marker to confirm the expected amplicon size, the amplification of the 16S rRNA gene. The gel was visualized under ultraviolet (UV) transillumination using a Gel Doc imaging system (Bio-Rad Universal hood II Gel Doc Imaging system).

3.7 Nanopore Library Preparation and Sequencing using Native Barcode Kit

The DNA amplicons generated from the PCR reactions were subjected to library preparation using the Native Barcoding Kit 24 V14 (SQK-NBD114.24, Oxford Nanopore Technologies, UK), which facilitates the multiplexing of up to 24 samples in a single sequencing run.

- DNA Quantification and Input

The concentration and quality of DNA were determined using a Qubit fluorometer and agarose gel electrophoresis. 130 ng of DNA per sample was used as an input for library preparation.

- End-Repair and dA-Tailing

DNA amplicons were subjected to end-repair and dA-tailing using the NEBNext End Repair/dA-Tailing Module following the manufacturer's protocol. This step ensures that DNA fragments have blunt ends and a 3′ adenine tail, to enable efficient ligation of the native barcodes.

- Native Barcode Ligation

Each DNA sample was ligated to a unique native barcode provided in the kit. Barcodes were handled carefully, and each well was used only once to prevent cross-contamination between the samples. The ligation reaction was performed under optimal

conditions as specified in the protocol, ensuring efficient attachment of barcodes to the DNA fragments.

- Adapter Ligation and Library Purification

Following barcode ligation, sequencing adapters were attached to the barcoded DNA fragments. The library was purified using (AMPure beads) magnetic beads based clean up to remove excess adapters, enzymes, and other reaction components. The purified libraries were stored temporarily at 4°C if used immediately, or at –20°C for longer-term storage.

- Flow Cell Preparation and Sequencing

Sequencing was performed on a Oxford Nanopore Sequencer, MinION Mk1C platform using R10.4.1 flow cells (FLO-MIN114). Prior to loading the library, the flow cells were checked and primed according to the manufacturer's protocol. Libraries were then loaded, and sequencing was initiated using MinKNOW software, which controls the sequencing run and performs real-time basecalling. Optionally, downstream analysis was performed using the Kraken2 platform.

3.8 Data Analysis

Data Processing and Quality Control

Raw sequencing reads were first assessed for quality using NanoQC, which provides a summary of read length distributions, quality scores, and sequencing yield. Low-quality reads and adapter sequences were removed using NanoFilt by applying a minimum quality score threshold of Q9 and filtering reads within the length range of 1200–1700 bp. After preprocessing, reads were demultiplexed based on their native barcodes to separate individual samples [32].

Taxonomic Classification and Analysis

Cleaned and demultiplexed reads were subjected to **Kraken 2**, a k-mer-based taxonomic classification tool, to assign reads to bacterial, archaeal, and fungal taxa. We worked with Centrifuge, a created species classification suite, to lower the computational time needed for species discovery. At the species level, Centrifuge could identify all bacteria included in the mock community, except *Actinomyces odontolyticus.*

The output was used to generate **microbial abundance profiles** at various taxonomic levels, providing insights into the composition of the microbial community in each sample. Sequencing depth, read length distributions, and classification statistics were carefully evaluated to ensure reliable results. Data visualization, including bar plots, upset plots, diversity analysis was performed to represent the relative abundance of different taxa across samples.

Bar plots were plotted using the MS office with required legends, margins and axis. Upset plots were plotted in the R platform. Diversity analysis including both alpha and beta metrics was performed for the obtained data [33].

Diversity Analysis

Diversity analysis was done for the microbiome data obtained from the different samples of native and crossbreed cows

Alpha Diversity metrics were calculated focusing on Shannon and Simpson indices using python and conda environment.

Beta Diversity was analyzed using Bray-Curtis and Mann-Whitney distances calculated with conda package. Principal Coordinate Analysis (PCoA) was performed using ggplot2 in R packages. Permutational Multivariate Analysis of Variance (PERMANOVA) was also conducted using R packages to evaluate group differences based on metadata variables [34].

AMR mecA gene analysis

Raw Nanopore FASTQ files generated for the mecA gene were first subjected to quality control using NanoFilt to ensure high-quality reads. Reads with a minimum quality score (Q score) of ≥12 was retained, and length filtering was applied to include only sequences between 800–2600 bp. The filtered FASTQ files were then converted into FASTA format using seqtk (seqtk seq -a) for downstream clustering analysis.

De novo clustering of sequences was performed using vsearch (cluster_fast) at a 90% sequence similarity threshold (id 0.90) to group similar reads into clusters. To improve reliability and remove unreliable clusters, clusters containing fewer than five reads were removed. For each retained cluster, the relationship between Cluster ID and Read ID was extracted, and Read IDs were mapped back to their respective Sample IDs to determine sample-wise distribution.

A cluster × sample abundance table was generated by calculating the number of reads per cluster in each sample. To account for differences in sequencing depth across samples, normalization was performed using Reads Per Million (RPM), calculated as: RPM = (Reads per cluster / Total reads in the sample) × 1,000,000.

Samples were then categorized into two groups: native breeds and crossbred cattle for comparative analysis. Representative centroid sequences from each cluster were annotated using BLASTn against the CARD (Comprehensive Antibiotic Resistance Database) nucleotide database. Matches were considered significant based on the following criteria: percentage identity ≥90%, query coverage ≥80%, and e-value ≤1e-10. Finally, CARD metadata were integrated to extract the corresponding ARO (Antibiotic Resistance Ontology) terms, gene names, and associated organisms, enabling functional characterization of the identified mecA gene clusters.

To visualize shared and unique mecA gene clusters among different cattle breed samples, an UpSet plot was generated using the R statistical platform (UpSetR package), enabling graphical representation of cluster intersections and comparative abundance across the groups.

4. Results

The survey responses showed that 69.6% of the stakeholders have concerns regarding the use of antibiotics in dairy cows, which outlines the widespread awareness of the problem. But there is only 35.7% reported awareness about the existence of specific guidelines for the antibiotics prescription and indicated about the aligning the prescription practices with the WHO guidelines and standards.

Questionnaires	**Percentage**
There are concerns to antibiotic use in dairy cows	69.60%
There are specific guidelines to prescribe antibiotics in India	35.70%
Ensurement of antibiotic prescribing align to WHO guidelines	35.70%
Need to educate cattle owners about responsible use of antibiotics	83.90%
Cattle owners are aware regarding antibiotic use and resistance in India	16.10%
Vets are monitoring effectiveness of antibiotic treatments in disease management	42.90%
Vets in India are knowledgeable about AMR and its implications	48.20%
Responsible use of antibiotics is being addressed in animal health in India	35.70%
Animals can be raised without antibiotics	57.10%
Dairy cattle health practitioners could encourage more responsible use of antibiotics	75%
Antibiotics can be recommended as growth promotors	75%
Antibiotics in animal feed plays a major role for the development of AMR	64.30%
One should collaborate with specialists when making decisions about antibiotic use	84%
There is a difference b/w reducing use of antibiotics and reducing the need for antibiotics	85.70%
There can be devastating result from overuse of antibiotics in livestock	87.50%

We must decrease the use of antibiotics in livestock	85.70%
Banning of antimicrobials could have negative effects on animal welfare.	37.50%
There is a need to engage with the expert veterinary community to share best practices regarding antibiotic use	89.30%
Ethno-veterinary based recipes can be an alternative to antibiotics	75%
There is a need for continuous veterinary education on antibiotic use	83.90%
Farmers should be able to buy antibiotics over counter	16.10%
The dosage and course of the antibiotics are strictly followed by vets in dairy sector in India	50%
Minimizing the use of antibiotics is very crucial in containing AMR	76.80%
Antibiotics should only be prescribed by a veterinarian	89.30%
Overuse of antibiotics can lead to AMR	86.20%
Human health is depending on animal and environmental health	92.90%
We can manage many infectious conditions without using antibiotics	66.10%

Table 3. Survey-Based evaluation of Antibiotic Use Awareness, Supervision Practices, and Perceptions of Antimicrobial Resistance in the Indian Dairy Sector

The 50% of the respondents reported that veterinarians strictly adhere to the dosage and administration protocols of antibiotics in the dairy sector in India, in terms of monitoring, 42.9% indicated that veterinary practitioners evaluate antibiotic treatment in disease management.

There are 85.7% responses claims differences between merely reducing antibiotic use and reducing the need for antibiotics. Also 57.1% respondents noted that animals can be raised without antibiotics and 66.1% believed many infectious conditions are manageable without antibiotics, encouraging the alternatives and restricted use of antibiotics are feasible.

The 75% participants demonstrated the ethno-veterinary based recipes could serve as alternatives. There are pronounced concerns about the devastating effect of antibiotics, responses scaling up to 87.5%, there is an identifying link between overuse and AMR with the response of 86.2%.

Eventually, 76.8% align with minimizing the antibiotic use is essential for managing AMR. 89.3% states that antibiotics should only be prescribed by a veterinary practitioner, emphasizing the importance on the insights of controlling the frequency, type and dosage of administering antibiotics.

Clinical conditions as per the response	Percentage of response
Mastitis	86.79%
Metritis and reproductive disorders	45.94%
Fever with no symptoms	37.93%
Foot and mouth disease	37.93%
Dermatitis	28.57%

Table 4. Frequency (in %) of major clinical conditions treated with antibiotics as reported by veterinarians in the survey, showing mastitis as the predominant cause of antibiotic usage.

The survey unveils mastitis as the most widely treated condition through antibiotics treatment by showing 86.79% responses report for it, followed by metritis and reproductive disorders reporting 45.94%, followed by fever without symptoms, foot and mouth disease voting upto 37.93%.

Antibiotics prescribed by the veterinarians	No of responses w.r.t recommendation of veterinarians (%) (42 responses received)
Tetracycline	47.6
Penicillin	30.9
Gentamycin	38.0
Enrofloxacin	33
Amoxycillin	7.1
Sulphadimidine	4.7
cefriaxone	7.1
Otc (Oxytetracycline)	16.6
Ceftriaxone	9.5
cephalosporins	4.7

Table 5: Major antibiotics (in %) commonly prescribed by the veterinarians for the disease management of cattle.

Antibiotics prescription patterns by the veterinary respondents unfolds 47.6% recommendation of tetracyclines, followed by gentamycin comprising 38%, then enrofloxacin of 33%, and penicillin by 30.9%

The data demonstrate the reliance on broad-spectrum agents, predominantly tetracyclines and penicillin for disease management in dairy cattle. Regardless of the observed discrepancies in prescription practices, awareness of the guidelines, monitoring highlights the need for transformation of antibiotics stewardship protocols within the dairy sector.

Microbiome

Milk samples after collected were segregated for curd and starter culture preparation, microbiome studies and physicochemical analysis. The pH of the milk was assessed using pH meter.

Milk samples	pH (mean ± SD)	Moisture content (%) (mean ± SD)
Malnad gidda	6.73 ± 0.02	84.97 ± 0.01
Sahiwal	6.83 ± 0.02	89.72 ± 0.01
Gir	6.73 ± 0.02	89.01 ± 0.01
Jersey	6.79 ± 0.03	82.55 ± 0.01
Holstein Friesian	6.76 ± 0.03	90.78 ± 0.01

Table 6. pH and moisture content readings of the milk samples from different cattle breeds

After the starter culture and curd was prepared, pH of the starter culture and curd was assessed and obtained the readings as follows.

Samples	Starter culture pH	Curd pH (mean ± SD)	Curd Moisture content (%) (mean ± SD)
Malnad gidda	5.62	4.72 ± 0.01	83.86 ± 0.0
Sahiwal	5.58	4.71 ± 0.01	86.70 ± 0.0
Gir	5.53	4.61 ± 0.01	85.51 ± 0.0
Jersey	5.47	4.59 ± 0.01	83.90 ± 0.0
Holstein Friesian	5.49	4.61 ± 0.01	90.61 ± 0.0

Table 7. pH and moisture content readings of curd samples from different cattle breeds

The texture analysis obtained the readings as below.

Samples	Hardness	Adhesiveness	Resilience	Cohesiveness	Springiness	Gumminess	Chewiness
Malnad gidda	157.37 ± 40.52	0.29 ± 0.05	0.20± 0.06	0.52 ± 0.07	3.39 ± 0.26	110.43 ± 44.77	3.64 ± 1.35
Sahiwal	48.57 ± 7.05	0.46 ± 0.46	0.05 ± 0.02	0.36 ± 0.10	8.12 ± 0.95	24.47 ± 7.06	1.98 ± 0.79
Gir	24.13 ± 2.37	0.12 ± 0.02	0.16 ± 0.03	0.42 ± 0.08	6.81 ± 0.88	14.43 ± 0.90	0.97 ± 0.18
Jersey	29.7 ± 3.68	0.15 ± 0.08	0.12 ± 0.05	0.32 ± 0.15	7.49 ± 1.67	38.27 ± 38.07	2.69 ± 2.46
Holstein Friesian	34.03 ± 3.10	0.19 ± 0.05	0.14 ± 0.06	0.44 ± 0.02	5.33 ± 0.63	19.9 ± 1.18	1.05 ± 0.17

Table 8. TPA (Texture Profile Analysis) of curd samples

After the DNA extraction from the milk, starter culture and curd samples using the manufacturer's protocol, DNA concentration and quality was assessed using the Nanodrop Spectrophotometry. PCR amplification was subsequently carried out, and the amplified products were checked by agarose gel electrophoresis by visualizing the presence and expected amplicon size and checked the nanodrop quantification, followed by Qubit fluorometer analysis.

Samples	Sample Type	gDNA CONCENTRATION ng/µl	A260/280	A260/230
Malnad gidda	Milk	5	2.5	0.18

Sahiwal	Milk	10.9	1.04	0.14
Gir	Milk	55.4	1.5	0.11
Jersey	Milk	15.3	1.47	0.04
Holstein Friesian	Milk	10	1.16	0.13
Malnad gidda	Curd	10.5	1.05	0.02
Sahiwal	Curd	13.6	1.63	0.04
Gir	Curd	33.4	1.23	0.31
Jersey	Curd	37.2	1.89	0.07
Holstein Friesian	Curd	37.2	1.89	0.09
Malnad gidda	Starter culture	18.6	1.8	1.28
Sahiwal	Starter culture	7.1	1.84	0.19
Gir	Starter culture	32.7	1.41	0.15
Jersey	Starter culture	16.8	1.71	0.46
Holstein Friesian	Starter culture	15	1.6	0.5

Table 9. Nanodrop-based assessment of DNA concentration and purity in milk, curd, and starter culture samples from different cattle breeds

Table 9 summarizes the concentration and genomic DNA purity values of the milk, curd and starter cultures of 5 different cattle breeds in which DNA concentration was analysed in ng/µl. DNA concentration and purity were varied across the different breeds of milk, curd and starter culture samples, however the Gir milk sample yields highest concentration of DNA ranging at 55.4 ng/µl, although curd samples were providing higher concentrations and better quality absorbance range than milk. Among the starter culture samples Malnad gidda and Jersey starter culture showed highest A260/230 absorbance range. Overall, the curd and starter culture samples were optimal for obtaining high-quality DNA.

The readings of the PCR nanodrop readings can be seen in the table.

Samples	Sample Type	PCR product CONCENTRATION ng/µl	A260/280	A260/230
Malnad gidda	Milk	922.4	1.8	1.81
Sahiwal	milk	866	1.8	1.56
Gir	Milk	742.8	1.88	1.26
Jersey	Milk	599.2	1.84	1.32
Holstein Friesian	Milk	775.8	1.79	1.76
Malnad gidda	Curd	486.9	1.86	0.96
Sahiwal	Curd	665.7	1.88	1.5
Gir	Curd	601.2	1.85	1.51
Jersey	Curd	861.4	1.82	1.27
Holstein Friesian	Curd	673.9	1.85	1.67
Malnad gidda	Starter culture	556.7	1.85	1.85
Sahiwal	Starter culture	728.1	1.81	1.77
Gir	Starter culture	614.5	1.83	1.55
Jersey	Starter culture	671.6	1.83	1.74
Holstein Friesian	Starter culture	765.7	1.84	1.81

Table 10. QC (nanodrop) of milk, curd and starter culture PCR product samples 16srRNA milk and curd samples

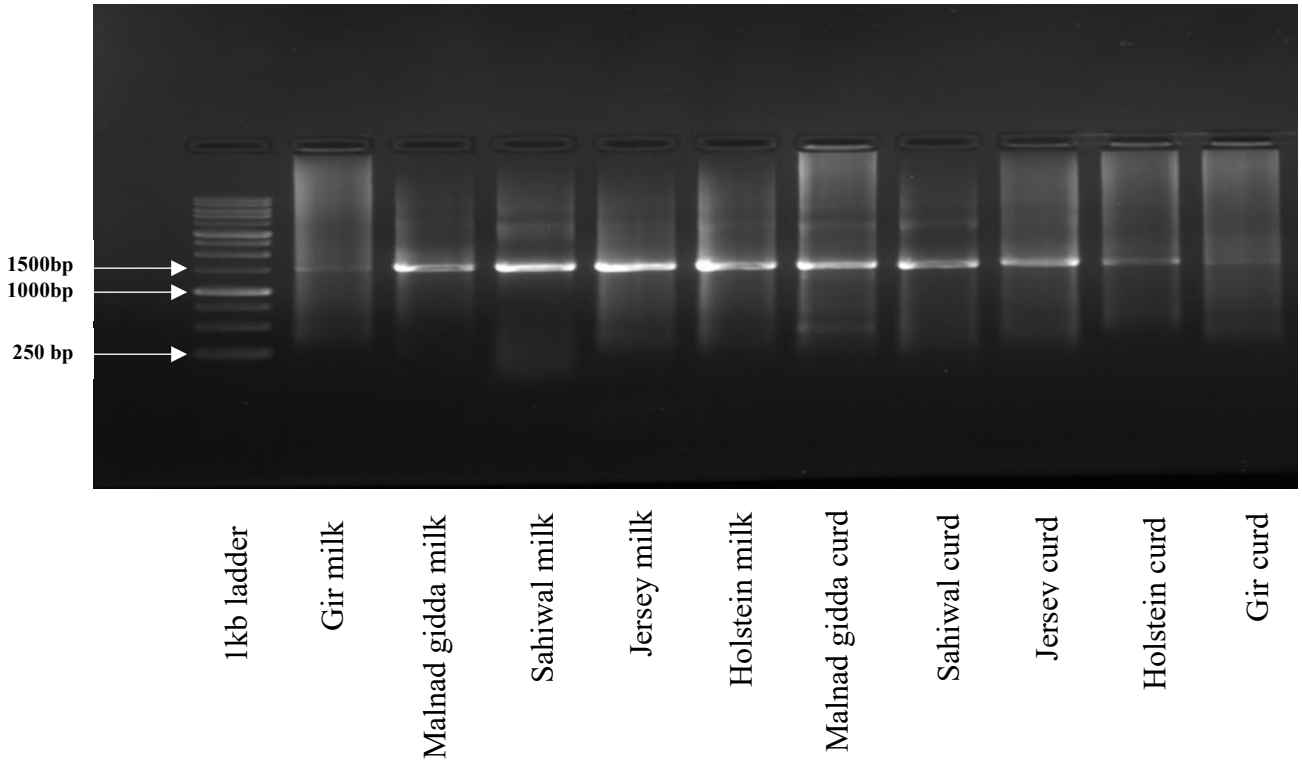


Figure 3. PCR amplification of 16S rRNA gene from milk and curd samples of different breeds of cow.

1 kb DNA ladder (250–10,000 bp), with bands at 250, 500, 750, 1,000, 1,500, 2,000, 3,000, 4,000, 5,000, 6,000, 8,000, and 10,000 bp. Figure 3 represents a distinct band observed at 1500bp corresponds to the expected amplicon size of the 16S rRNA gene.

Gel electrophoresis image representing the successful PCR amplification of the 16S rRNA gene from milk and curd samples of different breeds of cow amplified at 1500 bp

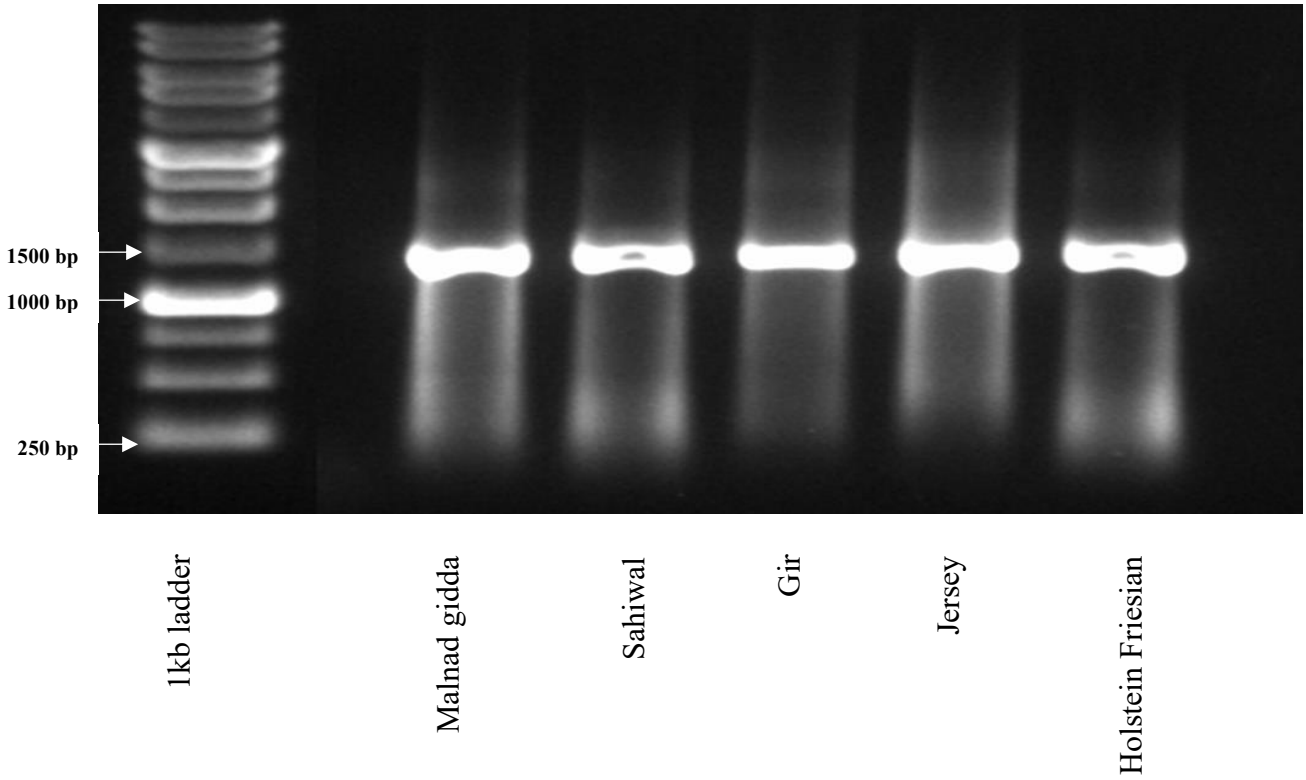


Figure 4. PCR amplification of 16S rRNA gene from starter culture samples of different breeds of cow

1 kb DNA ladder (250–10,000 bp), with bands at 250, 500, 750, 1,000, 1,500, 2,000, 3,000, 4,000, 5,000, 6,000, 8,000, and 10,000 bp. Figure 4 represents a distinct band observed at 1500bp corresponds to the expected amplicon size of the 16S rRNA gene.

Gel electrophoresis image representing the successful PCR amplification of the 16S rRNA gene from starter culture samples of different breeds of cow amplified at 1500 bp

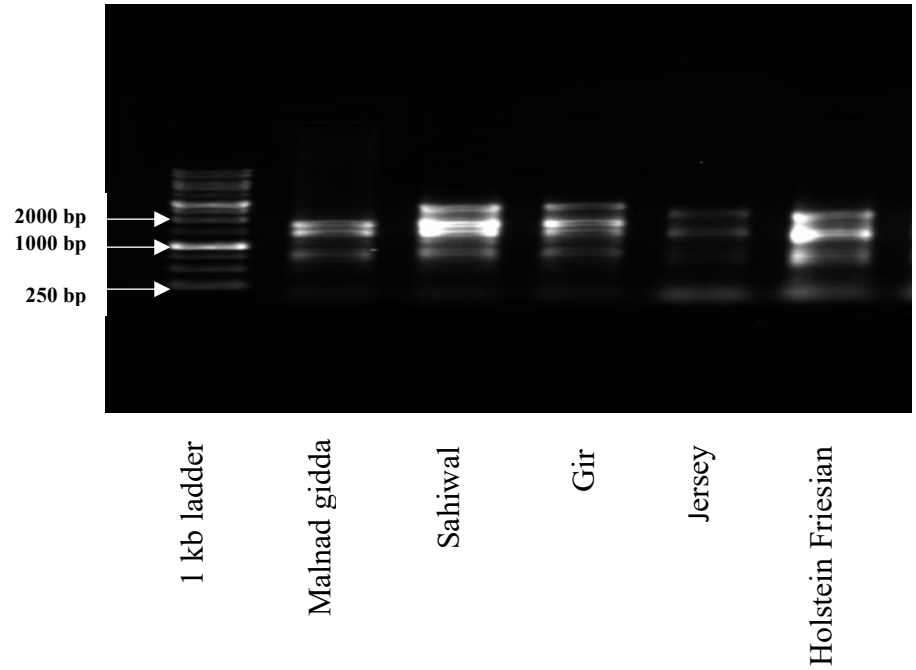


Figure 5. PCR amplification of AMR mecA gene from milk samples of different breeds of cow

1 kb DNA ladder (250–10,000 bp), with bands at 250, 500, 750, 1,000, 1,500, 2,000, 3,000, 4,000, 5,000, 6,000, 8,000, and 10,000 bp. Figure 5 represents, a distinct band observed at 1900bp corresponds to the expected amplicon size of the inhouse designed AMR *mecA* gene.

Gel electrophoresis image representing the successful PCR amplification of the AMR gene mecA (methicillin resistance gene) from milk samples of different breeds of cow amplified at 1900 bp for the inhouse designed primer.

Sample name	**Sample Type**	**PCR product- Qubit Reading**	**Qubit Reading-End Prep library ng/µl**
Malnad gidda	Milk	20.8	12.2
Sahiwal	milk	29.6	13.6
Gir	Milk	98.4	21.4
Jersey	Milk	24.8	5.06
Holstein Friesian	Milk	42	6.58

Malnad gidda	Curd	62.2	4.88
Sahiwal	Curd	61.4	4.08
Gir	Curd	150	5.88
Jersey	Curd	31.8	4.18
Holstein Friesian	Curd	56.2	3.98
Malnad gidda	Starter culture	34.2	5.26
Sahiwal	Starter culture	68.6	3.68
Gir	Starter culture	106	9.48
Jersey	Starter culture	50.8	5.18
Holstein Friesian	Starter culture	64.6	5.34

Table 11. QC of Qubit readings (End Prep library) ng/µl

Then library preparation was carried out using an Oxford Nanopore Technologies sequencing kit following the manufacturer's protocol and sequencing was completed. After nanopore sequencing, the table 11 represents read statistics of milk, curd and starter culture samples from 5 different breeds of cow, which includes raw and processed read counts followed by their read lengths. Raw reads count differs widely among the milk and curd samples respectively providing a comprehensive overview of sequencing depth and quality across sample types and breeds, allowing for the subsequent microbiome analysis.

Samples	Sample Type	Total Raw reads	Mean raw read length (bp)	Total Processed reads	Mean processed read length (bp)
Malnad gidda	Milk	140037	765.8	16151	1575.6
Sahiwal	Milk	308401	630.2	26066	1508.6
Gir	Milk	130504	715.1	9912	1465.1
Jersey	Milk	517902	482.4	10601	1576.6
Holstein Friesian	Milk	441818	543.3	23805	1594.3

Malnad gidda	Curd	390206	683.8	69725	1600.7
Sahiwal	Curd	260330	847.1	64090	1582.8
Gir	Curd	74484	664.2	2496	1518.2
Jersey	Curd	335310	709.7	52057	1584.4
Holstein Friesian	Curd	673875	567.7	86709	1590.4
Malnad gidda	Starter culture	542743	529.1	56877	1601.1
Sahiwal	Starter culture	383313	624.5	43097	1595.5
Gir	Starter culture	142473	669.8	8077	1567.5
Jersey	Starter culture	296597	631.1	34821	1589.5
Holstein Friesian	Starter culture	366069	622.2	41696	1567

Table 12. A review of the raw and processed read counts and lengths for milk, curd, and starter samples from five different cattle breeds is provided by the sequencing findings summary

Classification	Gir			Holstein Friesian			Jersey			Malnad gidda			Sahiwal		
	Milk	Curd	SC	Milk	Curd	SC	Milk	Curd	SC	Milk	Curd	SC	Milk	Curd	SC
Domain	2	2	2	2	2	4	2	2	2	2	2	2	2	2	2
Phylum	14	7	8	4	3	8	4	4	4	5	6	3	11	4	4
Class	25	13	12	7	4	11	9	9	10	12	8	7	15	9	8
Order	58	21	17	10	6	22	19	8	10	20	6	6	31	13	8
Family	88	32	25	18	15	36	28	15	19	33	14	16	41	24	22
Genus	112	49	33	24	22	72	37	17	25	47	14	20	56	31	30
Species	86	81	60	38	60	72	79	54	40	89	48	55	84	54	52

Table 13. Taxonomic distribution of microbial diversity (domain to species level) identified in milk, curd, and starter culture samples from five different cattle breeds, SC: Starter Culture

The table outlines the taxonomic diversity of microbial communities detected in milk, curd and starter culture samples from 5 different breeds at various levels (from domain to species level). Between the milk samples, in the genus the richness was seen highest in Gir milk comprising 112 genera and for the species Malnad gidda cow milk showed the highest richness of 89 species followed by Gir milk sample species with a less variation of 86 species count. Starter culture samples indicated lower diversity compared to 5 breeds of milk and curd samples yet contributed with notable counts across taxonomic classification.

The graphs were plotted for the microbial composition at various levels of taxonomic classification of milk, starter culture and curd samples.

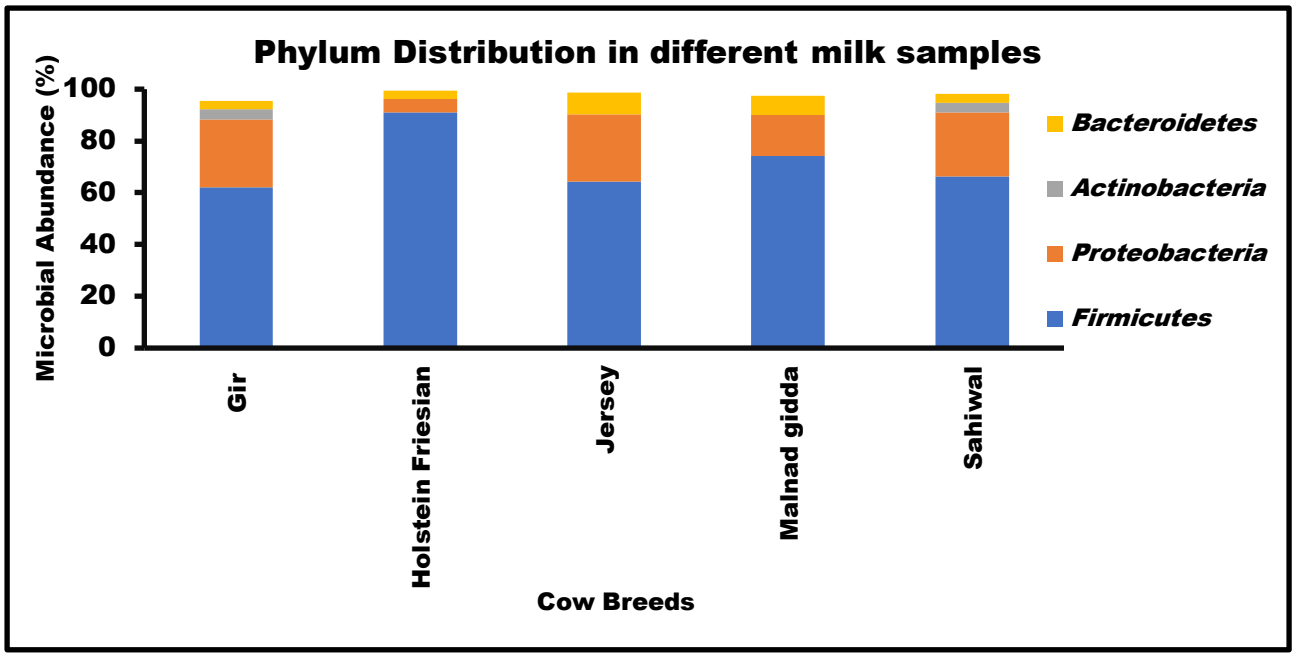


Figure 6. Microbial phylum distribution in the milk of different cow breeds

The graphical illustration depicts the analysis of the microbial composition at Phylum level displays the diverse microbial communities majorly including Firmicutes in the various breeds of milk samples.

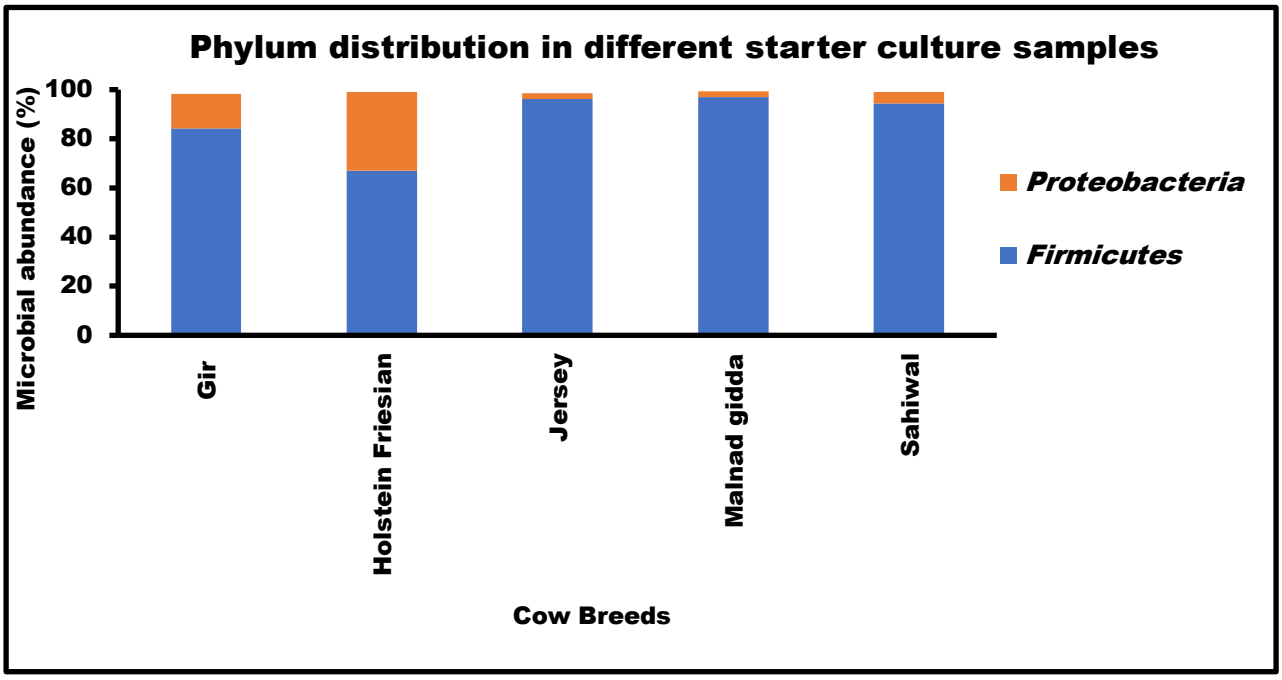


Figure 7. Microbial phylum distribution in the starter cultures of different cow breeds

The starter culture samples indicated that there is a clear dominance of the phylum Firmicutes in the starter culture samples of all the breeds of the cow attributing to the deliberate selection for lactic acid bacteria during starter culture preparation, leading to play a vital role in fermentation by also shaping the development of texture, taste and preservation of dairy products.

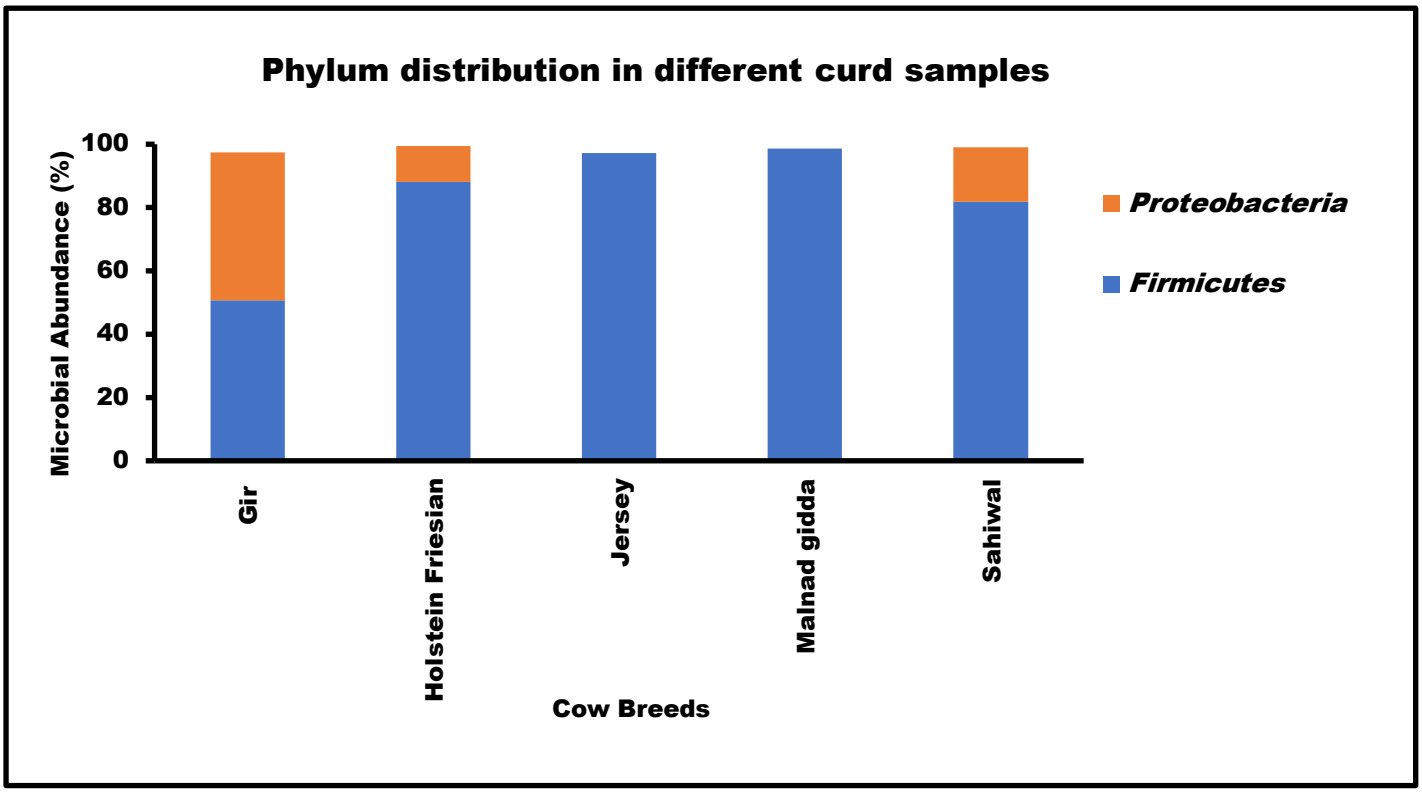


Figure 8. Microbial phylum distribution in the curd of different cow breeds.

In curd samples, however exclusively Firmicutes were dominated in most of the curd samples, signifying a selective enrichment of the phylum Firmicutes during fermentation process.

Genus level

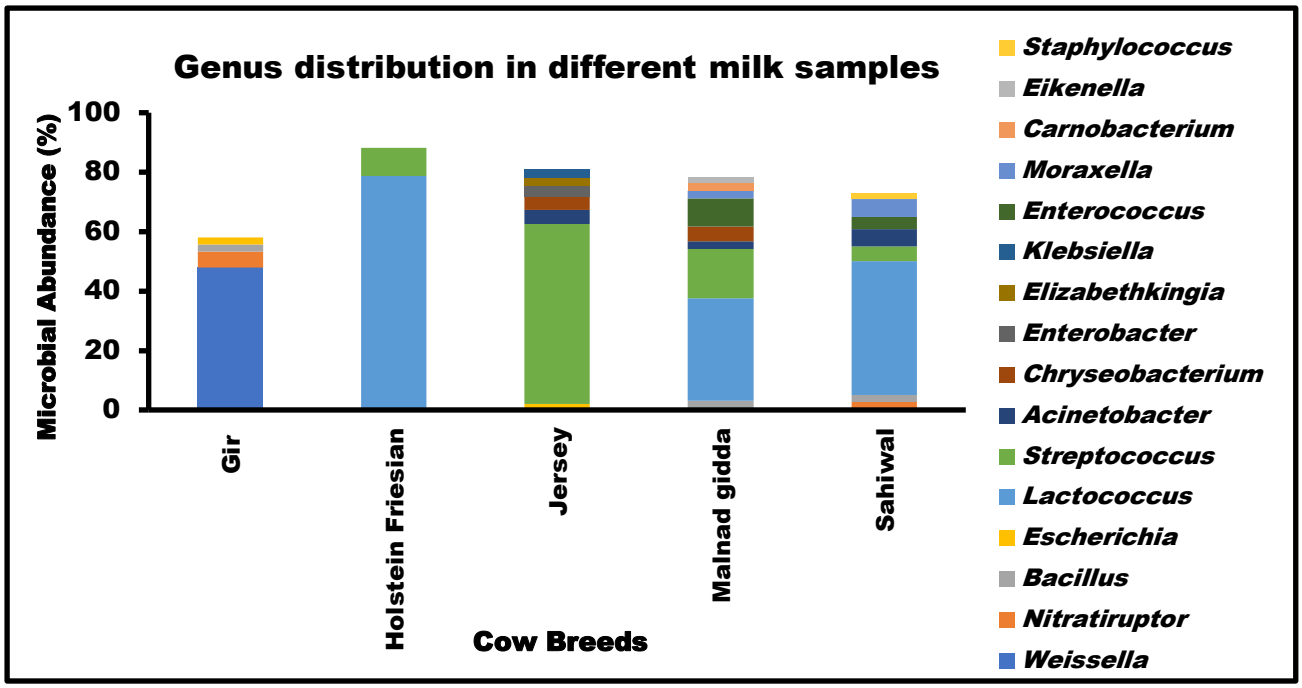


Figure 9. Microbial genus distribution in the milk of different cow breeds

The analysis of milk at genus level revealed distinct microbial genus profiles. Lactococcus was the dominant genus seen in Holstein Friesian, Malnad gidda and Sahiwal cow milk samples. The figure shows a breed-specific variation in milk microbiota.

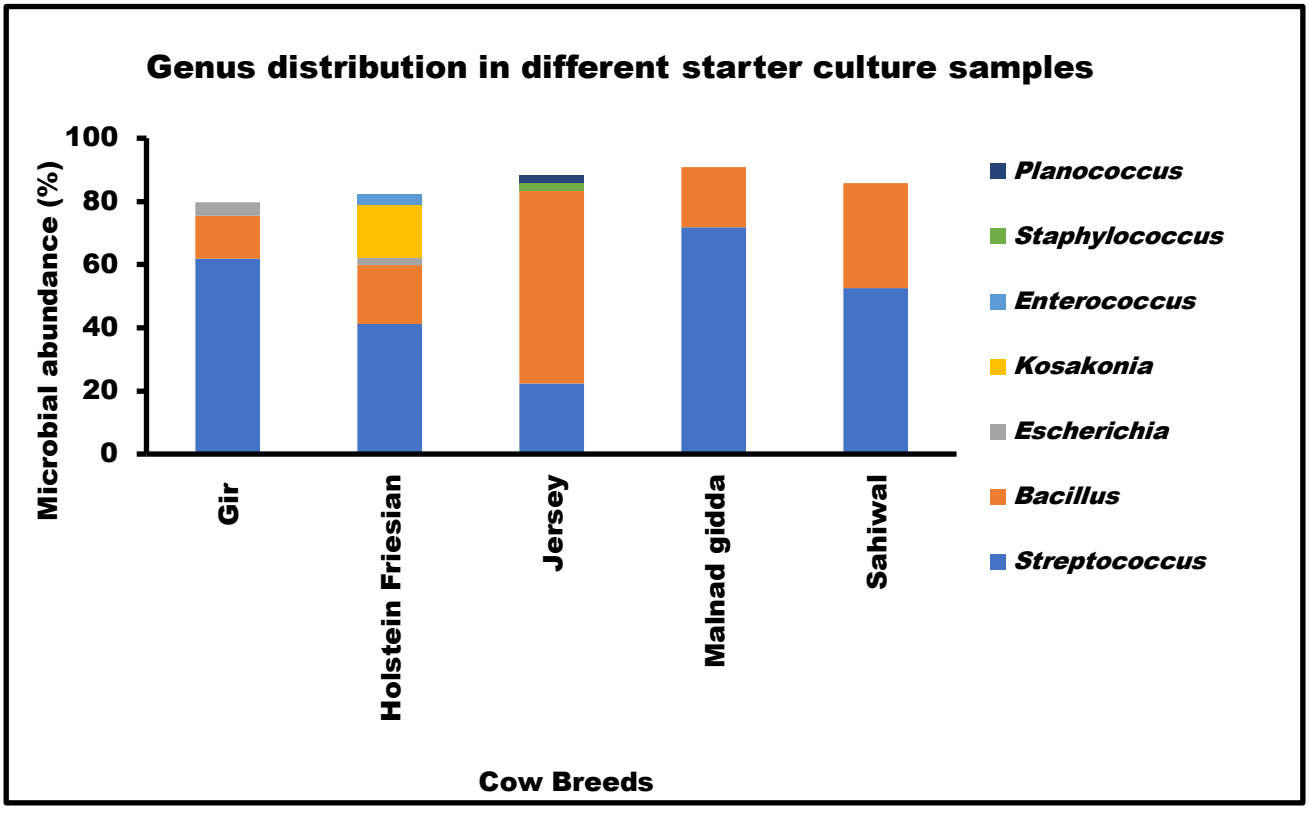


Figure 10. Microbial genus distribution in the starter culture of different cow breeds

The microbial genus distribution across the starter culture samples from 5 cow breeds is illustrated in the figure. The overall microbial profiles of starter culture samples were breed-specific, which indicates both native and crossbreed cows harbour distinct microbial distribution in starter culture, influencing fermentation and fermentation product characteristics. The native breeds showed the dominant presence of *Streptococcus* and *Bacillus* with *Planococcus* also prevalent, particularly in Gir and Sahiwal.

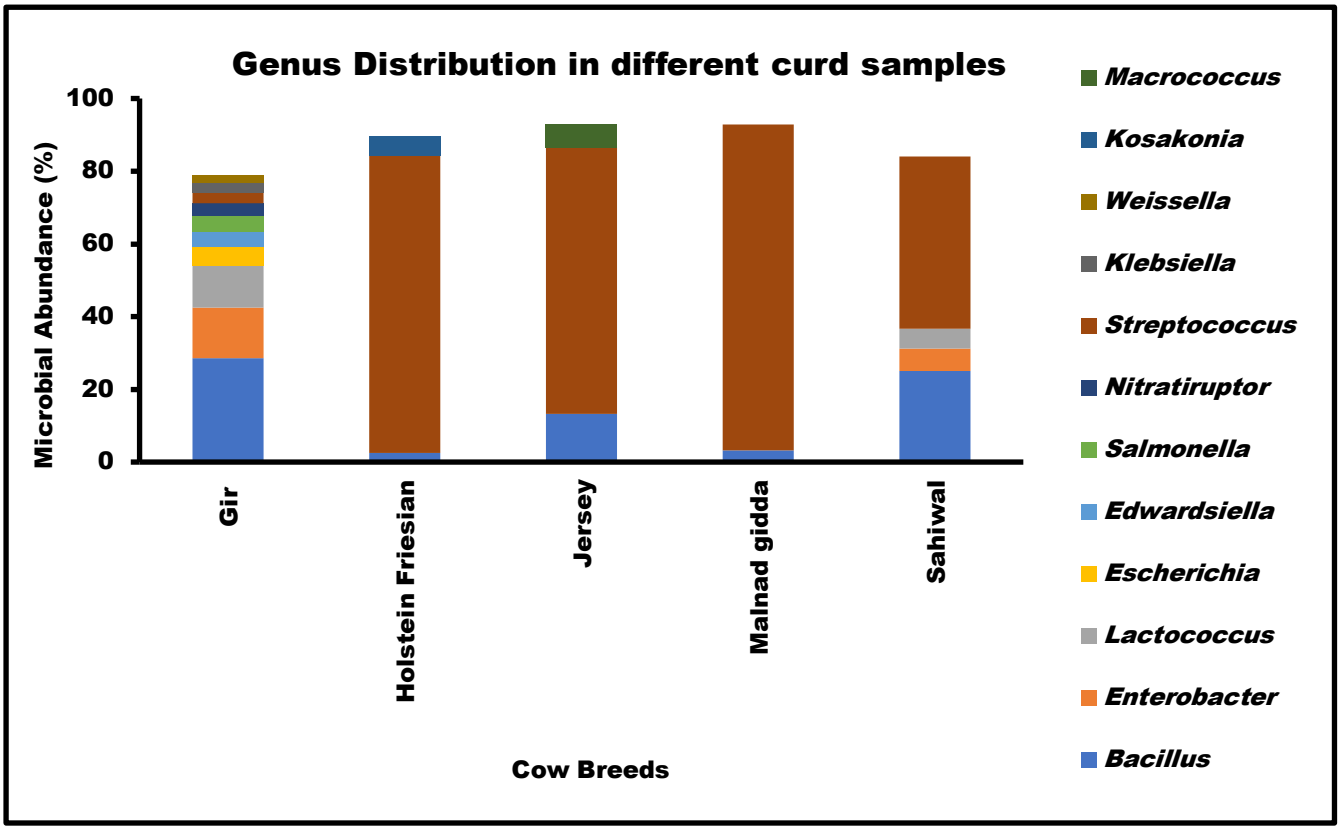


Figure 11. Microbial genus distribution in the curd of different cow breeds

Most of the curd samples microbial communities account for the genus *Streptococcus* dominance with the substantial proportions of *Bacillus* in Gir, Malnad gidda and Sahiwal curd samples. The presence of *Streptococcus* in these curd samples exclusively reflects the genus s level selectivity during the curd fermentation and formation.

Species level

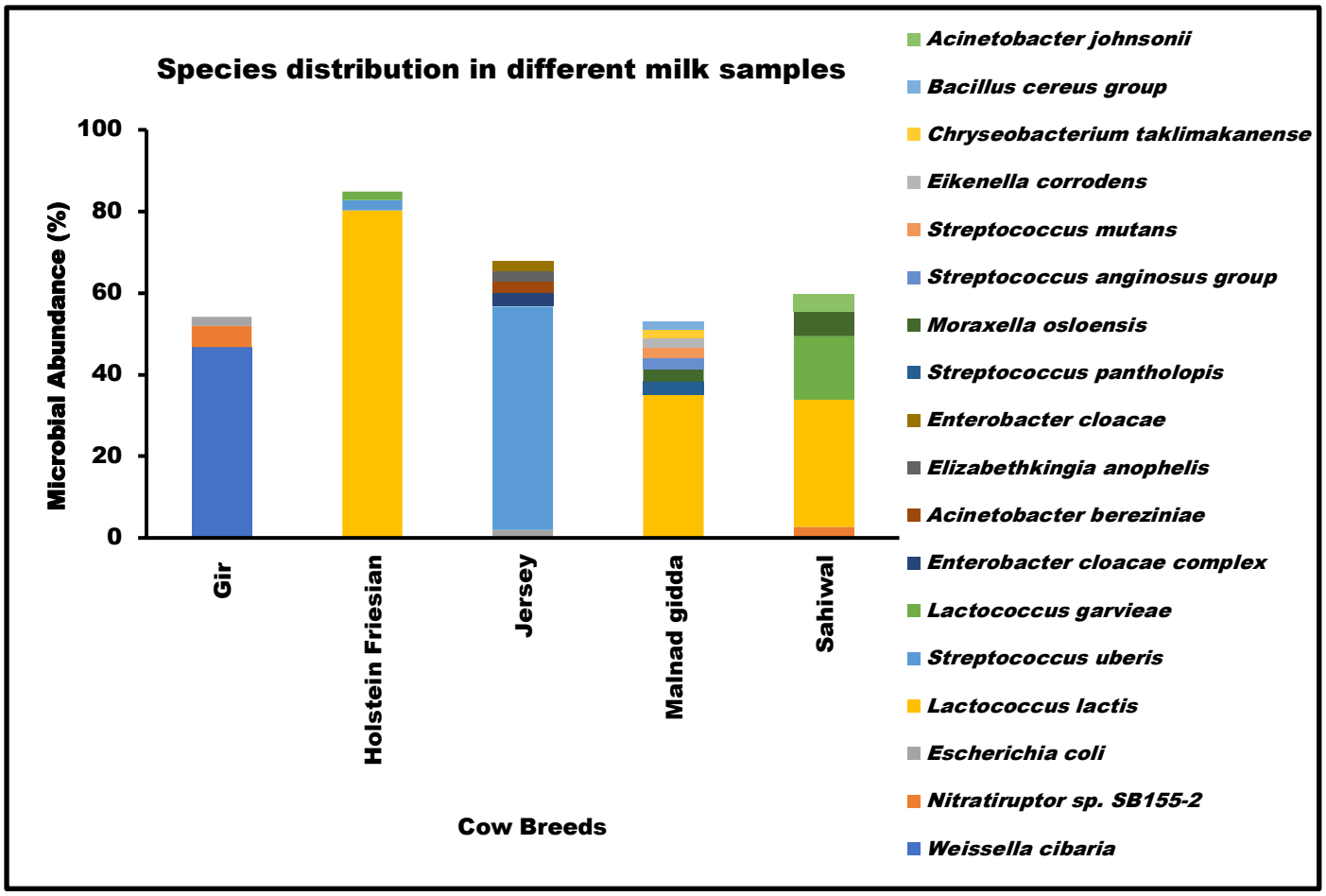


Figure 12. Microbial species distribution in the milk of different cow breeds

The microbial species profiles are distinct across the different breeds of milk samples. Malnad gidda milk samples exhibited broader species diversity, with moderate levels of multiple bacteria including *Streptococcus* and *Enterobacter* species. Gir and Jersey breeds milk showed high relative abundance of *Bacillus cereus* group. Holstein Friesian and Sahiwal cow milk marked the dominance of *Lactococcus lactis*.

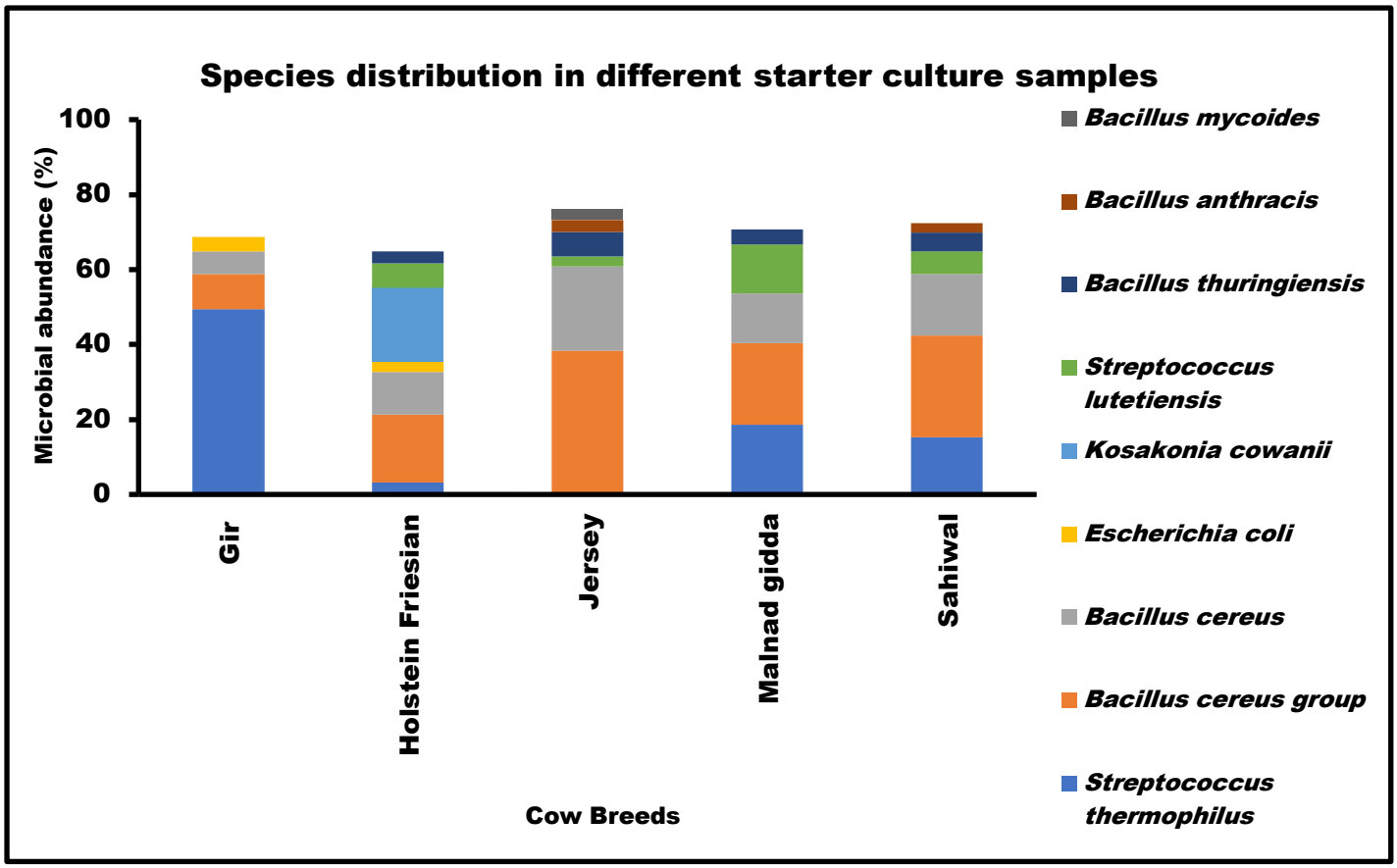


Figure 13. Microbial species distribution in the starter culture of different cow breeds

Starter cultures samples showed (fig) *Streptococcus thermophilus* and *Bacillus cereus* group in the native breed samples of Malnad gidda, Sahiwal and Gir. *Bacillus cereus* group is dominant in Jersey starter culture. Holstein samples are characterized by moderate *Streptococcus thermophilus* levels with the presence of notable *Bacillus cereus* species. The species composition varied distinctly among breeds impacting fermentation efficiency and final product microbial quality and composition.

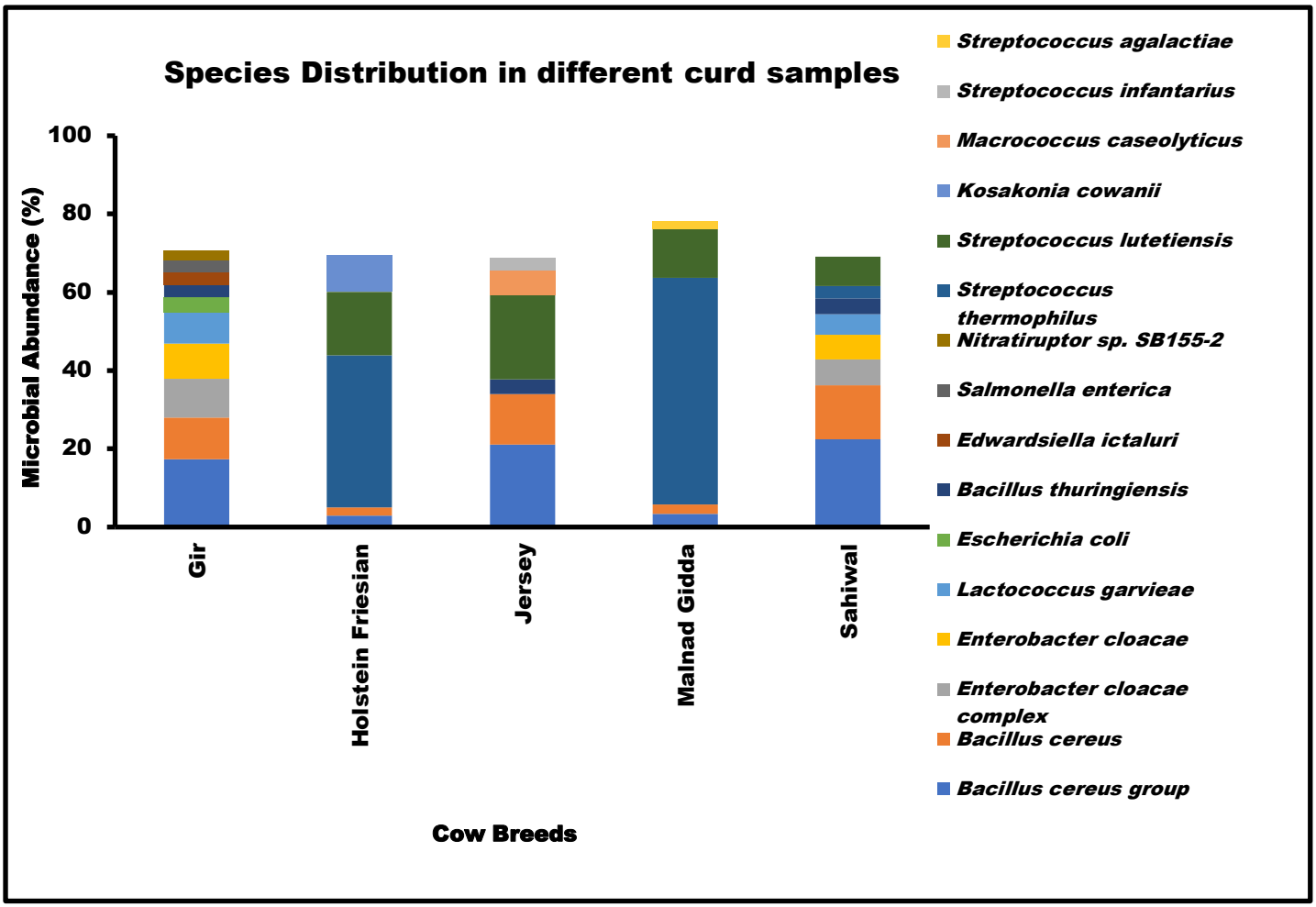
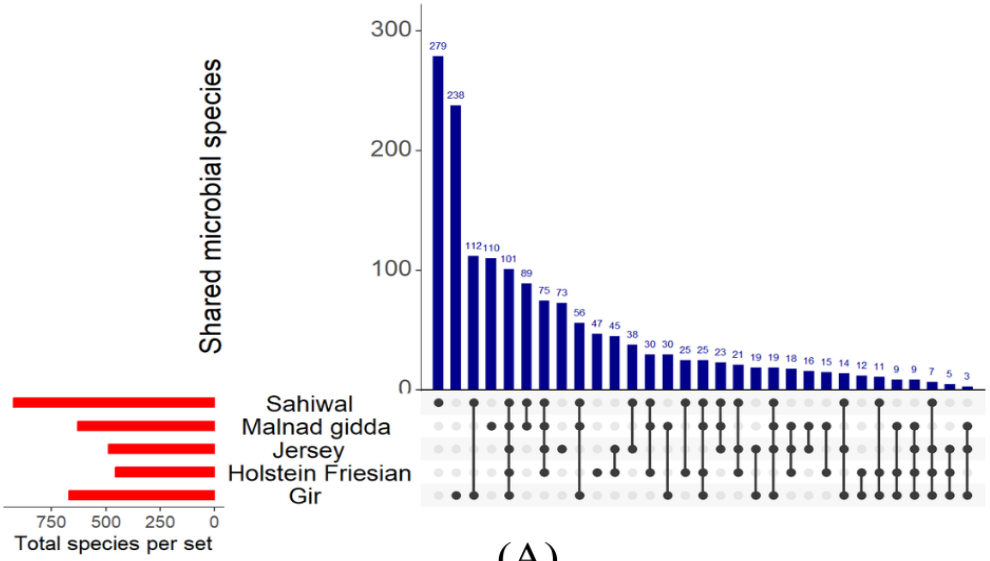
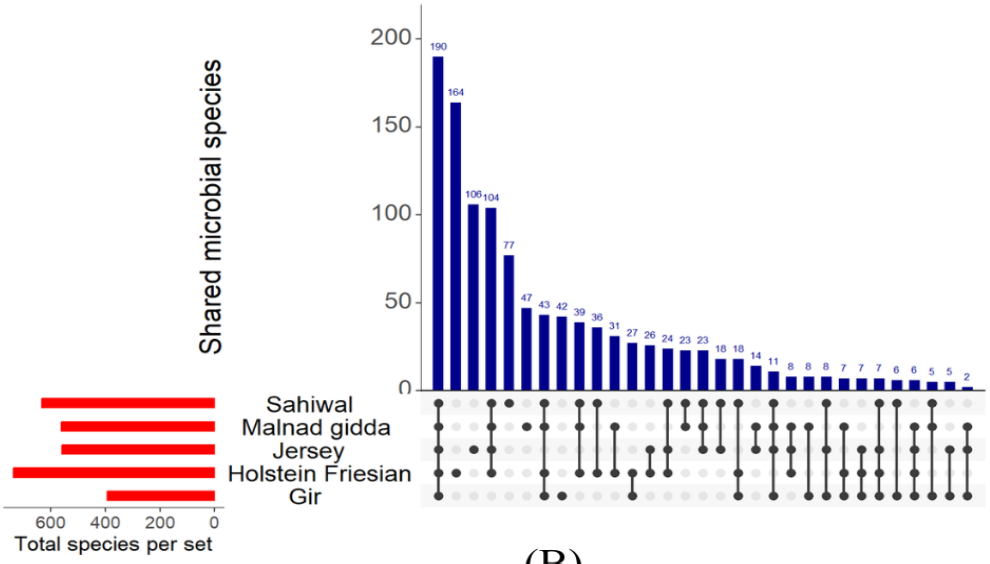


Figure 14. Microbial species distribution in the curd of different cow breeds

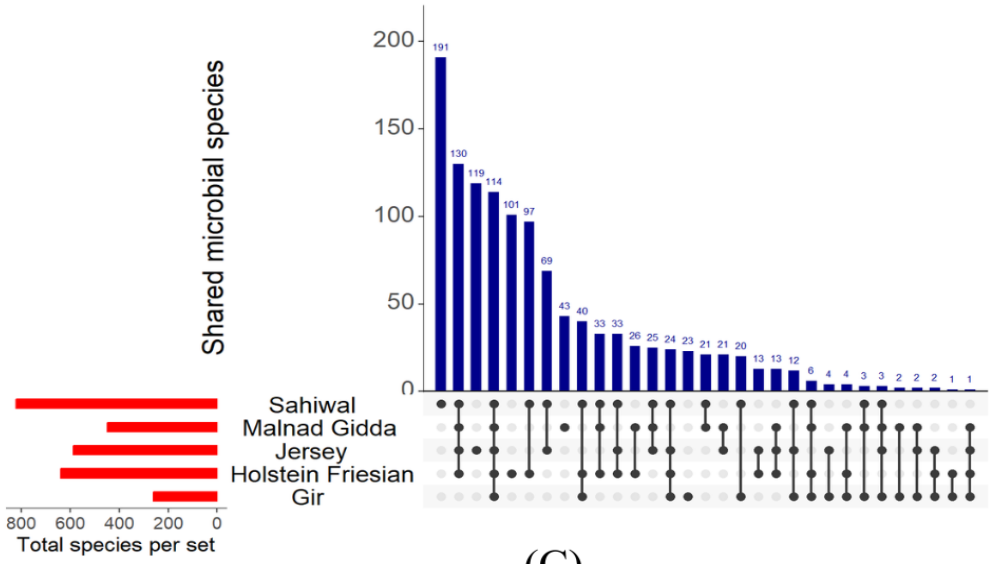
The graph plot depicts the various bacterial species demonstrating the differences in microbial abundance in curd samples. *Bacillus cereus* group is the dominant species in Holstein friesian, Jersey, Malnad gidda and Sahiwal curd samples. Gir curd showed a more diverse bacterial distribution.


Shared microbial species
300
200
100
0
279
238
112
110
101
89
75
73
56
47
45
38
30
30
25
25
23
21
19
19
18
16
15
14
12
11
9
9
7
5
3
Sahiwal
Malnad gidda
Jersey
Holstein Friesian
Gir
750
500
250
0
Total species per set

(A)


Shared microbial species
200
150
100
50
0
190
164
106
104
77
47
43
42
39
36
31
27
26
24
23
23
18
18
14
11
8
8
8
7
7
7
6
6
5
5
2
Sahiwal
Malnad gidda
Jersey
Holstein Friesian
Gir
600
400
200
0
Total species per set

(B)


Shared microbial species
200
150
100
50
0
191
130
119
114
101
97
69
43
40
33
33
26
25
24
23
21
21
20
13
13
12
6
4
4
3
3
2
2
2
1
1
Sahiwal
Malnad Gidda
Jersey
Holstein Friesian
Gir
800
600
400
200
0
Total species per set

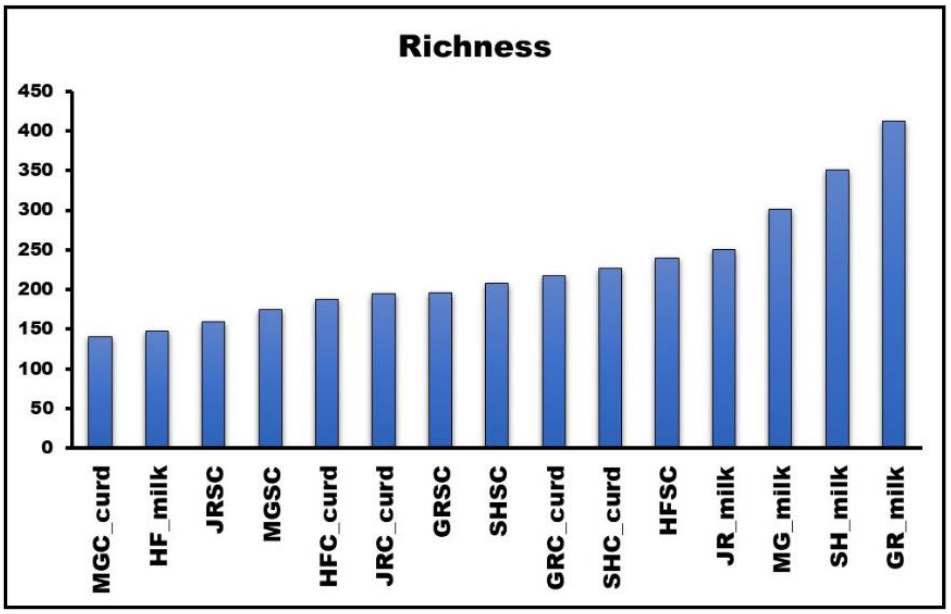
(C)

Figure 15. UpSet plots depicting the number of shared and unique microbial species among (A)milk samples (B) Starter culture samples and (C) curd samples from five cattle breeds

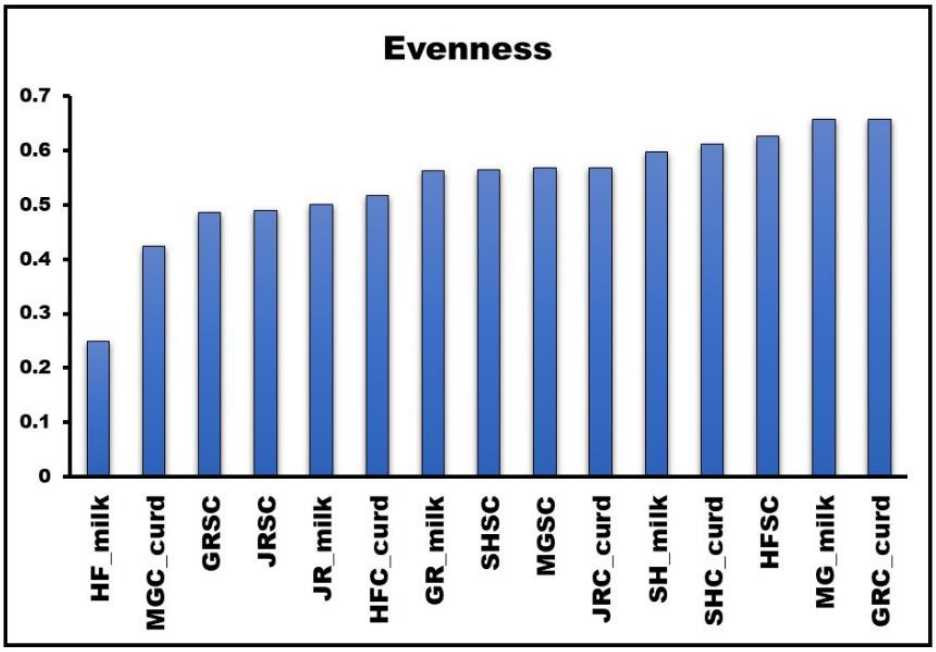
The microbial analysis was further elucidated using the Upset plots to visualize the distribution of the bacterial species across the different samples of different breeds of cows. The Upset plot figures highlight the common species and core species across the breeds with respective sample types. Upset plot revealed that the starter culture samples foster the highest number of 190 commonly shared microbial species among five different breeds followed by curd comprising 114 species and milk of 101 species. The data demonstrate considerable microbial diversity with core and common microbial communities varying across the cattle breeds and samples types.

Diversity analysis

The diversity analysis was carried out for the comprehensive understanding of the obtained microbiome data of the various breeds and all the different samples respectively.



(a)



(b)

Figure 16. Microbial community diversity in milk, curd, starter culture samples of different cattle breeds shown through (a) Richness indices (b) Evenness indices

Richness plot from diversity analysis from above (figure) revealed the highest species counts in milk samples from native breeds, preferably Gir and Sahiwal, showing substantial microbial diversity. But the curd and starter culture samples on the other hand showed reduced richness respectively. The observation of reduction in richness from milk to fermented product (curd) reflects selective microbial growth during the fermentation process.

Evenness analysis from diversity analysis from above (figure) revealed significant differences among the breeds and simple types. Holstein friesian milk sample had essentially low evenness, while Malnad gidda milk and Gir curd samples among the different breed, maintained high evenness, displaying greater microbial diversity and balance with regard to milk samples.

Also, diversity analysis indicated Shannon and Simpson indices displaying highest microbial diversity from milk samples of native cow breeds along with curd and starter culture samples, showing elevated diversity values. Holstein milk demonstrated reduced diversity, with the consistently lower in richness and evenness. While other starter culture and curd samples tend to maintain moderate diversity suggesting selective enrichment during fermentation.

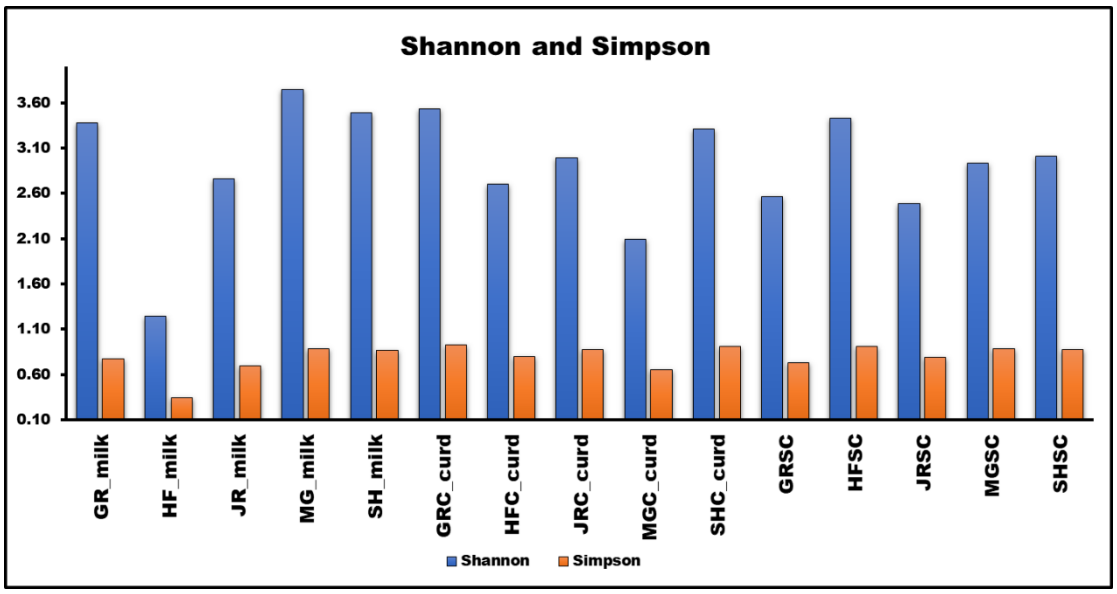


Figure 17. Shannon and Simpson diversity indices depicting microbial diversity in milk, curd and starter culture samples across different cattle breeds

The PCoA plot signifies no specific differences in the microbial communities of the milk, starter culture and curd samples from the different cattle breeds.

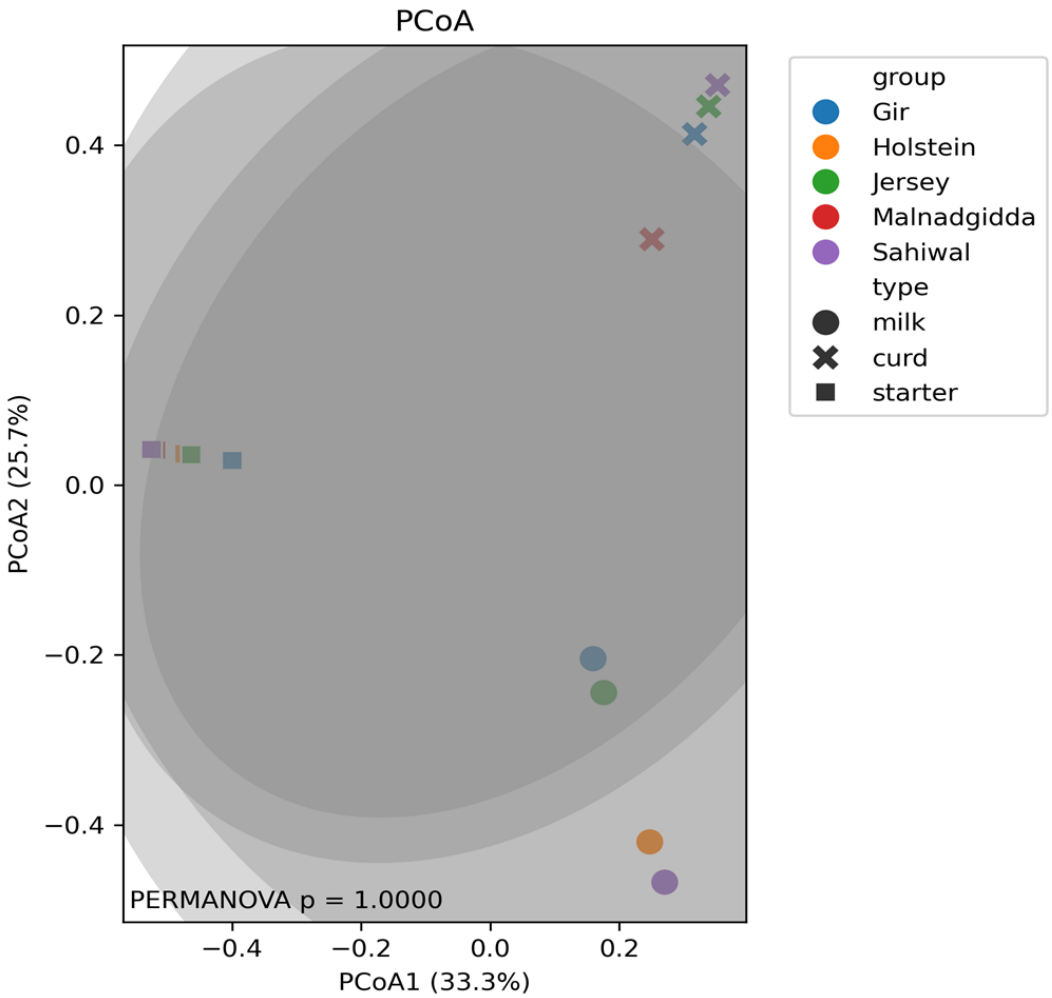


Figure 18. Microbial communities in milk, curd, and starter samples from different cattle breeds show similar compositions with no significant differences, as visualized by the PCoA plot

The PCoA plot signifies no specific differences in the microbial communities of the milk, starter culture and curd samples from the different cattle breeds.

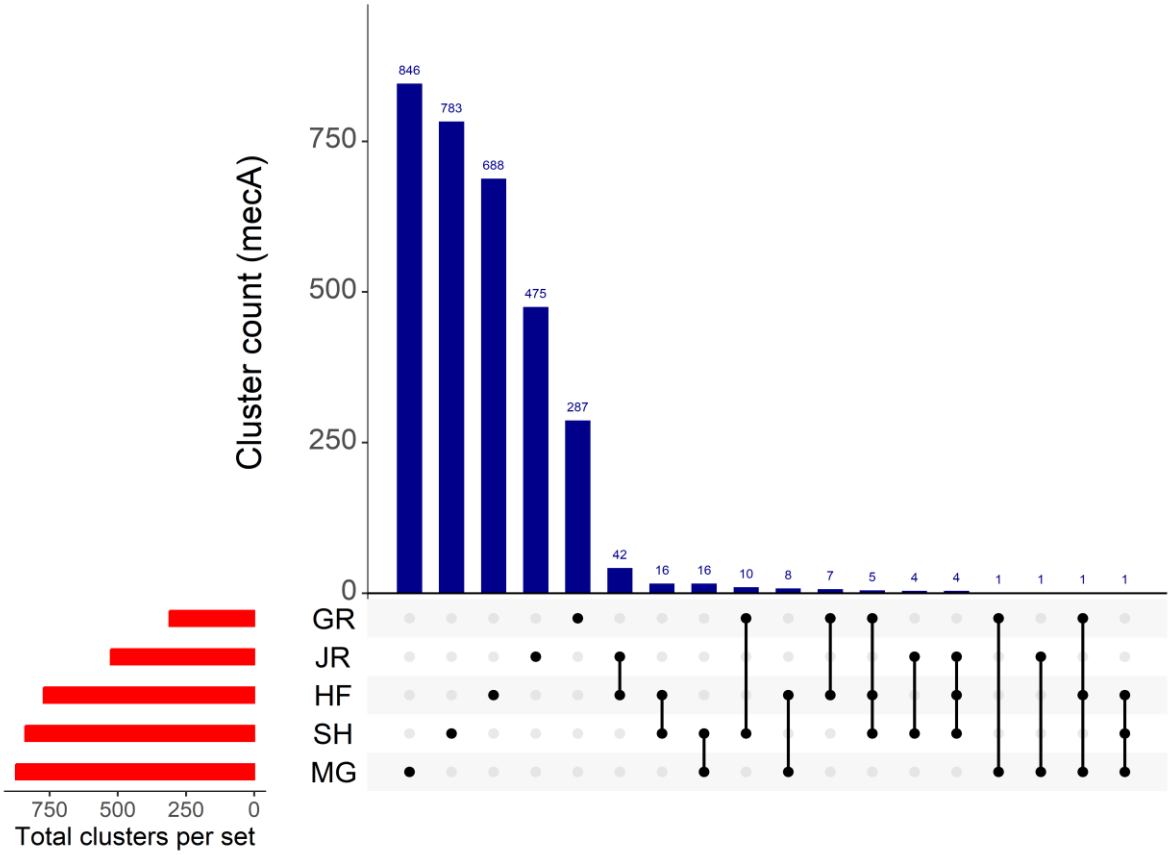


Figure 19. Upset plot showing the distribution of mecA gene clusters across cattle breeds, GR- Gir, JR- Jersey, HF- Holstein Friesian, SH- Sahiwal, MG- Malnad gidda

Most clusters were breed-specific, indicating distinct mecA profiles within breeds. The highest shared clusters were observed between Holstein Friesian and Jersey (42), suggesting greater similarity between these crossbred breeds. Overall, the plot highlights both unique and shared mecA cluster patterns among native and crossbred cattle.

The resistome analysis revealed the presence of the *mecA* gene (ARO:3000617) in samples annotated to *Staphylococcus capitis*. The *mecA* gene encodes an altered penicillin-binding protein (PBP2a), which has reduced affinity for β-lactam antibiotics, including methicillin, penicillin, and other cephalosporins. The presence of *mecA* is a well-established molecular marker of methicillin resistance in staphylococci.

5.1 Discussion

Milk and dairy products encompass an extensive variety of nutrients, enabling consumers to acquire adequate nutritional levels and consume food of high dietary quality. Global health authorities acknowledge the benefits of dairy products for health and recommend including them as part of a balanced and healthy diet [1] .
Since 1998, India has been the world's largest producer of milk. India often serves as the oyster of the global dairy Industry. India produced 187.96 million tonnes of milk in 2018–19, an increase over 17 million tonnes (MT) in 1950–51. About 22.29% of the world's dairy comes from India production.

To facilitate toward increasing milk yield, crossbreeding programs between native and foreign cattle were started in India in the 1950s, mostly with Holstein Friesian (HF) and Jersey.
But crossbreed cows are more prone to infectious diseases like foot and mouth disease, mastitis, babesiosis, theileriosis, milk fever and also exhibit increased susceptibility to heat stress and shock.
Third-generation cephalosporins and quinolones were the most frequently administered antibiotics for mastitis. And the most often used antibiotics for metritis were third-generation cephalosporins, tetracycline and quinolones.
The survey findings expressed concerns with respect to the use of antibiotics in dairy cows, revealing significant awareness of the possible risks associated with the misuse of antibiotics in Indian dairy farming. The study reported that concerns could be due to the antibiotics use by failing to adhere to the treatment period and below the recommended dosage are the major two factors promoting to AMR, with the proof of previous research where there was an incident of the community altering in the dosage with their own initiative and discontinuity without considering the treatment period range [35].
There is indication that common mastitis pathogens are evolving into increasingly resistant to antibiotics. It is essential that we analyze the methods and beliefs of the dairy farmers and managers who choose to treat with antibiotics because numerous studies show that mastitis pathogens are becoming resistant to the widely used and accessible antibiotics as well as the spread of resistant bacteria in the dairy industry [36].
Deepthi Vijay et al. (2021) reported that 74.0% of veterinarians reported using alternative treatments such herbal medicines, while 67.2% used probiotics, 43.8% used homeopathic medicine, and 2.4% utilized traditional remedies for various diseases [37].
The World Health Organization reports that at least 80% of people in developing nations depend significantly on traditional methods to prevent and treat several types of diseases that affect both people and their animals. Ethnoveterinary methods are easily accessible, simple to prepare, and administer, and cost the farmer little or nothing. All livestock species and all veterinary specialties are covered by these traditional practices [38].

The World Health Organization implemented a list of highly significant drugs whose use in animals should be restrained in order to sustain their efficacy. The WHO Southeast Asia region's health ministers adopted the "Jaipur Declaration on Antimicrobial Resistance" in 2011. Among other things, they agreed to establish a

comprehensive and integrated national strategy to combat AMR, create multi-sectoral national alliances against AMR, control the use of antimicrobial agents, and enhance capacity for effective AMR surveillance. The National Program on Containment of AMR (2012–2017) was established in 2011 with the goals of strengthening infectious disease control guidelines, encouraging the prudent use of antibiotics, and assisting in the establishment of an AMR surveillance system (with an initial aim of 30 network laboratories).

On the other hand, analysis of the microbiota in milk and curd from native versus crossbred dairy cows revealed notable differences in composition and diversity, emphasizing the influence of breed-specific factors and management practices including antibiotic exposure on beneficial LAB populations like *Lactococcus lactis*.

"Lactic acid bacteria (LAB) comprise a diverse group of Gram-positive, catalase-negative, non-spore-forming cocci or rod-shaped bacteria that are primarily facultative anaerobes."

Lactococcus lactis, one of the most well-known and characterized LAB species, acts as a model organism for studying LAB. L. lactis has long been utilized in milk fermentation, both in controlled industrial settings and on a small scale in traditional operations.

Additionally, L. lactis is a vital microbe in the dairy food fermentation sector since it aids in acidification, flavour development, and the production of various dairy products such as cheese, fermented butter, and so on [39].

Weissella, a genus of Gram-positive, facultatively anaerobic lactic acid bacteria, plays a vital role in human microbiota and has numerous biotechnological and therapeutic applications.

Weissella species produce bioactive metabolites such as exopolysaccharides (EPS), bacteriocins, and organic acids with antibacterial, antioxidant, and anti-inflammatory properties [40].

These bacteria have potential in food fermentation, probiotic applications, and therapeutic interventions for gut health, obesity, and inflammation.

Streptococcus thermophilus is a Gram-positive, homofermentative lactic acid bacteria belonging to the Firmicutes phylum. It is known for its probiotic qualities and contributes to human health.

S. thermophilus stimulates the synthesis of short-chain fatty acids (SCFAs) including butyrate and acetate, which are crucial for maintaining gut barrier integrity and immunological function. Clinical evidence shows its effectiveness in lowering

Antibiotic-associated diarrhoea (AAD) and inflammatory bowel disease symptoms, including ulcerative colitis and Crohn's disease, can be alleviated by restoring microbial balance and lowering intestinal inflammation.

S. thermophilus, a lactic acid bacteria, exhibits metabolic flexibility and functional properties that enable its widespread use in food processing, health enhancement, and

biotechnology.
The reported data says that S. thermophilus produces various bioactive metabolites, including organic acids (lactic, acetic, and formic acids), EPS, aromatic compounds (acetaldehyde and phenylacetic acid), and bacteriocins (thermophilins) [41].

This study shows that LAB starters reduce B. cereus from 10^3-10^4 CFU/ml to 10^2-10^3 CFU/ml during rice fermentation (pH lowers to <4.0 in 8 hours). The study suggests that occasional contamination (less than 10^4 CFU/ml) can be prevented, indicating control but not concluding that final levels are safe for eating [42].

The previous study investigates B. cereus spore survival (0.7 log reduction after steaming), growth, and enterotoxin production in fresh rice noodles stored at 4°C, 22°C, and 32°C. Enterotoxins occur after 12 hours at 32°C or 24 hours at 22°C, with growth above 5 log CFU/g; no safe intake levels are specified; instead, it cautions of dangers after 24 hours of room temperature storage [43].

The findings of a study support previous data that S. uberis is an opportunistic pathogen whose pathogenicity is heavily impacted by environmental exposure, host characteristics, and management approaches. To reduce infection and reinfection, effective control methods should focus enhanced home hygiene, bedding management, teat sanitation, and dry-period interventions, as well as judicious antimicrobial usage [44, 45].

PCR amplification of the milk samples, followed by agarose gel electrophoresis for amplicon visualization, the presence of antimicrobial resistance (AMR) gene *mecA* in both native and crossbred dairy cow samples were seen on the gel.

The extensive use of antibiotics in dairy farming has significant implications for milk microbiota, dairy product quality, and the emergence of antimicrobial resistance (AMR). This study aimed to analyze and compare antibiotic usage patterns, milk and curd microbiota, and the occurrence of antimicrobial resistance genes in native and crossbred dairy cows.

Antibiotic usage data were collected to evaluate the frequency, type, and dosage of antibiotics administered to the dairy cattle. Milk samples were obtained from the native and crossbred dairy cows, and curd was prepared using natural starter culture under standardized conditions. The composition and diversity of milk and curd microbiota were assessed using high-throughput sequencing technique, The Oxford Nanopore sequencer. In addition, physicochemical properties of milk and curd, including pH, moisture content, and texture parameters, were analyzed. The occurrence of antimicrobial resistance genes in milk samples was detected using molecular methods to determine the impact of antibiotic practices on the spread of resistance.

The study revealed notable differences in antibiotic usage across the dairy cows, with the dairy cattle showing higher frequency and dosage of antibiotic administration. Microbial diversity and composition of milk and curd differed between the native and crossbreed dairy cows, indicating the influence of breed type and antibiotic exposure on dairy microbiota. Furthermore, milk from crossbred cows exhibited a higher occurrence of AMR genes, suggesting a potential link between intensive antibiotic use and AMR dissemination.

5.2 Limitation of the study

Although with the broad lens view inculcated in this study, a few limitations have to be acknowledged.
Foremost, the milk sample collection from the dairy farm was very challenging, to convince the cattle farmers to make them understand the purpose and about the study. So, awareness and education about dairy farming to the dairy farmers could serve as the key.
The study did not include the fungal identification of the samples (ITS). The fungal analysis contributes to the complete analysis of the samples and analysis to be carried out to serve the complete view.
The study has covered only 3 native cow breeds and 2 crossbreed cows, which could be extended to more breeds and sample size for better understanding of the microbiome of the samples.
Other AMR gene identification and investigation have to be performed further.
The study focused on the selected cattle breeds and protocols. Differences arising from samples from different animals of same breeds, different farm locations, variability among different breeds was not completely examined.

5.3 Future Directions

- To develop breed specific microbiome profiling to identify beneficial, core microbial taxa consistently associated with native breeds.
- Can use culturing technique to grow probiotic bacteria and isolate it, then could be commercialized into a lyophilized starter culture for curd preparations.
- Establish microbial biomarkers that can be used to assess herd health and antibiotic exposure status.
- Design of Indigenous Probiotic and Starter Cultures, then isolate and characterize native milk and curd associated beneficial microbes
- Ayurveda informed translational research: Scientifically validate traditional dairy practices as microbiome preserving interventions.
- The study can be carried out with more sample size and breed specific microbiome profiling to evaluate in depth.

5.4 Conclusion

The study is expected to conclude that patterns of antibiotic use differ between native and crossbred dairy cows in terms of frequency, type, and dosage, and these practices are closely linked with variations in milk and curd microbiota. Differences in microbial composition and diversity, along with variations in pH, moisture content, and texture, may indicate that native breeds potentially harbour more diverse or beneficial microbiota compared to crossbreds. Furthermore, the detection and comparison of antimicrobial resistance genes in milk samples will likely reveal an association between higher or indiscriminate antibiotic usage and increased occurrence of resistance determinants. Overall, the findings would highlight the importance of prudent antibiotic administration, preservation of breed-specific microbiota, and continuous monitoring of antimicrobial resistance to safeguard animal health, product quality, and public healthcare.

6. References

[1] Bréjon M, Tavares F, Florença SG, Gonçalves JC, Barroca MJ, Guiné RP. Milk: A Nutritive and Healthy Food? Consumer Perspective from French and Portuguese Participants. Applied Sciences. 2024 Apr 24;14(9):3577.

[2] Wakchaure R, Ganguly S, Para PA, Praveen PK, Kumar A, Sharma S. Development of crossbred cattle in India: a review. International Journal of Emerging Technology and Advanced Engineering. 2015;5(10):75-7.

[3] Kumar L, Singh Y, Nainwal K, Bissu P, Kuri R. Major Cross Breeds of Cattle and Their Impact on India's Dairy Productivity. Agri Articles. 2024;4(04):657-660.

[4] Pires AJ, Pereira G, Fangueiro D, Bexiga R, Oliveira M. When the solution becomes the problem: a review on antimicrobial resistance in dairy cattle. Future Microbiology. 2024 Jul 2;19(10):903-29.

[5] Li W, Ren M, Duo L, Li J, Wang S, Sun Y, Li M, Ren W, Hou Q, Yu J, Sun Z. Fermentation characteristics of Lactococcus lactis subsp. lactis isolated from naturally fermented dairy products and screening of potential starter isolates. Frontiers in microbiology. 2020 Aug 4;11:1794.

[6] Sarang SK, Sreekumar D, Sejian V. Indigenous cattle biodiversity in India: Adaptation and conservation. Reproduction and Breeding. 2024 Dec 1;4(4):254-66.

[7] Liu S. The microbiology of milk and its functional significance. J Food Microbiol Saf Hyg. 2023;8(4).

[8]. Kuhnert P, Loosli N, Brodard I, Lindtke D, Jores J. Resistance of zebu cattle (Bos indicus) to colonization by major ruminant hoof pathogens. Veterinary Microbiology. 2024 Sep 1; 296:110184.

[9]. Patbandha TK, Sabapara GP, Savaliya BD, Dash SK, Parikh SS, Ali M. Physical characteristics and production performance of Gir cattle in India. International Journal of Livestock Research. 2020;10(8):1-1.

[10]. Mukesh M, Sodhi M, Bhatia S, Mishra BP. Genetic diversity of Indian native cattle breeds as analysed with 20 microsatellite loci. Journal of Animal Breeding and Genetics. 2004 Dec;121(6):416-24.

[11]. Gajbhiye PU, Gadariya MR, Ahlawat AR, Dongre VB. Present status and future prospects of Gir cow. The Indian Journal of Animal Sciences. 2016 May 1;86(5):553-8.

[12]. Prasanna JS, Rao ST, Prakash MG, Rathod S, Kalyani P, Sharma MR. Production and reproduction performance of sahiwal and HF× sahiwal cows. Indian Journal of Animal Research. 2023;57(6):698-701.

[13]. Singh PK, Pundir RK, Manjunath VK, Rudresh BH, Govindaiah MG. Features and status of miniature indigenous germplasm of cattle-Malnad Gidda. Indian Journal of Animal Sciences. 2008 Oct 1;78(10):1123-26.

[14]. Huson HJ, Sonstegard TS, Godfrey J, Hambrook D, Wolfe C, Wiggans G, Blackburn H, VanTassell CP. A genetic investigation of Island Jersey cattle, the foundation of the Jersey breed: Comparing population structure and selection to Guernsey, Holstein, and United States Jersey cattle. Frontiers in Genetics. 2020 Apr 17;11:366.

[15]. Kőrösi ZJ, Holló G, Bene S, Bognár L, Szabó F. Association of Production and Selected Dimensional Conformation Traits in Holstein Friesian Cows. Animals. 2024 Sep 23;14(18):2753.

[16]. Ojemaye MO, Adefisoye MA, Okoh AI. Nanotechnology as a viable alternative for the removal of antimicrobial resistance determinants from discharged municipal effluents and associated watersheds: A review. Journal of Environmental Management. 2020 Dec 1;275:111234.

[17]. Dey TK, Shome BR, Bandyopadhyay S, Goyal NK, Lundkvist Å, Deka RP, Shome R, Venugopal N, Grace D, Sharma G, Rahman H. Molecular characterization of methicillin-resistant staphylococci from the dairy value chain in two Indian States. Pathogens. 2023 Feb 17;12(2):344.

[18] Dey TK, Shome BR, Bandyopadhyay S, Goyal NK, Lundkvist Å, Deka RP, Shome R, Venugopal N, Grace D, Sharma G, Rahman H. Molecular characterization of methicillin-resistant staphylococci from the dairy value chain in two Indian States. Pathogens. 2023 Feb 17;12(2):344.

[19] Aidara-Kane A, Angulo FJ, Conly JM, Minato Y, Silbergeld EK, McEwen SA, Collignon PJ, WHO Guideline Development Group Balkhy Hanan Collignon Peter Conly John Friedman Cindy Hollis Aidan Kariuki Samuel Kwak Hyo-Sun McEwen Scott Moulin Gérard Ngandjio Antoinette Rollin Bernard Rossi Flávia Wallinga David. World Health Organization (WHO) guidelines on use of medically important antimicrobials in food-producing animals. Antimicrobial Resistance & Infection Control. 2018 Jan 17;7(1):7.

[20] Pol M, Ruegg PL. Relationship between antimicrobial drug usage and antimicrobial susceptibility of gram-positive mastitis pathogens. Journal of dairy science. 2007 Jan 1;90(1):262-73.

[21] Kumar R, Sharma A. Prevalence, etiology and antibiogram of mastitis in cows and buffaloes in Hisar, Haryana. Indian journal of animal sciences. 2002 May 1;72(5):361-3.

[22] Llanos-Soto SG, Vezeau N, Wemette M, Bulut E, Safi AG, Moroni P, Shapiro MA, Ivanek R. Survey of perceptions and attitudes of an international group of veterinarians regarding antibiotic use and resistance on dairy cattle farms. Preventive veterinary medicine. 2021 Mar 1; 188:105253.

[23] Chantziaras I, Boyen F, Callens B, Dewulf J. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. Journal of Antimicrobial Chemotherapy. 2014 Mar 1;69(3):827-34.

[24] Schar D, Sommanustweechai A, Laxminarayan R, Tangcharoensathien V. Surveillance of antimicrobial consumption in animal production sectors of low-and middle-income countries: Optimizing use and addressing antimicrobial resistance. PLoS medicine. 2018 Mar 1;15(3): e1002521.

[25] Taponen S, McGuinness D, Hiitiö H, Simojoki H, Zadoks R, Pyörälä S. Bovine milk microbiome: a more complex issue than expected. Veterinary Research. 2019 Jun 6;50(1):44.

[26] Oikonomou G, Addis MF, Chassard C, Nader-Macias ME, Grant I, Delbès C, Bogni CI, Le Loir Y, Even S. Milk microbiota: what are we exactly talking about? Frontiers in microbiology. 2020 Feb 14; 11:60.

[27] Addis MF, Tanca A, Uzzau S, Oikonomou G, Bicalho RC, Moroni P. The bovine milk microbiota: insights and perspectives from-omics studies. Molecular biosystems. 2016;12(8):2359-72.

[28] Kerek Á, Németh V, Szabó Á, Papp M, Bányai K, Kardos G, Kaszab E, Bali K, Nagy Z, Süth M, Jerzsele Á. Monitoring Changes in the Antimicrobial-Resistance Gene Set (ARG) of Raw Milk and Dairy Products in a Cattle Farm, from Production to Consumption. Veterinary Sciences. 2024 Jun 8;11(6):265.

[29] Catozzi C, Ceciliani F, Lecchi C, Talenti A, Vecchio D, De Carlo E, Grassi C, Sánchez A, Francino O, Cuscó A. Milk microbiota profiling on water buffalo with full-length 16S rRNA using nanopore sequencing. Journal of dairy science. 2020 Mar 1;103(3):2693-700.

[30] Shinozuka Y, Kawai K, Kurumisawa T, Shimizu Y, Imanishi T, Ohno A, Takahashi M, Kaneko S, Suzuki N. Examination of the microbiota of normal cow milk using MinIONTM nanopore sequencing. Journal of Veterinary Medical Science. 2021;83(11):1620-7.

[31] Chandran U, Mehendale NE, Tillu GI, Patwardhan BH. Network pharmacology: an emerging technique for natural product drug discovery and scientific research on ayurveda. InProc Indian Natn Sci Acad 2015 Jun (Vol. 81, No. 3, pp. 561-8).

[32] Shinozuka Y, Kawai K, Kurumisawa T, Shimizu Y, Imanishi T, Ohno A, Takahashi M, Kaneko S, Suzuki N. Examination of the microbiota of normal cow milk using MinIONTM nanopore sequencing. Journal of Veterinary Medical Science. 2021;83(11):1620-7.

[33] Mitsuhashi S, Kryukov K, Nakagawa S, Takeuchi JS, Shiraishi Y, Asano K, Imanishi T. A portable system for rapid bacterial composition analysis using a nanopore-based sequencer and laptop computer. Scientific reports. 2017 Jul 18;7(1):5657.

[34] Macip G, Soler-Comas A, Palomeque A, Motos A, Llonch B, Canseco-Ribas J, Bueno-Freire L, Calabretta D, Kiarostami K, Cabrera R, Ferrer M. Comparative analysis of illumina and oxford nanopore sequencing platforms for 16S rRNA profiling of respiratory microbial communities. Scientific Reports. 2025 Sep 29;15(1):33688.

[35] Hirwa EM, Mujawamariya G, Shimelash N, Shyaka A. Evaluation of cattle farmers' knowledge, attitudes, and practices regarding antimicrobial use and antimicrobial resistance in Rwanda. Plos one. 2024 Apr 11;19(4): e0300742.

[36] Casseri E, Bulut E, Llanos Soto S, Wemette M, Stout A, Greiner Safi A, Lynch R, Moroni P, Ivanek R. Understanding antibiotic resistance as a perceived threat towards dairy cattle through beliefs and practices: a survey-based study of dairy farmers. Antibiotics. 2022 Jul 25;11(8):997.

[37] Vijay D, Bedi JS, Dhaka P, Singh R, Singh J, Arora AK, Gill JP. Knowledge, attitude, and practices (KAP) survey among veterinarians, and risk factors relating to antimicrobial use and treatment failure in dairy herds of India. Antibiotics. 2021 Feb 22;10(2):216.

[38] Balaji NS, Chakravarthi PV. Ethnoveterinary practices in India-a review.

[39] Kondrotiene K, Zavistanaviciute P, Aksomaitiene J, Novoslavskij A, Malakauskas M. Lactococcus lactis in dairy fermentation—Health-promoting and probiotic properties. Fermentation. 2023 Dec 25;10(1):16.

[40] Ma W, Liu X, Jing Y, Zhang M, Zhang X, Wang C, Khan MZ, Zhu M. Weissella: From Beneficial Probiotics to Opportunistic Pathogens—A Review. Nutrients. 2025 Oct 7;17(19):3162.

[41] Ghailan AZ, Niamah AK. Streptococcus thermophilus: metabolic properties, functional features, and useful applications. Applied Microbiology. 2025 Sep 23;5(4):101.

[42] Yang Y, Tao WY, Liu YJ, Zhu F. Inhibition of Bacillus cereus by lactic acid bacteria starter cultures in rice fermentation. Food Control. 2008 Feb 1;19(2):159-61.

[43] Mohammadi B, Reyes ME, Smith SA. Survival, growth, and toxin production of Bacillus cereus during cooking and storage of fresh rice noodles. Journal of Food Protection. 2024 Mar 1;87(3):100239.

[44] Krömker V, Reinecke F, Paduch JH, Grabowski N. Bovine Streptococcus uberis intramammary infections and mastitis. Clinical Microbiology: Open Access. 2014;3.

[45] Di Domenico EG, Toma L, Prignano G, Pelagalli L, Police A, Cavallotti C, Torelli R, Sanguinetti M, Ensoli F. Misidentification of Streptococcus uberis as a human pathogen: a case report and literature review. International Journal of Infectious Diseases. 2015 Apr 1; 33:79-81.

Annexure 1
Survey on antibiotic use in Indian Dairy

The scope of the survey

To generate data on common infectious conditions of dairy cows where most of the antibiotics are used

Common antibiotics in clinical practice

Knowledge attitude and practice towards use and abuse of antibiotics, global status and alternatives to antibiotics.

1. Demographic details and general interpretation of veterinary practitioners

Part-A

Name

_____

2. Age

_____

3. Educational background

Mark only one oval.

BVSc
MVSc
PhD

4. Years of clinical practice
5. Full address

_____
_____
_____
_____
_____

6. Specialization

Mark only one oval.

Gynaecology
Surgery
Oncology
General medicine and pharmacology

Other:

_____

7. Animals of Treatment

Mark only one oval.

Cow
Dog
Goats
Horse
Wildlife

Other:

_____

8. Area of Interest

Mark only one oval.

Infertility
Udder health
Surgery
Preventive health

9. Practicing species

10. Email

11. WhatsApp number

12. State

13. Organization

14. Date

Example: January 7, 2019

15. Place:

16. Signature:

17. Part-B

Top 10 clinical conditions where you use antibiotics. (Practice based) Order the diseases where most antibiotics are used in your practice (1 to 10, where 1 is most 5 is the most often and 10 is least)

Mastitis, Enteritis, Arthritis and hoof disorders, Metritis and other reproductive disorders, Respiratory diseases, Fever with no symptoms, Foot and mouth disease,

Dermatitis, Wounds/Maggot wounds, Lumpy skin disease

18. Common antibiotics used to treat dairy cows in the field. Please list the antibiotics you use in dairy cows your field practice. Preference of order: 1 is the antibiotic you use most 5 is the often, 10 the least. Please indicate brand

name and how it is administered (for injection, intra-mammary tubes, oral etc)

Antibiotics	**Condition for which it is used**

Instruction: Please indicate 10 if you agree, indicate 1 if you disagree 1 2 3 4 5 6 7 8 9 10 agree (for question number 19 to 45)

19. There are concerns related to antibiotic use in dairy cows.
20. There are specific guidelines available to prescribe antibiotics in India
21. I ensure that my antibiotic prescribing practices align with WHO regulations
22. There is a need to educate cattle owners about the responsible use of antibiotics
23. Cattle owners are aware regarding antibiotic use and resistance in India
24. Vets are monitoring the effectiveness of antibiotic treatments in disease management
25. Veterinarians in India are knowledgeable about antimicrobial resistance (AMR) and its implication
26. Responsible use of antibiotics is being addressed in animal health in India
27. Animals can be raised without antibiotics
28. Dairy cattle health practitioners could encourage more responsible use of antibiotics
29. Antibiotics can be recommended as growth promotors.
30. Antibiotics in animal feed places a major role for the development of AMR
31. One should collaborate with specialists when making decisions about antibiotic use
32. There is a difference between reducing use of antibiotics and reducing the need for antibiotics?
33. There can be devastating result from overuse of antibiotics in livestock
34. We must decrease the use of antibiotics in livestock
35. Banning of antimicrobials could have negative effects on animal welfare
36. There is a need to engage with the expert veterinary community to share best practices regarding antibiotic use
37. Ethno-veterinary based recipes can be an alternative to antibiotics
38. There is a need for continuous veterinary education on antibiotic use
39. Farmers should be able to buy antibiotics over counter

40. The dosage and course of the antibiotics are strictly followed by vets in dairy sector in India
41. Minimizing the use of antibiotics is very crucial in containing AMR
42. Antibiotics should only be prescribed by a veterinarian
43. Overuse of antibiotics can lead to AMR
44. Human health is depending on animal and environmental health
45. We can manage many infectious conditions without using antibiotics