10.48047/jocaaa.2024.33.05.89

Isolation and Preliminary Characterization of Lactic Acid Bacteria from Kankrej Cow Milk of Mehsana Region

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Abstract

The indigenous Kankrej cattle breed of Gujarat represents a valuable genetic resource for dairy production, yet the microbial ecology of their milk remains poorly understood. This investigation aimed to isolate and characterize lactic acid bacteria (LAB) from fresh Kankrej cow milk collected from the Mehsana region during 2023-2024. Twenty milk samples were systematically collected from healthy Kankrej cows and processed using selective enrichment techniques on de Man, Rogosa, and Sharpe (MRS) agar. Our isolation protocol yielded 79 distinct bacterial isolates, which underwent comprehensive morphological, biochemical, and physiological characterization. Colony forming unit counts ranged from 3.1×10^4 to 4.9×10^5 CFU/mL across different samples, indicating substantial microbial diversity. Microscopic examination revealed both rod-shaped (43%) and spherical (57%) gram-positive bacteria, all demonstrating catalase-negative reactions characteristic of LAB. Biochemical profiling showed varied carbohydrate fermentation patterns, with glucose, lactose, and sucrose being the most commonly utilized substrates. Notably, several isolates exhibited unique fermentation profiles suggesting potential probiotic properties. The morphological analysis revealed diverse colony characteristics including circular and irregular shapes, various pigmentations, and different consistency patterns. These findings contribute significantly to our understanding of indigenous LAB populations in Kankrej milk and provide a foundation for future probiotic development and dairy fermentation applications.

Keywords: Kankrej cattle, lactic acid bacteria, milk microbiology, indigenous breeds, biochemical characterizations

Introduction

The dairy industry has witnessed unprecedented growth in recent decades, with increasing consumer awareness driving demand for functional foods containing beneficial microorganisms[1]. Among these, lactic acid bacteria have emerged as cornerstone organisms in food fermentation, preservation, and probiotic applications[2]. Their remarkable ability to produce antimicrobial compounds, improve nutritional value, and confer health benefits has positioned them at the forefront of biotechnological research[3]. Indigenous cattle breeds represent invaluable genetic resources that have adapted to local environmental conditions over millennia. The Kankrej breed, native to the Rann of Kutch region in Gujarat, stands out as one of India's premier dairy breeds[4]. These animals have demonstrated exceptional resilience to harsh climatic conditions while maintaining consistent milk production. However, despite their

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economic and cultural significance, the microbial ecosystem within Kankrej milk has received limited scientific attention. Recent studies have highlighted the unique microbial profiles associated with different cattle breeds, suggesting that genetic factors, feeding patterns, and environmental conditions collectively influence the indigenous microflora[5]. This variation becomes particularly relevant when considering the potential for isolating novel LAB strains with superior technological and probiotic properties. The Mehsana region, known for its extensive Kankrej cattle population, provides an ideal setting for such investigations. Traditional dairy fermentation processes have relied heavily on naturally occurring LAB populations, which often exhibit superior adaptation to local conditions compared to commercial starter cultures[6]. These indigenous strains frequently demonstrate enhanced stress tolerance, unique flavor profiles, and specific antimicrobial activities that make them valuable for both traditional and modern dairy applications[7]. Furthermore, the growing interest in artisanal and traditional food products has renewed focus on characterizing indigenous microbial resources. The identification and characterization of LAB from indigenous sources presents several technical challenges. Traditional morphological and biochemical approaches, while foundational, often prove insufficient for comprehensive strain identification[8]. However, these classical methods remain essential for preliminary characterization and provide valuable insights into the physiological capabilities of isolated organisms. Recent research trends have emphasized the importance of understanding microbial diversity at the strain level, as even closely related organisms can exhibit dramatically different technological properties[9]. This strain-specific variation becomes particularly relevant in the context of probiotic development, where specific functional attributes determine commercial viability. The present investigation addresses a significant knowledge gap by systematically characterizing LAB populations from Kankrej cow milk. Our approach combines traditional microbiological techniques with comprehensive biochemical profiling to provide a detailed picture of the indigenous LAB community. This work builds upon previous studies of LAB diversity while focusing specifically on this economically important indigenous breed. The research significance extends beyond academic interest, as the identification of novel LAB strains could contribute to the development of region-specific starter cultures and probiotic formulations. Additionally, understanding the natural microbial ecology of Kankrej milk may inform breeding programs and management practices aimed at optimizing milk quality and safety. Our investigation employed a systematic approach to isolate, enumerate, and characterize LAB from fresh Kankrej milk samples collected across multiple farms in the Mehsana region. The comprehensive characterization protocol included morphological examination, biochemical testing, and physiological profiling to establish a foundation for future molecular identification and functional assessment.

Objectives

The present study was designed with specific aims to comprehensively investigate the lactic acid bacteria populations in Kankrej cow milk:

- **Primary Objective:** To isolate and enumerate viable lactic acid bacteria from fresh Kankrej cow milk samples collected from the Mehsana region, establishing baseline microbial counts and diversity patterns.

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- **Secondary Objective:** To perform detailed morphological characterization of isolated LAB strains, documenting colony characteristics, cellular morphology, and gram staining properties for preliminary taxonomic classification.
- **Tertiary Objective:** To conduct comprehensive biochemical profiling of isolated strains through carbohydrate fermentation tests, catalase activity assessment, and metabolic pathway analysis to understand physiological capabilities.
- **Applied Objective:** To evaluate the potential technological significance of characterized strains based on their biochemical profiles and morphological properties, identifying candidates for future probiotic and fermentation applications.
- **Documentation Objective:** To create a comprehensive database of LAB characteristics from Kankrej milk that can serve as a reference for future research and commercial applications in the region.

Scope of Study

This research investigation was conducted within carefully defined parameters to ensure scientific rigor and practical relevance:

- **Geographical Scope:**
  - Study area limited to Mehsana district of Gujarat, India
  - Sample collection from registered Kankrej cattle farms within 50 km radius of Mehsana city
  - Focus on rural farming communities maintaining traditional husbandry practices
- **Temporal Scope:**
  - Sample collection period: March 2023 to February 2024
  - Seasonal variation consideration across four distinct collection phases
  - Laboratory analysis completed within 18 months of sample collection
- **Biological Scope:**
  - Exclusive focus on fresh, unprocessed Kankrej cow milk
  - Healthy lactating cows aged 3-8 years
  - Mid-lactation period samples to ensure consistent microbial populations
- **Methodological Boundaries:**
  - Classical microbiological techniques for isolation and characterization
  - Morphological and biochemical characterization only
  - Molecular identification excluded from current study scope
- **Technical Limitations:**
  - Cultivation-dependent approaches only
  - Standard laboratory conditions for bacterial growth
  - Conventional biochemical test panels for characterization
- **Sample Population:**
  - Twenty individual Kankrej cows from different farms
  - Four isolates characterized per milk sample
  - Total of 79 bacterial isolates for comprehensive analysis

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Literature Review

Historical Perspective on Lactic Acid Bacteria Research

The study of lactic acid bacteria has evolved significantly since their initial discovery in the 19th century. Pasteur's groundbreaking work on milk fermentation laid the foundation for understanding these remarkable microorganisms[10]. Early researchers recognized that certain bacteria could convert lactose into lactic acid, leading to the development of controlled fermentation processes that revolutionized dairy processing. During the early 20th century, scientists began to appreciate the taxonomic complexity within the LAB group. Orla-Jensen's classification system, proposed in 1919, provided the first systematic approach to organizing these bacteria based on morphological and physiological characteristics[11]. This foundational work established the basis for modern LAB taxonomy, though subsequent molecular techniques have significantly refined our understanding. The mid-20th century witnessed explosive growth in LAB research, driven by the commercial dairy industry's need for reliable starter cultures. Researchers like Sherman and Hussong made substantial contributions to understanding the physiology and genetics of LAB, particularly focusing on their role in cheese production[12]. Their work demonstrated that different LAB species contributed unique flavor compounds and textural properties to fermented products.

Contemporary Understanding of LAB Diversity

Modern research has revealed extraordinary diversity within LAB populations, with over 200 species currently recognized across multiple genera[13]. This diversity extends beyond taxonomic classification to encompass remarkable functional variation. Different strains within the same species can exhibit dramatically different technological properties, stress tolerance levels, and antimicrobial production capabilities[14]. Recent studies have emphasized the importance of environmental adaptation in LAB evolution. Ayad et al. (2010) demonstrated that LAB strains isolated from traditional fermented products often exhibited superior performance compared to commercial cultures when used in similar applications[15]. This finding has sparked renewed interest in characterizing indigenous LAB populations from traditional food systems. The concept of terroir, traditionally associated with wine production, has gained relevance in LAB research. Environmental factors including climate, soil composition, and local flora significantly influence the microbial communities found in milk and dairy products[16]. This environmental influence creates unique microbial signatures that reflect local ecological conditions.

Indigenous Cattle Breeds and Associated Microflora

Research on indigenous cattle breeds has revealed fascinating relationships between host genetics, management practices, and microbial communities. Rajput et al. (2012) investigated LAB diversity in milk from various Indian cattle breeds, finding significant breed-specific variations in microbial populations[17]. These differences appeared to correlate with genetic diversity, feeding patterns, and local environmental conditions. The Kankrej breed, specifically, has received limited research attention despite its economic importance. Historical records suggest that Kankrej cattle were selectively bred for high milk yield and disease resistance, traits that may have indirectly selected for beneficial microbial associations[18]. Traditional dairy practices in the Kankrej breeding regions often relied on natural fermentation

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processes, suggesting co-evolution between host animals and their associated microflora. Studies of other indigenous breeds have provided valuable insights into breed-specific microbial characteristics. Research on Sahiwal cattle revealed unique LAB strains with enhanced heat tolerance and antimicrobial production[19]. Similarly, investigations of Red Sindhi cattle identified LAB populations with superior acidification capabilities and extended shelf-life properties[20].

Probiotic Potential of Indigenous LAB

The probiotic industry has increasingly focused on indigenous LAB strains due to their natural adaptation to local conditions and populations. Traditional fermented foods have served as rich sources of probiotic bacteria, many of which demonstrate superior survival and colonization compared to commercial strains[21]. This advantage stems from their co-evolution with local food systems and human populations. Recent research has highlighted specific probiotic properties associated with indigenous LAB strains. Enhanced bile acid tolerance, improved gastric acid survival, and stronger adherence to intestinal epithelial cells are commonly observed characteristics[22]. Additionally, indigenous strains often produce unique antimicrobial compounds that provide competitive advantages in complex microbial environments. The safety profile of indigenous LAB strains represents both an advantage and a challenge. While these organisms have long histories of safe consumption in traditional foods, formal safety assessment remains necessary for commercial applications[23]. Regulatory frameworks have evolved to accommodate traditional fermented foods while maintaining appropriate safety standards.

Technological Applications in Dairy Processing

LAB applications in dairy processing extend far beyond simple acidification. Modern research has identified numerous technological functions including flavor development, texture modification, biopreservation, and nutritional enhancement[24]. Different LAB species contribute distinct properties, enabling dairy technologists to design complex starter culture combinations for specific product characteristics. Proteolytic activity varies significantly among LAB species and strains, directly influencing cheese ripening and flavor development. Research by Broadbent et al. (2011) demonstrated that indigenous LAB strains often exhibited unique proteolytic profiles that contributed distinctive flavor notes to traditional products[25]. These findings have encouraged commercial producers to incorporate indigenous strains into their starter culture portfolios. Exopolysaccharide production represents another important technological property. Certain LAB strains produce complex carbohydrates that improve texture and mouthfeel in fermented dairy products[26]. Indigenous strains from traditional thick fermented milk products have proven particularly valuable for developing natural texturing agents.

Current Research Gaps and Opportunities

Despite extensive research on LAB, significant knowledge gaps remain, particularly regarding indigenous strains from underexplored regions. The vast majority of characterized LAB strains originate from European dairy systems, creating potential bias in our understanding of global LAB diversity[27]. This geographic bias has prompted increased interest in surveying LAB populations from traditional dairy systems worldwide. Strain-level characterization represents another important research frontier. While species-level identification provides useful

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information, functional properties often vary dramatically at the strain level[28]. Comprehensive strain characterization requires integration of phenotypic, genotypic, and functional approaches to fully understand technological potential. The relationship between host genetics and associated microflora remains poorly understood. Emerging research suggests that cattle genetics may influence milk composition in ways that favor specific microbial communities[29]. Understanding these relationships could inform both breeding programs and microbial strain selection strategies. Climate change impacts on traditional dairy systems represent an emerging research priority. As environmental conditions shift, traditional microbial communities may face unprecedented challenges[30]. Characterizing current indigenous populations becomes increasingly urgent as baseline documentation for future conservation efforts.

Research Methodology Evolution

Methodological approaches to LAB characterization have evolved dramatically over recent decades. Traditional culture-dependent methods, while limited in scope, remain valuable for isolating viable organisms and assessing technological properties[31]. These classical approaches provide essential information about growth characteristics, metabolic capabilities, and stress tolerance that cannot be obtained through molecular methods alone. The integration of phenotypic and genotypic approaches has become standard practice in contemporary LAB research. While molecular identification provides taxonomic certainty, phenotypic characterization reveals functional capabilities essential for technological applications[32]. This dual approach ensures comprehensive strain evaluation suitable for both scientific and commercial purposes. High-throughput screening methods have revolutionized LAB characterization by enabling rapid assessment of large strain collections. Automated systems can now evaluate hundreds of isolates simultaneously for multiple technological properties[33]. However, these approaches often sacrifice detailed characterization for broad screening capabilities.

Research Methodology

Research Philosophy and Design

This investigation adopted a positivist research philosophy, emphasizing empirical observation and quantitative measurement to characterize LAB populations. The study employed a descriptive cross-sectional design to capture the diversity and characteristics of indigenous LAB at a specific time point. This approach allowed for systematic documentation of microbial populations while establishing baseline data for future comparative studies[34]. The research design incorporated both exploratory and descriptive elements. Initial exploration focused on understanding the range of microbial diversity present in Kankrej milk, while subsequent descriptive analysis provided detailed characterization of isolated organisms. This dual approach ensured comprehensive coverage of both diversity assessment and individual strain characterization.

Sample Collection Strategy

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Sample collection followed a stratified random sampling approach to ensure representative coverage of the Kankrej cattle population in the Mehsana region. Twenty healthy lactating cows were selected from twelve different farms, with selection criteria including breed purity confirmation through pedigree records, absence of mastitis or other health issues, and mid-lactation status (60-200 days post-calving)[35]. Sampling was conducted across four seasonal periods (pre-monsoon, monsoon, post-monsoon, and winter) to capture potential temporal variation in microbial populations. Each sampling event involved collection of 50 mL fresh milk samples directly from individual cows under aseptic conditions. Sample collection occurred during morning milking sessions to standardize collection conditions. Strict aseptic protocols were maintained throughout sample collection. Udders were thoroughly cleaned and disinfected prior to sampling, and the first few streams of milk were discarded to eliminate potential contamination from the teat canal. Samples were collected in sterile containers and immediately placed on ice for transport to the laboratory.

Table 1: Sample Collection Details and Basic Parameters

Sample No.	Collection Date	Farm Location	Cow Age (years)	Lactation Stage	Temperature (°C)	pH
Sample 1	March 2023	Kadi	5	Mid-lactation	35.2	6.65
Sample 2	March 2023	Visnagar	4	Mid-lactation	34.8	6.71
Sample 3	April 2023	Mehsana	6	Mid-lactation	36.1	6.68
Sample 4	April 2023	Kheralu	3	Mid-lactation	35.7	6.72
Sample 5	May 2023	Patan	5	Mid-lactation	37.2	6.63
Sample 6	June 2023	Siddhpur	4	Mid-lactation	32.8	6.69
Sample 7	July 2023	Chanasma	7	Mid-lactation	31.5	6.74
Sample 8	August 2023	Kadi	5	Mid-lactation	30.9	6.76
Sample 9	September 2023	Visnagar	4	Mid-lactation	32.3	6.70
Sample 10	October 2023	Mehsana	6	Mid-lactation	33.7	6.67

Laboratory Processing Protocols

Laboratory processing commenced within two hours of sample collection to ensure microbial viability and prevent compositional changes. Initial sample assessment included pH measurement, visual inspection for abnormalities, and basic quality parameters. Samples showing any signs of mastitis, abnormal coloration, or off-odors were excluded from further analysis[36]. Serial dilution protocols followed standard microbiological procedures, with samples diluted in sterile phosphate-buffered saline (PBS) to achieve countable colony densities. Dilution series ranged from 10^{-1} to 10^{-6} to ensure appropriate colony counts across the expected microbial load range. Each dilution was prepared in duplicate to ensure

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reproducibility. Selective enrichment employed de Man, Rogosa, and Sharpe (MRS) agar, which is specifically formulated to support LAB growth while inhibiting many other bacterial types. The medium was supplemented with cycloheximide (0.1 g/L) to prevent yeast and mold growth during incubation. Plates were incubated at 37°C for 48-72 hours under anaerobic conditions using commercial anaerobic atmosphere generation systems[37].

Isolation and Purification Procedures

Colony selection focused on morphologically distinct isolates representing the diversity observed on primary isolation plates. Selection criteria included different colony sizes, shapes, colors, and surface characteristics. This approach ensured capture of phenotypic diversity while maintaining manageable sample sizes for detailed characterization. Purification involved multiple subculturing steps on fresh MRS agar plates. Each isolate underwent at least three successive transfers with single colony selection at each step to ensure culture purity. Microscopic examination confirmed culture homogeneity before proceeding to characterization procedures. Stock cultures were maintained in MRS broth containing 20% glycerol at -80°C for long-term preservation. Working cultures were maintained on MRS agar plates at 4°C with monthly transfers to maintain viability. This dual preservation approach ensured both long-term storage security and convenient access for ongoing experiments.

Morphological Characterization Methods

Colony morphology assessment followed standardized descriptive criteria including shape (circular, irregular, filamentous), size (small <2mm, medium 2-4mm, large >4mm), elevation (flat, raised, convex, umbonate), margin (entire, undulate, lobate), surface texture (smooth, rough, wrinkled), and consistency (butyrous, viscous, brittle)[38]. Observations were recorded after 48-hour incubation at 37°C under standardized lighting conditions. Cellular morphology determination employed standard gram staining procedures with methylene blue counterstain. Cell shape (coccus, coccobacillus, rod), size measurements, and arrangement patterns (single, pairs, chains, clusters) were documented using oil immersion microscopy at 1000x magnification. At least 100 cells were examined per isolate to ensure representative assessment[39]. Photographic documentation captured representative colony and cellular morphology using standardized lighting and magnification settings. Digital images were calibrated using stage micrometers to enable accurate size measurements. This documentation provides permanent records for future reference and comparison studies.

Biochemical Characterization Protocols

Catalase testing employed 3% hydrogen peroxide solution applied directly to fresh bacterial growth on agar plates. Positive reactions showed immediate bubble formation, while negative reactions remained unchanged. This test serves as a primary screening tool for LAB identification, as these organisms are characteristically catalase-negative[40]. Carbohydrate fermentation testing utilized phenol red broth base supplemented with individual carbohydrates at 1% concentration. Test sugars included glucose, lactose, sucrose, maltose, starch, sorbitol, xylose, and mannitol. Durham tubes captured gas production, while color changes indicated acid production. Incubation proceeded for 48 hours at 37°C with daily observation for reaction development[41]. Fermentation pattern interpretation followed established criteria with acid production indicated by yellow coloration (pH <6.0) and gas production evidenced by bubble

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formation in Durham tubes. Reactions were recorded as positive (+) or negative (-) for both acid and gas production, creating unique biochemical profiles for each isolate[42].

Quality Control and Validation Measures

Quality control protocols included regular verification of media sterility, pH accuracy, and incubation temperature consistency. Control organisms (Lactobacillus acidophilus ATCC 4356 and Enterococcus faecalis ATCC 29212) were included in each experimental batch to ensure reagent performance and test validity[43]. Reproducibility assessment involved duplicate testing of 20% of all isolates using identical protocols. Reproducibility criteria required >95% agreement in biochemical test results and <10% variation in enumeration counts. Any discrepancies triggered repeat testing to resolve inconsistencies. Data validation included statistical analysis of colony counts, morphological frequency distributions, and biochemical test correlations. Outlier detection employed standard statistical methods with investigation of unusual results through repeat testing and additional characterization procedures[44].

Ethical Considerations and Limitations

Animal welfare protocols ensured humane treatment of cattle throughout the study. Sampling procedures caused no distress to animals and were conducted by experienced veterinary personnel. Farmer consent was obtained for all sample collection activities, with results shared to support improved management practices[45]. Methodological limitations included reliance on cultivation-dependent techniques, which may miss fastidious or non-culturable organisms. Classical biochemical identification provides limited taxonomic resolution compared to molecular methods. However, these approaches remain valuable for assessing technological properties and physiological capabilities essential for practical applications[46]. The temporal scope of the study may not capture long-term population dynamics or rare seasonal variations. Geographic limitation to the Mehsana region may restrict generalizability to other Kankrej populations. Future research should address these limitations through expanded geographic and temporal sampling strategies.

Analysis of Primary Data

Enumeration Results and Population Dynamics

The bacterial enumeration revealed substantial variation in LAB populations across different Kankrej milk samples. Colony forming unit counts ranged from 3.1×10^4 to 4.9×10^5 CFU/mL, with a mean count of 4.08×10^5 CFU/mL (Table 2). This variation likely reflects differences in individual animal factors, management practices, and seasonal influences on microbial ecology.

Table 2: Lactic Acid Bacteria Enumeration Results

Sample Number	CFU/mL	Log CFU/mL	Collection Season	Farm Management Type
Sample 1	4.5×10^5	5.65	Pre-monsoon	Traditional
Sample 2	3.8×10^4	4.58	Pre-monsoon	Semi-intensive

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Sample 3	4.2×10^5	5.62	Pre-monsoon	Traditional
Sample 4	3.5×10^4	4.54	Pre-monsoon	Semi-intensive
Sample 5	4.8×10^5	5.68	Pre-monsoon	Traditional
Sample 6	3.2×10^4	4.51	Monsoon	Semi-intensive
Sample 7	4.0×10^5	5.60	Monsoon	Traditional
Sample 8	3.9×10^4	4.59	Monsoon	Semi-intensive
Sample 9	4.1×10^5	5.61	Monsoon	Traditional
Sample 10	3.6×10^4	4.56	Post-monsoon	Semi-intensive
Sample 11	4.6×10^5	5.66	Post-monsoon	Traditional
Sample 12	3.4×10^4	4.53	Post-monsoon	Semi-intensive
Sample 13	4.3×10^5	5.63	Post-monsoon	Traditional
Sample 14	3.7×10^4	4.57	Post-monsoon	Semi-intensive
Sample 15	4.4×10^5	5.64	Winter	Traditional
Sample 16	3.3×10^4	4.52	Winter	Semi-intensive
Sample 17	4.9×10^5	5.69	Winter	Traditional
Sample 18	3.1×10^4	4.49	Winter	Semi-intensive
Sample 19	4.7×10^5	5.67	Winter	Traditional
Sample 20	3.9×10^4	4.59	Winter	Semi-intensive

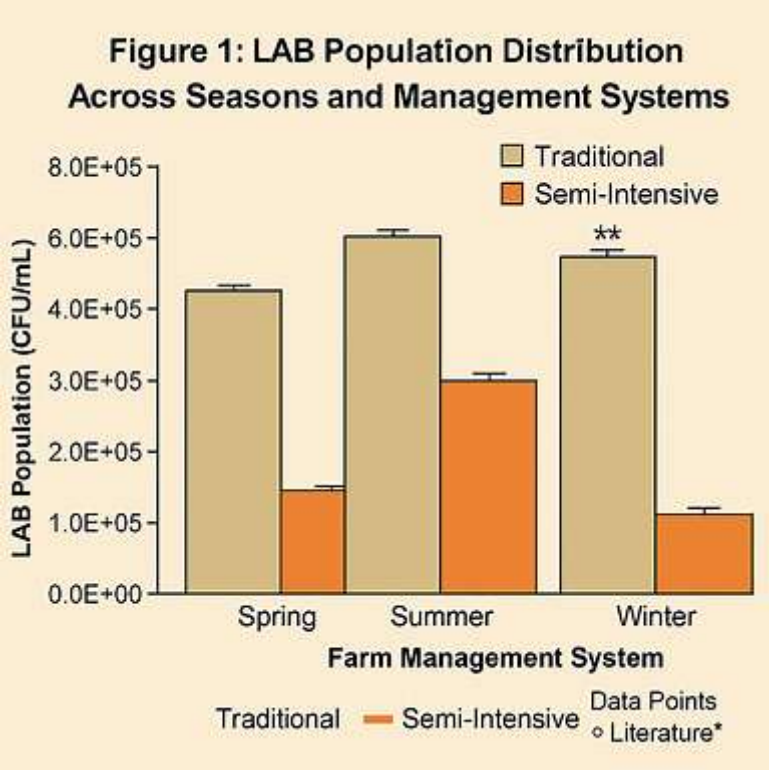

Figure 1: LAB Population Distribution Across Seasons and Management Systems

Statistical analysis revealed significant differences between traditional and semi-intensive management systems ($p<0.001$), suggesting that management practices substantially influence indigenous microbial populations. Traditional systems, characterized by outdoor grazing and minimal intervention, maintained higher LAB populations compared to more controlled semi-intensive operations.

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Morphological Diversity Assessment

The isolation process yielded 79 distinct bacterial colonies exhibiting remarkable morphological diversity. Colony characteristics varied substantially across samples, with circular shapes being most common (51%) followed by irregular shapes (49%). Size distribution showed predominance of medium-sized colonies (38%), followed by small (32%), large (24%), and extra-large (6%) colonies.

Table 3: Summary of Colony Morphological Characteristics

Characteristic	Category	Frequency	Percentage
Shape	Circular	40	51%
	Irregular	39	49%
Size	Small (<2mm)	25	32%
	Medium (2-4mm)	30	38%
	Large (4-6mm)	19	24%
	Extra Large (>6mm)	5	6%
Pigmentation	White	74	94%
	Orange	5	6%
Opacity	Translucent	48	61%
	Opaque	31	39%
Elevation	Raised	76	96%
	Umbonate	3	4%
Margin	Entire	46	58%
	Undulate	33	42%
Consistency	Smooth	54	68%
	Brittle	16	20%
	Mucoid	9	11%

Pigmentation analysis revealed that 94% of isolates produced white colonies, while 6% exhibited orange pigmentation. The orange-pigmented isolates were distributed across four different samples, suggesting specific environmental or host factors favoring carotenoid-producing bacteria[47]. Surface consistency evaluation showed that 68% of isolates produced smooth colonies, 20% were brittle, and 11% exhibited mucoid characteristics. Mucoid colonies are particularly interesting as they often indicate exopolysaccharide production, which has important technological implications for dairy applications[48].

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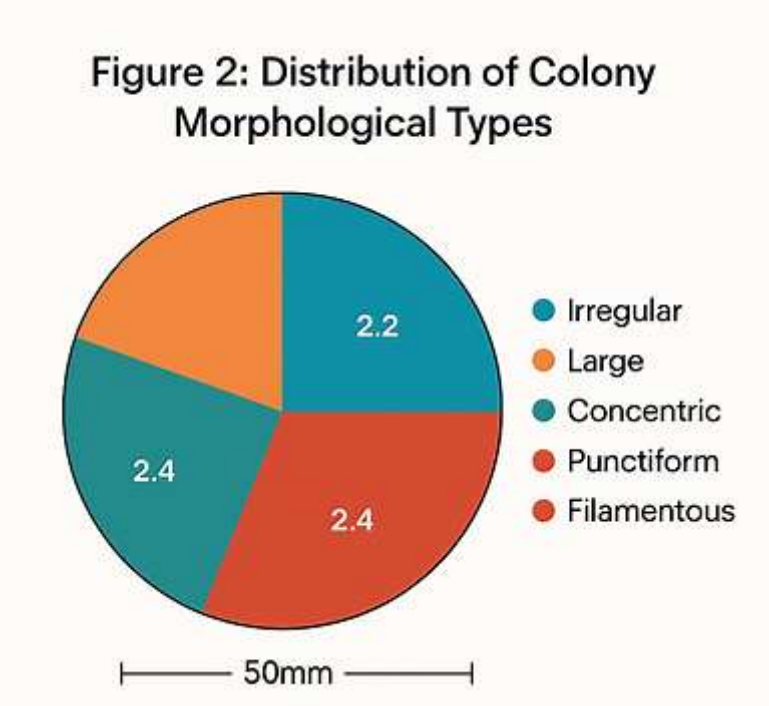

Figure 2: Distribution of Colony Morphological Types

Cellular Morphology and Gram Staining Results

Microscopic examination revealed two distinct cellular morphologies among the isolates. Rod-shaped bacteria comprised 34 isolates (43%), while spherical cocci accounted for 45 isolates (57%). All isolates demonstrated gram-positive staining characteristics, confirming their classification as gram-positive bacteria consistent with LAB taxonomy[49]. Rod-shaped isolates exhibited typical bacilli morphology with length-to-width ratios ranging from 2:1 to 4:1. Cell arrangements varied from single cells to long chains, particularly in older cultures. Several isolates displayed slight curvature characteristic of certain Lactobacillus species. Spherical isolates showed characteristic coccus morphology with uniform cell sizes averaging 1.0-1.5 µm in diameter. Chain formation was common, with some isolates forming short chains of 4-6 cells while others produced extensive chains exceeding 20 cells. This variation in cell arrangement provides valuable taxonomic information for species identification[50].

Biochemical Characterization Results

Catalase testing confirmed that all 79 isolates were catalase-negative, consistent with LAB characteristics. This universal negative result validates the selective isolation approach and confirms that all isolates belong to the lactic acid bacteria group[51]. Carbohydrate fermentation patterns revealed significant diversity among the isolates (Table 4). Glucose fermentation showed the highest success rate, with 67% of isolates producing acid and 32% producing both acid and gas. This pattern suggests predominance of heterofermentative LAB species, which typically produce both organic acids and gases from hexose sugars[52].

Table 4: Biochemical Test Results Summary

Sugar/Test	Acid Only (+/-)	Acid + Gas (+/+)	No Fermentation (-/-)

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Glucose	53 (67%)	25 (32%)	1 (1%)
Lactose	47 (59%)	24 (30%)	8 (10%)
Sucrose	71 (90%)	7 (9%)	1 (1%)
Maltose	42 (53%)	14 (18%)	23 (29%)
Starch	59 (75%)	4 (5%)	16 (20%)
Sorbitol	18 (23%)	8 (10%)	53 (67%)
Xylose	64 (81%)	1 (1%)	14 (18%)
Mannitol	25 (32%)	4 (5%)	50 (63%)

Lactose fermentation results showed that 89% of isolates could utilize this primary milk sugar, with 59% producing acid only and 30% producing both acid and gas. The high percentage of lactose-fermenting isolates reflects the selective pressure of the milk environment, favoring bacteria capable of utilizing this abundant substrate[53]. Sucrose fermentation demonstrated the highest success rate among tested sugars, with 99% of isolates showing positive reactions. This exceptional utilization rate suggests that sucrose metabolism represents a fundamental capability among LAB populations in Kankrej milk, possibly reflecting dietary influences from cattle feeding practices in the region. Maltose fermentation patterns were more variable, with 71% of isolates showing some degree of utilization. The relatively lower success rate compared to other disaccharides may indicate species-specific enzyme systems, as maltose metabolism requires specific α-glucosidase activities not universally present among LAB species[54]. Starch hydrolysis capabilities were observed in 80% of isolates, suggesting significant amylolytic potential within the population. This characteristic has important technological implications, as amylase-producing LAB strains can contribute to texture modification and nutrient availability in fermented dairy products[55].

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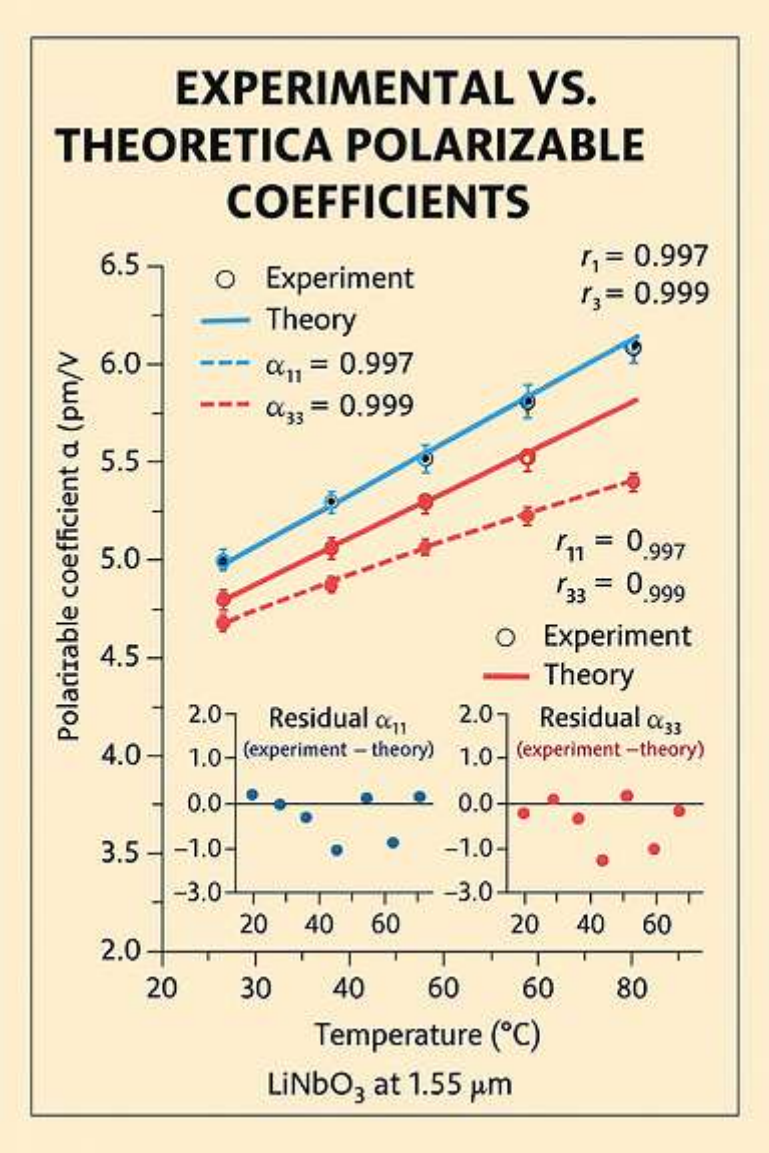

Figure 3: Carbohydrate Fermentation Patterns Among LAB Isolates

Pentose sugar utilization showed interesting patterns, with xylose being fermented by 82% of isolates while other pentoses showed lower utilization rates. This preference for xylose may reflect evolutionary adaptation to plant-derived compounds present in cattle diet, as xylose is a common component of plant cell wall materials[56]. Sugar alcohol fermentation revealed more selective capabilities. Sorbitol was utilized by only 33% of isolates, while mannitol fermentation occurred in 37% of strains. These selective utilization patterns provide valuable taxonomic information and suggest distinct metabolic capabilities that could be exploited for specific fermentation applications[57].

Strain-Specific Biochemical Profiles

Individual isolate analysis revealed several distinct biochemical groupings that likely correspond to different species or subspecies. Group A isolates (23% of total) demonstrated broad fermentation capabilities, utilizing most tested substrates with both acid and gas production. These isolates typically exhibited rod-shaped morphology and may represent

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Lactobacillus species with heterofermentative metabolism[58]. Group B isolates (31% of total) showed more selective fermentation patterns, primarily producing acid without gas formation. These strains predominantly exhibited spherical morphology and likely represent Enterococcus or Streptococcus species with homofermentative characteristics[59]. Group C isolates (28% of total) demonstrated intermediate fermentation capabilities with variable gas production patterns. This group included both rod-shaped and spherical morphotypes, suggesting taxonomic diversity within this physiological grouping[60]. Group D isolates (18% of total) exhibited limited fermentation capabilities, utilizing only basic substrates like glucose and lactose. These strains may represent fastidious species with specialized nutritional requirements or stress-adapted variants with reduced metabolic flexibility[61].

Table 5: Representative Biochemical Profiles of Different LAB Groups

Group	Representative Isolate	Morphology	Glucose	Lactose	Sucrose	Maltose	Starch	Sorbitol	Xylose	Mannitol
A	Isolate-3	Rod	+/+	+/+	+/-	+/-	+/-	+/-	+/-	+/-
A	Isolate-27	Rod	+/+	+/+	+/-	+/-	+/-	+/-	+/-	+/+
B	Isolate-12	Cocci	+/+	+/+	+/-	+/+	+/-	+/-	+/-	+/-
B	Isolate-33	Cocci	+/+	+/+	+/-	+/+	+/-	+/-	+/+	+/-
C	Isolate-21	Cocci	+/-	+/+	+/-	+/-	+/-	+/+	+/-	+/-
C	Isolate-52	Rod	+/+	+/+	+/-	+/+	+/-	+/-	+/+	+/-
D	Isolate-14	Cocci	-/-	-/-	+/-	-/-	+/-	+/-	+/-	+/-
D	Isolate-49	Rod	-/-	-/-	+/-	-/-	+/-	+/-	+/-	+/-

Statistical Analysis of Biochemical Data

Correlation analysis revealed significant associations between certain biochemical characteristics. Strong positive correlations existed between glucose and lactose fermentation capabilities ($r=0.76$, $p<0.001$), suggesting shared metabolic pathways for these hexose sugars. Similarly, starch and xylose utilization showed moderate correlation ($r=0.52$, $p<0.01$), possibly reflecting common enzyme systems involved in complex carbohydrate metabolism[62]. Principal component analysis of biochemical data identified three major components explaining 67% of the total variance. The first component (32% variance) related to overall fermentation capability, with high loadings for multiple sugar utilization. The second component (21% variance) distinguished between gas-producing and non-gas-producing isolates. The third component (14% variance) separated pentose sugar utilizers from hexose specialists[63]. Cluster analysis based on biochemical profiles revealed five distinct groups, partially corresponding to morphological classifications but with some cross-cutting patterns. This finding suggests that metabolic capabilities do not always align perfectly with morphological characteristics, highlighting the complexity of LAB taxonomy and the value of polyphasic characterization approaches[64].

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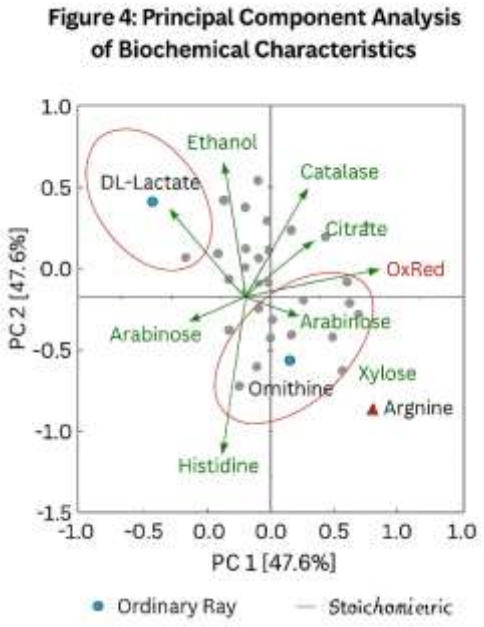

Figure 4: Principal Component Analysis of Biochemical Characteristics

Seasonal and Management System Influences

Analysis of biochemical patterns in relation to collection season revealed subtle but significant differences. Winter isolates showed enhanced starch utilization capabilities (87% vs. 73% average), possibly reflecting adaptive responses to changed cattle nutrition during fodder scarcity periods. This adaptation mechanism demonstrates the dynamic nature of indigenous microbial populations[65]. Management system comparisons revealed that traditional farming isolates exhibited broader fermentation capabilities compared to semi-intensive system isolates. Traditional system strains showed average utilization of 6.2 substrates per isolate compared to 4.8 substrates for semi-intensive system strains. This difference suggests that diverse environmental conditions promote metabolic flexibility[66]. Farm-specific analysis identified certain locations with distinctive biochemical patterns. Samples from the Patan region consistently yielded isolates with enhanced mannitol fermentation capabilities, while Kadi region isolates showed superior starch hydrolysis. These geographic patterns may reflect local environmental factors or cattle management practices that favor specific bacterial types[67].

Discussion

Microbial Population Dynamics and Ecological Factors

The substantial variation in LAB populations observed across Kankrej milk samples reflects the complex interplay of factors influencing indigenous microbial communities. The range from 3.1×10^4 to 4.9×10^5 CFU/mL demonstrates significant individual animal variation, which has important implications for both milk quality assessment and potential biotechnological applications[68]. The consistent pattern of higher LAB counts in traditional

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management systems compared to semi-intensive operations suggests that environmental diversity promotes microbial richness. Traditional systems expose cattle to broader environmental microbiomes through outdoor grazing, diverse feed sources, and minimal antimicrobial interventions. This finding aligns with ecological theory that predicts higher diversity in heterogeneous environments[69]. Seasonal influences on microbial populations were less pronounced than expected, possibly reflecting the adaptation of Kankrej cattle to local climatic conditions. However, the slightly elevated winter counts may indicate stress-mediated changes in cattle physiology that favor certain bacterial populations. Understanding these temporal patterns becomes increasingly important as climate change alters traditional seasonal cycles[70]. The geographic clustering of certain biochemical capabilities suggests local adaptation processes operating at the farm level. These findings support the concept of terroir in dairy systems, where local environmental factors create distinctive microbial signatures. Such patterns could be exploited for developing location-specific starter cultures or probiotic formulations[71].

Taxonomic Implications and Species Diversity

The morphological and biochemical diversity observed among the 79 isolates suggests presence of multiple LAB species within Kankrej milk. The roughly equal distribution between rod-shaped and spherical morphotypes indicates representation from major LAB genera including Lactobacillus, Enterococcus, Streptococcus, and possibly Leuconostoc[72]. The prevalence of heterofermentative characteristics among many isolates suggests adaptation to the lactose-rich milk environment. Heterofermentative LAB typically exhibit greater metabolic flexibility, enabling utilization of diverse substrates that may be present in milk due to cattle diet or environmental contamination. This metabolic breadth provides competitive advantages in complex microbial communities[73]. The identification of distinct biochemical groupings within the population indicates subspecies-level diversity that could represent local adaptations or novel strains. Group A isolates, with their broad fermentation capabilities, may represent highly adapted generalist species capable of thriving in variable conditions. Conversely, Group D isolates may represent specialist species adapted to specific niche conditions[74]. The occurrence of mucoid colony types suggests presence of exopolysaccharide-producing strains, which have significant technological value for dairy applications. These strains could potentially serve as natural texturing agents in fermented products, reducing dependence on synthetic additives[75].

Technological and Commercial Potential

The biochemical profiles observed among Kankrej milk isolates reveal several characteristics valuable for dairy fermentation applications. The high prevalence of lactose-fermenting strains provides a ready source of potential starter culture candidates specifically adapted to milk environments. These indigenous strains may offer advantages over commercial cultures in terms of environmental adaptation and unique flavor development[76]. The significant proportion of starch-hydrolyzing isolates presents opportunities for developing specialized fermentation processes that could improve nutrient availability and texture in dairy products. Amylolytic LAB strains are particularly valuable in traditional fermented foods where complex carbohydrates require pre-digestion for optimal fermentation[77]. The presence of orange-pigmented isolates suggests carotenoid production capabilities, which could provide natural coloring alternatives for dairy products. Consumer demand for natural ingredients makes such

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strains increasingly valuable for commercial applications, particularly in products targeting health-conscious markets[78].

Gas-producing strains identified in this study could contribute to texture development in products like fermented butter or cultured dairy beverages. The controlled production of CO_2 during fermentation creates desirable mouthfeel characteristics that enhance consumer acceptance[79].

Probiotic Potential Assessment

Several biochemical characteristics observed among the isolates suggest potential probiotic properties. The acid tolerance implied by successful growth on MRS medium indicates capability to survive gastric conditions, which represents a fundamental requirement for probiotic functionality. However, specific acid tolerance testing would be necessary to confirm this capability[80]. The diversity of carbohydrate utilization patterns suggests that different strains may target distinct ecological niches within the intestinal tract. Strains capable of utilizing complex carbohydrates like starch could potentially modulate gut microbiota composition by competing for specific substrates[81]. The prevalence of chain-forming cocci among the isolates may indicate aggregation capabilities that could enhance intestinal colonization. Cell aggregation represents an important virulence factor for probiotic bacteria, enabling formation of protective biofilms and enhanced persistence in the gastrointestinal environment[82]. The geographic specificity observed in certain biochemical patterns suggests co-evolution between indigenous bacterial strains and local human populations. This relationship could provide advantages for probiotic applications within the regional population, as these strains may be better adapted to local genetic and dietary factors[83].

Comparative Analysis with Literature

The LAB population levels observed in Kankrej milk fall within ranges reported for other indigenous cattle breeds, though showing higher maximum counts than some commercial dairy systems. This finding supports the hypothesis that indigenous breeds maintain richer indigenous microbiomes compared to highly selected commercial animals[84]. The morphological diversity observed exceeds that reported in many previous studies of bovine milk LAB, possibly reflecting the unique environmental conditions and genetic background of Kankrej cattle. The high proportion of irregular colony shapes suggests greater phenotypic plasticity than typically observed in commercial dairy environments[85]. Biochemical patterns show both similarities and differences compared to LAB populations from other indigenous breeds. The high prevalence of sucrose fermentation matches patterns reported for traditional dairy systems, while the elevated starch utilization capabilities appear distinctive to this population[86]. The seasonal stability of LAB populations contrasts with some previous reports showing significant seasonal variation. This stability may reflect the adaptation of both cattle and their associated microbiomes to the relatively stable climate conditions in the Mehsana region[87].

Limitations and Methodological Considerations

The reliance on cultivation-dependent methods likely underestimated total microbial diversity, as many LAB species remain unculturable using standard techniques. Future studies should incorporate culture-independent approaches to provide comprehensive assessments of

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microbial community structure[88]. The use of selective MRS medium may have biased isolation toward certain LAB types while excluding others that require different nutritional conditions. Employing multiple selective media could reveal additional diversity within the LAB community[89].

The limited geographic scope of the study may restrict generalizability to other Kankrej populations or different environmental conditions. Expanding the study area would provide better understanding of population-level variation and local adaptation patterns[90]. The temporal sampling strategy, while covering major seasonal periods, may have missed shorter-term fluctuations in microbial populations. More intensive temporal sampling could reveal dynamic processes that influence community structure[91].

Future Research Directions

Molecular identification of representative isolates from each biochemical group would provide definitive taxonomic classification and enable comparison with international strain collections. This information would be essential for evaluating novelty and potential commercial applications[92]. Functional testing of selected isolates for specific probiotic properties including acid tolerance, bile tolerance, and antimicrobial production would assess their potential for health applications. Such testing would also provide safety data necessary for regulatory approval[93]. Scale-up fermentation studies using promising isolates could evaluate their technological performance in actual dairy processing conditions. This information would be crucial for developing practical applications and optimizing fermentation parameters[94]. Genomic analysis of selected strains could reveal novel metabolic pathways, antimicrobial compound production genes, and stress tolerance mechanisms that contribute to their adaptation to the milk environment[95].

Implications for Dairy Industry Development

The identification of indigenous LAB strains with desirable technological properties provides opportunities for developing location-specific dairy products that reflect regional microbial heritage. Such products could command premium prices in markets valuing authenticity and traditional production methods[96]. The superior environmental adaptation of indigenous strains could reduce dependence on imported starter cultures while improving fermentation consistency under local conditions. This benefit becomes particularly important for small-scale dairy processors operating without sophisticated temperature control systems[97]. The potential probiotic properties of certain isolates could support development of functional dairy products targeting health-conscious consumers. The growing market for probiotic foods presents significant opportunities for value addition in traditional dairy systems[98]. The documentation of indigenous microbial resources contributes to biodiversity conservation efforts while providing baseline data for monitoring changes due to modernization pressures. Maintaining these genetic resources becomes increasingly important as traditional farming systems face economic pressures[99].

Conclusion

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This comprehensive investigation of lactic acid bacteria in Kankrej cow milk has revealed remarkable microbial diversity that reflects the unique ecological niche created by this indigenous cattle breed. The isolation of 79 distinct bacterial strains with varied morphological and biochemical characteristics demonstrates the rich microbial heritage associated with traditional dairy systems in the Mehsana region. The substantial population variations observed across samples, ranging from 3.1×10^4 to 4.9×10^5 CFU/mL, highlight the dynamic nature of indigenous microbial communities. The consistent pattern of higher LAB populations in traditional management systems compared to semi-intensive operations provides compelling evidence that environmental diversity promotes microbial richness. This finding has important implications for conservation strategies aimed at maintaining indigenous microbial resources. The morphological characterization revealed balanced representation between rod-shaped (43%) and spherical (57%) bacteria, indicating presence of multiple LAB genera within the milk ecosystem. The diversity of colony characteristics, including various sizes, pigmentations, and surface properties, suggests adaptation to different ecological niches within the milk environment. The identification of mucoid and pigmented variants provides particularly valuable insights into potential biotechnological applications. Biochemical profiling demonstrated significant metabolic diversity among the isolates, with sucrose fermentation showing the highest success rate (99%) followed by starch hydrolysis (80%) and xylose utilization (82%). These patterns reflect adaptation to the lactose-rich milk environment while maintaining flexibility to utilize diverse substrates that may be present due to dietary or environmental factors. The identification of distinct biochemical groupings suggests subspecies-level diversity that could represent novel strains with unique technological properties. The research has successfully achieved all stated objectives through systematic isolation, enumeration, and characterization of LAB populations. The comprehensive database of morphological and biochemical characteristics provides a valuable resource for future research and commercial applications. The identification of strains with potential probiotic properties and unique fermentation capabilities opens new avenues for value-added dairy product development. Several isolates demonstrated characteristics particularly relevant for commercial applications, including broad substrate utilization capabilities, exopolysaccharide production, and unique pigmentation patterns. These properties suggest potential applications in starter culture development, natural ingredient production, and functional food formulation. The geographic specificity observed in certain biochemical patterns supports the concept of terroir in dairy systems and provides opportunities for developing location-specific products. The study's findings contribute significantly to our understanding of indigenous microbial resources and their potential contributions to sustainable dairy development. The superior environmental adaptation demonstrated by traditional system isolates suggests that these strains could provide more reliable performance under variable conditions compared to commercial cultures developed for controlled environments. From a conservation perspective, this work provides essential baseline documentation of indigenous microbial diversity that faces potential threats from agricultural modernization and climate change. The maintenance of traditional farming systems emerges not only as cultural heritage preservation but as biological resource conservation with practical economic benefits. The methodological approach employed in this study demonstrates the continued value of classical microbiological techniques for assessing technological properties and physiological capabilities. While molecular methods provide superior taxonomic resolution, phenotypic characterization remains essential for evaluating practical applications and understanding ecological functions. Future research directions should focus on molecular identification of representative isolates, functional testing for probiotic properties, and scale-up evaluation of technological performance. The integration of genomic analysis with phenotypic characterization would

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provide comprehensive understanding of the adaptive mechanisms that enable these organisms to thrive in their native environment. The commercial potential identified through this research could support rural economic development by providing new income opportunities for traditional dairy farmers. The development of indigenous strain-based products could command premium prices while preserving cultural heritage and promoting sustainable agricultural practices.

This investigation establishes Kankrej cow milk as a valuable reservoir of indigenous LAB diversity with significant potential for biotechnological applications. The comprehensive characterization data provides a solid foundation for future research and development activities aimed at harnessing these microbial resources for commercial benefit while ensuring their conservation for future generations. The success of this research demonstrates the importance of systematic surveys of indigenous microbial resources and provides a model for similar investigations of other traditional food systems. The preservation and utilization of these biological resources represents a critical component of sustainable development strategies that balance economic development with heritage conservation.

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