https://doi.org/10.17221/146/202-CJAS

Endogenous inhibitory compounds in bovine milk, their significance and methods of detection

Marcela Klimesova[1]*, *Ludmila Krizova*[2], *Hana Nejeschlebova*[1], *Oto Hanus*[1], *Lenka Vorlova*[3]

[1]*Dairy Research Institute, Prague, Czech Republic*
[2]*Department of Biochemistry, Faculty of Science, Masaryk University, Brno, Czech Republic*
[3]*Department of Animal Origin Food and Gastronomic Sciences, Faculty of Veterinary Hygiene and Ecology, University of Veterinary Sciences Brno, Brno, Czech Republic*

**Corresponding author: marcela.vyletelova@seznam.cz*

Citation: Klimesova M., Krizova L., Nejeschlebova H., Hanus O., Vorlova L. (2025): Endogenous inhibitory compounds in bovine milk, their significance and methods of detection. Czech J. Anim. Sci., 70: 499–517.

Abstract: Raw cow's milk is one of the most strictly controlled commodities in the food industry. Besides basic hygienic and nutritional parameters, the presence of inhibitory and contaminant substances plays a critical role in technological quality and safety. While the vast majority of inhibitory residues are represented by antibiotics originating from mastitis prevention and therapy, milk also contains a broad spectrum of naturally occurring native antimicrobial compounds. These include immunoglobulins, lactoferrin, lysozyme, lactoperoxidase, phosphatases, lipases, and proteases acting as integral elements of the innate immune system within the mammary gland. Although these substances are not legislatively regulated, they significantly influence milk processing, starter culture growth, cheese yield, and shelf-life of final dairy products. Their concentration is strongly associated with animal health status, especially mastitis, and may thus also serve as an indirect quality indicator. This review provides a comprehensive overview of native inhibitory compounds in bovine milk, their concentration ranges, biological significance, impact on dairy technology, and the analytical methods currently used for their detection.

Keywords: immunoglobulins; lactoferrin; lactoperoxidase; lipase; lysozyme; phosphatase; protease; raw milk

INTRODUCTION

Cow's milk is the main raw material for dairy production in the Czech Republic, while sheep's and goat's milk make up a minor share. It is one of the most strictly monitored commodities in the food industry. For use in dairy processing, it must comply with strict hygienic and nutritional standards, including established threshold values for total bacterial count, somatic cell count (SCC), and basic compositional parameters (fat, protein, lactose), as well as the absence of undesirable inhibitory

Supported by the National Agency for Agricultural Research of the Czech Republic (Project No. QK 21010212 and Grant No. MZe RO1425).

https://doi.org/10.17221/146/2025-CJAS

and contaminant substances. These requirements necessitate the application of analytical methods offering maximum precision, sensitivity, and reliability to ensure accurate detection and monitoring of all key quality parameters (Bogdanovicova et al. 2016; Visciano and Schirone 2022; Zahumenska et al. 2024).

Inhibitory substances are defined as agents with bactericidal or bacteriostatic activity that negatively influence the growth of dairy starter cultures during the processing of raw milk into dairy products (e.g. curds, yogurts, cheeses). Most of these substances are antibiotic residues, which enter milk prior to milking as part of mastitis prevention and treatment.

Antibiotics represent the greatest technological risks and account for more than 95% of all residues of inhibitory substances (Costa et al. 2024; Navratilova et al. 2024). They are classified as exogenous (non-natural) inhibitory substances, together with compounds such as bacterial enzymes, disinfectants and cleaning agents, and others. Another important category is made up of contaminant substances which are primarily associated with health risks for consumers. These include, for example, hormones, heavy metals, mycotoxins, pesticides, preservatives and antioxidants, microplastics, and other contaminant compounds.

A specific group of inhibitory substances is formed by endogenous inhibitory substances, naturally occurring antimicrobial compounds that originate within the mammary gland and function as part of its innate immune defense. These include immunoglobulins, lysozyme (LYZ), lactoferrin (LF), lactoperoxidase (LPO), alkaline and acid phosphatase (ALP and ACP), endogenous proteases and lipases, and other bacteriostatic agents. These naturally occurring substances are not subject to legislative control, since most of them lose their inhibitory activity during milk pasteurisation. Importantly, these natural inhibitors also have beneficial biological roles, supporting immunity and neonatal protection, which highlights their dual significance in milk (Navratilova 2002; Graf et al. 2021). The potential sources of endogenous and exogenous inhibitors and contaminant substances are presented in Figure 1.

This review focuses on selected endogenous inhibitory substances, their content in raw cow's milk, their impact on dairy production and consumer health, and the analytical methods available for their detection.

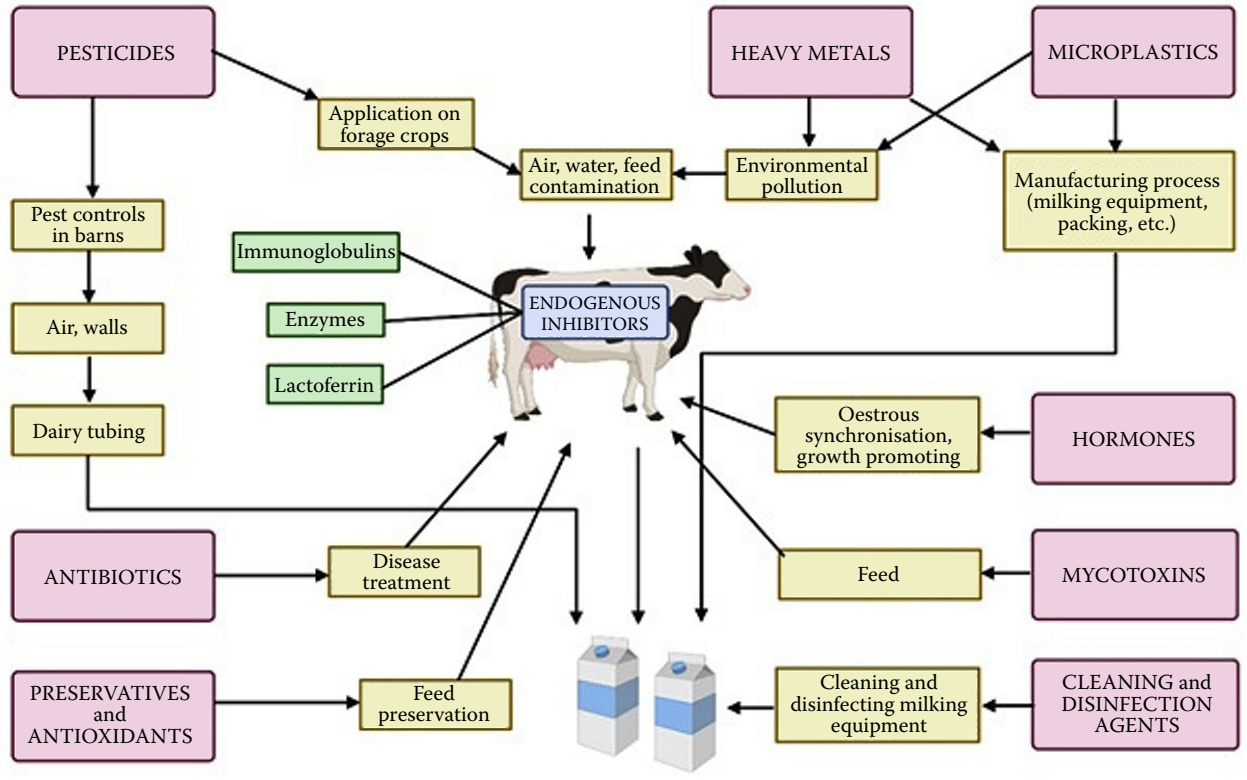

Figure 1. Sources of endogenous and exogenous inhibitors and contaminant substances (Created in BioRender)

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ENDOGENOUS INHIBITORS

Immunoglobulins

Characterisation. Ig are glycoproteins with antibody activity that are present in bovine milk. The predominant immunoglobulin IgG1 is transferred from the maternal bloodstream into colostrum and milk, whereas smaller amounts of IgA and IgM may also be produced locally in the mammary gland (Mehra et al. 2006; Godden et al. 2019). The high content of IgG1 in colostrum is essential for passive immunity in neonatal ruminants, as the bovine placenta does not allow transplacental transfer of maternal antibodies during pregnancy (Godden et al. 2019; Altvater-Hughes et al. 2023).

Occurrence in milk. Ig enter milk primarily by transfer from the bloodstream, and their concentration increases when the blood–milk barrier becomes more permeable, such as during mastitis or other inflammatory conditions (Mehra et al. 2006; Godden et al. 2019). Their occurrence is highest in colostrum, where they provide protection against neonatal diseases including diarrhoea, bronchopneumonia, umbilical disorders and septicaemia (Yalcintas et al. 2024). In healthy milk, Ig concentrations typically range from 0.15 g/l to 0.8 g/l (Mehra et al. 2006). In mastitic milk, Ig levels may increase substantially and, depending on severity, may reach up to 18.3 g/l (Navratilova et al. 2012; Table 1). During mastitis, IgG1 and IgG2 increase first as serum-derived components, whereas IgM and IgA rise later as part of the local immune response in the mammary gland (Gajdusek 1996).

Role in dairy processing. Although the increase in Ig during mastitis reflects a protective immune response, elevated Ig levels can adversely affect milk processing. High Ig concentrations are associated with increased SCC and reduced casein content, resulting in impaired milk coagulation and decreased cheese yield (Kitchen 1981; Gajdusek 1996; Piccinini et al. 2005). Some Ig fractions also contain lactenins, which exert agglutinating effects on certain starter cultures, potentially leading to defects in cheese manufacture (Holec 1994). IgG is relatively heat-stable, with minimal denaturation during pasteurisation at 63 °C for 30 min or 72 °C for 15 seconds. However, Ig are sensitive to acidic pH and higher temperatures; complete denaturation occurs at 80 °C within 60 min and at 100 °C within 10 min (Navratilova et al. 2012; Table 1).

Analytical methods. Determination of Ig in milk and colostrum is commonly performed using immunochemical techniques such as radial immunodiffusion (RID) and enzyme-linked immunosorbent assay (ELISA) (Skalka et al. 2014). Other analytical methods include chromatographic separation, nephelometry (NEP), and various electrophoretic methods (Copestake et al. 2006; Gapper et al. 2007; Abernethy et al. 2010). Advanced analytical techniques employ biosensors based on surface plasmon

Table 1. Content of immunoglobulins in bovine milk and their associated technological or physiological impacts

Milk type/ condition	Reported level/ activity	Technological/ physiological impact	References
Colostrum	IgG1 dominant; typically 50–100 g/l	provides passive immunity to calves	Godden et al. (2019); Altvater-Hughes et al. (2023)
Normal milk	0.15–0.8 g/l	physiological background, no technological issues	Mehra et al. (2006)
Mastitic milk	sequential increase of IgG1/IgG2 followed by IgM/IgA; Ig concentrations may rise up to ~18.3 g/l depending on severity	increased SCC, reduced casein, impaired coagulation, inhibition of starter cultures	Holec (1994); Gajdusek (1996); Navratilova et al. (2012)
Heated milk	Ig remain largely intact after 63 °C/30 min or 72 °C/15 s; denaturation occurs at 80 °C/60 min or 100 °C/10 min, especially at slightly acidic pH	high thermal stability under pasteurisation; denaturation and loss of activity under high heat or acidic conditions	Navratilova et al. (2012)

Ig = immunoglobulin; SCC = somatic cell count

https://doi.org/10.17221/146/2025-CJAS

resonance (SPR), which offer high sensitivity and rapid detection (Gapper et al. 2007; 2013), as well as newly developed immunosensors designed for IgG analysis (Campanella et al. 2009). In addition to these laboratory-based methods, indirect on-farm screening tools such as refractometry (BRIX) are widely used for rapid estimation of colostrum Ig concentrations (Costa et al. 2022). Recently, liquid chromatography-tandem mass spectrometry (LC-MS/MS) using peptide-based quantification has emerged as a selective and reproducible alternative for Ig analysis in complex dairy matrices (Ehling et al. 2025).

Lactoferrin

Characterisation. LF is an iron-binding glycoprotein (80 kDa) belonging to the transferrin family. LF has antimicrobial properties against many microorganisms (bacteria, viruses and fungi, including parasites). It is one of the non-specific components of the immune system and has anticancer, antioxidant and anti-inflammatory properties (Giansanti et al. 2016; Malaczewska et al. 2019). Several *in vitro* and animal studies have demonstrated a protective effect of LF against infections by intestinal microorganisms, including rotavirus, *Giardia*, *Shigella*, *Salmonella*, and diarrheagenic *E. coli* (Ochoa and Cleary 2009). In addition to iron transport, LF is involved in stimulating the immune system and in protecting tissues from oxidative damage caused by free radicals (Korhonen and Marnila 2003).

Occurrence in milk. Milk is the main dietary source of LF. In bovine colostrum, LF concentration typically ranges between 1 and 5.2 g/l, while in mature cow's milk it is considerably lower (0.07–0.12 g/l). Human milk contains markedly higher LF concentrations compared with other mammalian species, ranging from 0.1 g/l to 3.5 g/l, and even 7–16 g/l in colostrum (Korhonen and Marnila 2003; Wang 2016; Table 2).

Role in dairy processing. Similar to immunoglobulins, LF is not specifically regulated in most food legislation frameworks as a standalone component in milk or dairy products. Although LF inhibits the growth of beneficial intestinal microorganisms, it is interesting that it increases the growth of certain selected probiotic strains, such as bifidobacteria and lactobacilli, or has no effect on their growth at all (Vega-Bautista et al. 2019). From a technological perspective, LF is relatively heat sensitive. While HTST (high-temperature, short-time pasteurisation, 72 °C/15 s) and LTLT (low-temperature, long-time pasteurisation, 63 °C/30 min) cause minimal loss of activity, UHT treatment (ultra-high temperature, 135–150 °C/1–4 s) or prolonged heating at 85 °C results in significant denaturation and a marked reduction in antimicrobial function (Korhonen and Pihlanto 2006; Adlerova et al. 2008; Conesa et al. 2009, 2010; Table 2).

Analytical methods. Several analytical approaches are used for LF determination. Classical immunochemical methods include RID and ELISA, while high-performance liquid chromatographic techniques (HPLC), reversed-phase (RP) HPLC method, electrophoresis, and fluorescence-based

Table 2. Content of lactoferrin in bovine milk and theassociated technological or physiological impacts

Milk type/ condition	Reported lactoferrin level/activity	Technological/ physiological impact	References
Colostrum	1–5 g/l, occasionally higher	strong antimicrobial; immune support	Korhonen and Marnila (2003); Wang (2016)
Normal milk	0.07–0.12 g/l	immune defense; iron binding; minor direct technological impact	Korhonen and Marnila (2003)
Mastitic milk	LF markedly elevated: typically 1–5 g/l; may exceed 5 g/l in severe cases	marker of mastitis; increased SCC; effects on protein stability and processing quality	Kawai et al. (1999); Hagiwara et al. (2003)
Heated milk	stable during pasteurisation (63–72 °C); significant denaturation at ≥85 °C or during UHT	loss of antimicrobial activity; reduction of functional properties	Conesa et al. (2009); Adlerova et al. (2008)

LF = lactoferrin; SCC = somatic cell count; UHT = ultra-high temperature

https://doi.org/10.17221/146/202-CJAS

biosensors provide higher sensitivity (Tsakali et al. 2019). Novel strategies exploit electrochemical sensors, SPR, and mid-infrared Fourier-transform spectroscopy (MIR-FTIR) with multivariate analysis (Soyeurt et al. 2020; Nejeschlebova et al. 2024), as well as newly developed immunosensors, including an amperometric immunosensor for LF (Campanella et al. 2009). Despite this variety, no single method currently meets all the requirements for reproducibility, sensitivity, selectivity, and cost-effectiveness in routine analysis.

Enzymes

Enzymes represent a heterogeneous group of biologically active proteins in milk, divided into two subgroups: native (endogenous) and secondary (exogenous). The group of native enzymes includes LYZ, LPO, ALP, ACP, esterase, lipase, and protease. These enzymes perform various biological functions in milk, ranging from antimicrobial effects (e.g. LYZ and LPO) to the hydrolysis of fats and proteins (e.g. lipases and proteases).

Their enzymatic activity significantly changes during inflammatory conditions of the mammary gland, particularly mastitis, when the concentrations of several enzymes increase. Elevated enzymatic activity, especially lipolytic and proteolytic, can negatively affect the technological quality of milk and the sensory properties of dairy products such as cheese. Enhanced lipolysis of fats leads to reduced yield, formation of free fatty acids, and the development of taste defects, particularly rancidity (Navratilova 2002).

The optimal temperatures for enzymatic activity in most enzymes are found between 30–40 °C, which corresponds to the physiological environment of the mammary gland. Native enzymes exhibit a wide range of heat stability, with most being completely inactivated in the range of 70–100 °C, while some require treatment at higher temperatures for complete inactivation. For example, the lactoperoxidase system (LPS), which is based on the enzymatic activity of LPO, is known for its exceptional thermal resistance and remains active until temperatures exceed 80 °C (Deeth 2021). In contrast, enzymes of microbial origin, produced by bacteria contaminating raw milk, often exhibit greater thermal stability than native milk enzymes and may survive even severe heat treatments, such as ultra-high temperature (UHT) processing above 135 °C. These exogenous enzymes are therefore considered technologically and hygienically problematic (Navratilova 2002; Deeth 2021; Wisniewski et al. 2025). The legislation does not focus directly on the levels of individual enzymes, but their activity is indirectly monitored through thermal inactivation tests (e.g. the phosphatase test is used to verify the effectiveness of pasteurisation).

Lysozyme

Characterisation. LYZ plays an important role in innate immunity, providing protection against bacteria, viruses, and fungi (Micinski et al. 2013). LYZ (peptidoglycan *N*-acetylmuramoyl hydrolase) hydrolyses β(1–4) linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in bacterial peptidoglycan, particularly in Gram-positive organisms (Ragland and Criss 2017). In combination with detergents, chelators such as ethylenediaminetetraacetic acid (EDTA), nisin, or LF, it can also exert antimicrobial activity against Gram-negative bacteria (Branen and Davidson 2004). Beyond its natural antimicrobial function, LYZ has recognised clinical applications, including use in infection control, management of periodontal disease, prevention of dental caries, supportive therapy in oncology, and as an adjuvant that enhances the efficacy of selected antibiotics (Proctor et al. 1988).

Occurrence in milk. Two structurally distinct types of LYZ have been described: LYZ-c, originally identified in hen egg white, and LYZ-g, first isolated from goose egg white. Both isoforms consist of a single polypeptide chain. LYZ-c contains approximately 129–130 amino acid residues with a molecular mass of about 14 kDa, whereas LYZ-g is composed of 185 residues and exhibits a higher molecular mass in the range of 19–21 kDa.

In milk, LYZ from human, camel and mare's milk is classified as the c-type, and bovine milk contains only low concentrations of c-type lysozyme. According to Duman and Karav (2023) and Piccinini et al. (2005), LYZ concentrations are higher in cow's colostrum (0.14–0.7 mg/l) compared with mature milk (0.05–0.6 mg/l). Its activity increases with elevated SCC and during mastitis, where concentrations may reach 1–2 mg/l (Navratilova et al. 2012; Table 3).

https://doi.org/10.17221/146/2025-CJAS

Table 3. Content of lysozyme in bovine milk and theassociated technological or physiological impacts

Milk type/ condition	Reported lysozyme level/activity	Technological/ physiological impact	References
Colostrum	0.14–0.7 mg/l	antimicrobial effect; contributes to neonatal immune protection	Piccinini et al. (2005); Duman and Karav (2023)
Normal milk	0.05–0.6 mg/l	minor antimicrobial effect; limited technological influence	Piccinini et al. (2005)
Mastitic milk	1–2 mg/l	correlates with SCC; part of mammary immune defense	Navratilova et al. (2012)
Heated milk	largely stable at pasteurisation; ~25% activity loss at 80 °C/15 s; completely inactivated at 100 °C/3 min	partial retention of antimicrobial activity depending on heat load	Eitenmiller et al. (1976); Griffiths (1986)

SCC = somatic cell count

Beyond its antimicrobial role, LYZ also contributes to the nutritional value of milk for suckling offspring. In its presence, casein coagulated by gastric enzymes (chymosin or pepsin) forms soft, digestible floccules rather than firm curds, thereby improving protein digestibility. LYZ additionally exhibits bactericidal activity against intestinal enterococci and releases amino sugars from bacterial cell walls, which act as growth factors for *Lactobacillus bifidus* (Navratilova et al. 2012).

Role in dairy processing. In milk and dairy fermentation, LYZ has negligible inhibitory effects on lactic acid bacteria, and the addition of LYZ only slightly reduces the viscosity and acidity of fermented milk gels (Urbiene et al. 2006). However, LYZ has wide applications in the food industry, especially as a preservative for meats, seafood and other perishable foods, where it extends shelf life and prevents spoilage (Proctor et al. 1988; Cunningham et al. 1991). It is also permitted as a technological additive in cheese and beer production under EU regulations EC No. 1332/2008 (European Union 2008) and EU No. 471/2012 (European Union 2012) (Landschoot and Villa 2005). LYZ is relatively stable to heating at pH 7 and even more stable at pH 4. At pH 7, it was completely inactivated after approximately 3 min at 100 °C, whereas at pH 4 it was only 43% inactivated after 20 min at 100 °C (Eitenmiller et al. 1976). Griffiths (1986) further reported that heating at 80 °C for 15 s decreased LYZ activity by only about 25% (Table 3).

Analytical methods. LYZ quantification can be performed using a broad range of analytical techniques, including classical approaches such as radial diffusion (RD), NEP, turbidimetry (TM), ELISA, and immunofluorescence (IF). More advanced separation methods comprise HPLC and capillary electrophoresis (CE), while high-resolution identification relies on mass spectrometry techniques such as matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) and electrospray ionisation mass spectrometry (ESI-MS). Additional innovative strategies include surface-enhanced Raman spectroscopy (SERS), cyclic voltammetry (CV), electrochemical assays, and a colorimetric approach employing glutamic acid-functionalised silver nanoparticles (AgNPs/GA) as a selective biosensing probe for LYZ detection in milk (Bartunkova and Paulik 2011; Litzman et al. 2015; Shrivas et al. 2019).

Lactoperoxidase

Characterisation. LPO is one of the most stable naturally occurring enzymes in milk, accounting for approximately 1% of milk whey proteins. Together with its substrates, thiocyanate and hydrogen peroxide, LPO forms the LPS, which represents an important natural antimicrobial mechanism in raw milk. The LPS exhibits broad-spectrum antimicrobial activity against both Gram-negative and Gram-positive microorganisms, including *Pseudomonas* spp., coliforms, *Salmonella*, *Shigella*, *Listeria*, streptococci, *Staphylococcus aureus*, as well as certain yeasts and fungi (Wolfson and Sumner 1993; Beno et al. 2024).

Occurrence in milk. LPO is naturally present in cow's milk at concentrations sufficient to gen-

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erate antimicrobial activity. LPO content in mature cow's milk ranges from 10 mg/l to 30 mg/l, whereas in colostrum the concentration is considerably higher, typically reaching 50–100 mg/l (Reiter and Perraudin 1991; Korhonen and Marnila 2003; Navratilova et al. 2012). Korhonen and Marnila (2003) also reported similar concentrations for sheep's milk (10–15 mg/l), but considerably lower levels for goat's and human milk (8–10 and 1–3 mg/l, respectively). They further noted that LPO activity varies not only between species but also within individual animals, depending on factors such as stage of lactation, health status, and environmental conditions. The antimicrobial effect of LPS is based on the formation of oxidation products of thiocyanate (SCN^-) in the presence of hydrogen peroxide (H_2O_2), with effective inhibition of the growth of microorganisms occurring at LPO concentrations in the range of 10–20 mg/l (ppm) (Navratilova et al. 2012; Table 4).

Role in dairy processing. LPO is relatively heat-resistant in cow's milk and is only partially inactivated during short-term pasteurisation (72–74 °C), while retaining sufficient catalytic activity for the reaction between SCN^- and H_2O_2 (Reiter and Harnulv 1984). Although LPO is not an official indicator of pasteurisation, its thermal behaviour is sometimes evaluated as supplementary information on heat load in milk (Gajdusek 2003). Studies have shown that its activity decreases to approximately 90% after heating at 68 °C for 15 min or 72 °C for 2 min, and further declines to 64% and 36% after treatments at 74 °C and 76 °C for 40 s, respectively (Marin et al. 2003). Complete inactivation occurs at 78 °C for 15 s or 80 °C for 2.5 s (Griffiths 1986; Marks et al. 2001; Buys 2011), while Navratilova et al. (2012) reported complete inactivation at 75 °C after 30 min and at 80 °C after only 30 seconds. The calculated *z*-values, 3.1 °C (Marin et al. 2003) and 5.4 °C (Griffiths 1986), confirm that LPO is more sensitive to temperature than to exposure time (Table 4).

Due to its strong antimicrobial properties, LPS can be applied to extend the shelf life of milk and meat products, particularly in regions lacking adequate refrigeration (Wolfson and Sumner 1993; Lado and Yousef 2003; Beno et al. 2024). However, active LPS may also negatively influence fermentation by inhibiting starter cultures such as *Lactobacillus acidophilus*, leading to reduced lactic acid production, delayed coagulation, and impaired curd formation. The extent of inhibition depends on multiple factors, including milk type, concentrations of H_2O_2 and SCN^-, heating temperature and incubation conditions, as well as the starter culture used (Dillon 2014).

From a regulatory perspective, the intentional use of LPS for commercial purposes is prohibited under EU legislation. In contrast, Codex Alimentarius permits its controlled use in developing countries, setting specific conditions such as a maximum of 14 days of storage in the absence of a cold chain (FAO/WHO 1991).

Analytical methods. Various spectrophotometric assays, including those based on ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) and TMB (3,3'5,5'-tetramethylbenzidine), are commonly used to detect LPO activity in milk

Table 4. Content of lactoperoxidase in bovine milk and the associated technological or physiological impacts

Milk type/ condition	Reported LPO level/activity	Technological/ physiological impact	References
Colostrum	50–100 mg/l	strong antimicrobial activity	Reiter and Perraudin (1991); Korhonen and Marnila (2003)
Normal milk	10–30 mg/l	LPS antimicrobial effect; relatively stable to pasteurisation	Navratilova et al. (2012)
Mastitic milk	increased activity correlates with SCC and inflammatory status	supportive mastitis indicator	Silva et al. (2022)
Heated milk	partially stable at 72 °C; complete inactivation under ≥78–80 °C (time-dependent)	heat stability assessment; loss of antimicrobial activity	Griffiths (1986); Marks et al. (2001); Buys (2011)

LPO = lactoperoxidase; LPS = lactoperoxidase system

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(Lam and Smelt 1988). Silva et al. (2022) also applied this approach for the diagnosis of subclinical mastitis. In bovine and buffalo milk, the ABTS-based photometric assay has been standardised for routine determination of LPO activity, with optimisation of pH, linearity range and control of matrix effects (Kumar and Bhatia 1999; International Organisation for Standardisation 2011). For bovine LPO and related mammalian peroxidases, other chromogenic substrates such as guaiacol and o-dianisidine have been employed in quantitative assays that allow calculation of enzyme concentration from activity data (Pruitt et al. 1990). More recently, a sensitive microplate-based fluorescent assay using Amplex Red as the reporter substrate has been developed and validated for the determination of LPO activities in human, bovine, goat and camel milk, providing substantially lower detection limits and high-throughput analytical capacity (Zou et al. 2021). In human milk, LPO has also been purified and quantified by immunoaffinity chromatography followed by a specific sandwich ELISA, demonstrating the applicability of immunometric approaches to LPO determination in milk (Shin et al. 2001).

Phosphatase

Characterisation. Phosphatases are hydrolytic enzymes that catalyse the cleavage of phosphate groups from organic compounds. They are classified according to their pH optimum into ALP (active at pH > 7) and ACP (active at pH < 7). These enzymes are involved in the metabolism of phosphorus and calcium, as they facilitate their release from phosphate esters.

Occurrence in milk. Native ALP originates mainly from the mammary gland epithelium, blood, and cellular structures, but it is also produced by certain microorganisms, particularly the genera *Escherichia* and *Aerobacter*. In contrast, ACP is derived almost exclusively from leukocytes. In milk obtained from cows with mastitis, increased activity of several enzymes, including lipases and phosphatases, may occur. Neither ALP nor ACP directly participates in the hydrolysis of milk fat, which is the domain of lipases. Instead, their elevated activity is regarded as a marker of mammary tissue damage and increased cell permeability, processes that often coincide with enhanced lipolytic activity in mastitic milk and consequently with a higher concentration of free fatty acids (Shahani et al. 1973; Kitchen 1981; Fox and Kelly 2006). These changes negatively affect both the taste and shelf life of dairy products, such as butter.

The activity of ALP in raw bovine milk varies widely, with reported values ranging from approximately 2 mU/l to 5 000 mU/l, depending on the source and freshness of the milk (Klotz et al. 2008; Albillos et al. 2011; Table 5). Using the ISO 11816-1 reference method, a residual alkaline phosphatase activity ≤ 350 mU/l is generally considered indicative of adequate pasteurisation in cow's milk. According to EFSA's scientific assessment (EFSA 2021), higher species-specific indicative limits have been proposed for non-bovine milks, approximately 300 mU/l for goat's milk and 500 mU/l for sheep's milk. However, in mastitic milk the enzyme activity increases significantly. In cows, subclinical mastitic milk contained on average 123.4 mU/l compared with 30.3 mU/l in healthy controls, representing nearly a four-fold increase (El-Ashmawy et al. 2023). In buffaloes, ALP activity rose progressively with mastitis severity: from 16.5 U/l in normal milk to 28 U/l in subclinical (+), 38 U/l in subclinical (+++), and up to 42 U/l in clinical mastitis, corresponding to approximately a 2.5-fold elevation (Patil et al. 2015). These findings confirm that mastitic milk consistently exhibits higher ALP activity than normal milk, reflecting tissue damage and increased permeability of the mammary epithelium. For practical quality control, it is recommended that each dairy plant establish baseline ALP levels for its incoming raw milk and apply the threshold values consistently in monitoring the pasteurisation process (Cornell University 2022).

ACP occurs in milk at much lower levels, approximately 2–5% of the activity of ALP (Andrews and Alichanidis 1975; Andrews 1992). Its concentration in bovine milk has been reported to range from about 4.1 U/100 ml to 50 U/100 ml (Mullen 1950). ACP activity increases significantly in mastitic milk, often rising 4–10 fold compared to normal milk, which makes it a potential indicator of mammary inflammation and reduced technological quality (Kitchen 1981; Chandra et al. 2012; Table 5).

Role in dairy production. ALP is a heat-labile enzyme and therefore serves as a reliable indicator of proper pasteurisation; during correctly performed pasteurisation, ALP is reduced to levels below the regulatory detection limit (Gajdusek

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Table 5. Content of acid and alkaline phosphatase in bovine milk and theirassociated technological or physiological impacts

Milk type/ condition	Reported enzyme level/activity	Technological/ physiological impact	References
Alkaline phosphatase			
Colostrum	very high on day 1; markedly decreases during first postpartum week	high basal ALP interferes with routine pasteurisation testing; ALP test not valid for colostrum	McGrath et al. (2016); EFSA (2021)
Normal milk	2–5 000 mU/l (prepared by spiking pasteurised milk with raw milk)	marker of raw milk; pasteurisation control	Klotz et al. (2008); Albillos et al. (2011);
Mastitic milk	elevated ALP	reflects mammary tissue damage and increased epithelial permeability	Shahani et al. (1973); Kitchen (1981); Fox and Kelly (2006)
Heated milk	≤350 mU/l (ISO 11816-1 threshold for negative test)	indicator of adequate pasteurisation	European Union (2019); International Organisation for Standardisation (2024)
Acid phosphatase			
Colostrum	low in early colostrum; transient increase during first postpartum week; low in mature milk	variable background; not suitable as pasteurisation marker	McGrath et al. (2016)
Normal milk	ACP ≈ 2–5% of ALP activity	not suitable as pasteurisation marker due to high thermostability	Andrews (1992)
Mastitic milk	4–10 × higher than normal; 4.1–50 U/100 ml	SCC-related; impairs coagulation and protein stability	Mullen (1950); Andrews and Alichanidis (1975); Chandra et al. (2012)
Heated milk	80–90% retained at 62–72 °C	too thermostable for pasteurisation test	Mullen (1950); Andrews et al. (1987)

ACP = acid phosphatase; ALP = alkaline phosphatase; SCC = somatic cell count

2003). Some studies indicate that the use of the ALP test to verify pasteurisation in non-bovine milk may be limited, as thermal inactivation kinetics differ markedly among species (Tzereme et al. 2025). According to Commission Implementing Regulation (EU) 2019/627 (European Union 2019), the test result is considered negative if the residual activity in cow's milk does not exceed 350 mU/l (European Union 2019). Painter and Bradley (1997) investigated the thermal inactivation of ALP in milk with different fat contents under HTST (68–75 °C for 15 s) and LTLT (60–66 °C for 30 min) conditions. Their results showed that all time–temperature combinations at or above the legal minimum pasteurisation parameters reduced ALP activity to levels well below the regulatory cut-off of 350 mU/l.

Increased activity of ACP has been associated with impaired protein stability and reduced coagulation properties in cheesemaking, thereby limiting the suitability of mastitic milk for dairy processing (Shahani et al. 1973; Kitchen 1981; Chandra et al. 2012). In contrast to ALP, ACP is considerably more thermostable. Andrews et al. (1987) reported that after heating milk to 72 °C for 15 s, more than 90% of its activity remained. Similarly, Mullen (1950) showed that batch pasteurisation (62.8 °C for 30 min) preserved 80–90% of ACP activity. Complete inactivation requires substantially more severe heat treatments, such as 88.2 °C for 30 min, 93.3 °C for 12 min, or 100 °C for 1 min (Table 5). Due to this heat resistance, ACP cannot be applied as a marker of pasteurisation; however, its persistence may serve as an indicator of pathological conditions in milk.

Analytical methods. The CSN ISO 3356 (CSN 2011) standard is commonly used for the determination of ALP activity. This method is applicable

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not only to fresh milk, but also to milk powders, buttermilk, dried buttermilk, whey, and dried whey. Results are expressed as the amount of phenol (μg) released from 1 ml of sample under precisely defined conditions. The method is suitable for samples with an activity exceeding 1 μg of phenol/ml. In addition to colorimetric assays, phosphatase activity in milk can also be quantified using HPLC-based methods, which can be employed in accredited laboratories for advanced analytical verification of pasteurisation efficiency (Meena et al. 2021).

Lipase

Characterisation. Lipases are a group of water-soluble enzymes that catalyse the hydrolysis of fats (triglycerides) into free fatty acids. Lipoprotein lipase (LPL; EC 3.1.1.34) is a specific ester hydrolase that catalyses the hydrolysis of triacylglycerols, which are the major form of fat in bovine milk. The enzyme exhibits positional specificity, acting predominantly at the sn-1 and sn-3 positions of the glycerol backbone. As a result, the hydrolytic reaction yields two free fatty acids and one 2-monoacylglycerol (2-MAG) molecule (Korn 1955; Jensen 1989; Deeth 2006).

Occurrence in milk. In bovine milk, the most important lipase is the native LPL, although lipases of microbial origin may also be present (Vyletelova et al. 2000a). LPL is synthesised by the secretory cells of the mammary gland and subsequently passes into the milk. Its enzymatic activity is sufficient to cause rapid hydrolysis of milk fat. In reality, however, this hydrolysis does not occur because the membrane of the milk fat globule prevents lipase from accessing the fat. Lipolysis begins only when the fat globule membrane is disrupted by physical, chemical, or microbial factors (Deeth 2006).

The concentration of LPL in bovine milk has not been precisely quantified in terms of absolute protein content. Most studies report only enzymatic activity, as the enzyme is present in trace amounts but exerts a pronounced effect once the milk fat globule membrane is disrupted. Shahani and Sommer (1951) reported native milk lipase activities in the range of 0.2–1.0 U/ml (Table 6). Jensen (1989) described LPL as a "trace enzyme," occurring in very low concentrations (ng/ml range), but sufficient to catalyse significant lipolysis under favourable conditions. More recently, Deeth and Fitz-Gerald (2006) emphasised that reliable quantification of LPL concentration in milk remains challenging, since measured activity strongly depends on assay conditions and the physical state of the milk fat. Thus, from a technological perspective, even low levels of LPL activity are critical, as they can initiate lipolysis and rancidity once the protective fat globule membrane is compromised.

Role in dairy production. LPL in raw milk has little access to its substrate and therefore shows low activity; it becomes strongly active only when the milk fat globule membrane is disrupted or under favourable conditions, such as in the digestive

Table 6. Content of lipoprotein lipase in bovine milk and the associated technological or physiological impacts

Milk type/ condition	Reported LPL level/activity	Technological/ physiological impact	References
Colostrum	LPL activity detectable; generally low compared with mature milk	early-lactation changes in LPL activity may influence susceptibility to cold-storage lipolysis	Deeth (2006); McGrath et al. (2016)
Normal milk	native milk lipase activity 0.2–1.0 U/mL; LPL present as a trace enzyme (ng/ml range)	normally inactive due to fat globule membrane	Shahani and Sommer (1951); Jensen 1989
Mastitic milk	LPL activity often unchanged or decreased; microbial lipases frequently increased	rancidity due to membrane damage and microbial lipases	Kitchen (1981); Deeth and Fitz-Gerald (2006)
Heated milk	LPL rapidly inactivated by heat; HTST ≥72 °C for 15 s results in practically complete inactivation	prevention of lipolysis and rancidity; improved flavour stability and extended shelf life	Deeth (2006); Deeth (2021)

HTST = high-temperature, short-time (pasteurisation); LPL = lipoprotein lipase

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tract. Disruption of the fat globule membrane leads to spontaneous lipolysis and subsequent rancidity of milk fat. For this reason, lipase should be inactivated as soon as possible – usually by pasteurisation, which not only increases the hygienic quality of milk, but also extends its shelf life (Dickow et al. 2011; Deeth 2021). Deeth (2021) and Driessen (1989) summarised in their studies the effect of various heat treatments on LPL activity. Heating (thermisation) at 57–65 °C for 15 s typically inactivates approximately 50–70% of LPL activity, depending on the milk system and assay conditions, whereas HTST pasteurisation at 72 °C and higher temperatures for 15 s results in practically complete (>99%) inactivation of the enzyme (Table 6).

Analytical methods. Analytical methods for determining lipolytic activity in milk include classical titrimetric and spectrophotometric assays that quantify free fatty acids released from triglycerides, as originally applied to native milk lipases by Shahani and Sommer (Shahani and Sommer 1951). More specific assays were later developed, including radioisotope-based methods using radiolabeled triglycerides (Korn 1955) and fluorimetric or colorimetric assays employing synthetic substrates such as *p*-nitrophenyl esters (Jensen 1989). Immunochemical techniques, such as ELISA and Western blotting, provide direct detection of LPL protein with higher specificity (Peterson et al. 1991).

In recent years, proteomic approaches have provided new possibilities for studying LPL. Techniques such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) or matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS), often combined with immunoaffinity separation, allow precise identification and quantification of LPL within complex milk proteomes (Hettinga et al. 2011; Khan et al. 2025). These modern methods offer higher sensitivity and specificity, though they require advanced instrumentation and standardised protocols.

Protease

Characterisation. Proteases are proteolytic enzymes that hydrolyse proteins into peptides and free amino acids (Philipps-Wiemann 2018). Both native proteases (e.g. plasmin, elastase, cathepsin D, carboxy- and aminopeptidases) and proteases of microbial origin are present in milk (Vyletelova et al. 2000b). Native enzymes contribute to pre-intestinal hydrolysis of milk proteins, facilitating digestion and generating bioactive peptides that have been associated with sleep modulation, mucosal development, immunomodulation and regulation of gastrointestinal function (Hodgkinson et al. 2019).

Occurrence in milk. Native proteases are secreted into milk by the mammary gland, but their activity can also increase in relation to SCC and microbial contamination. Proteolytic activity in milk has been shown to increase with rising SCC, as demonstrated by Politis et al. (1989), who observed enhanced proteolysis in stored milk samples with higher SCC. More recently, Gautam et al. (2022) confirmed a direct influence of SCC on protease activity across different dairy species, including cows, buffaloes, and goats. The main native proteolytic enzyme in bovine milk is plasmin, a serine protease with an activity optimum at alkaline pH (~7.5). Plasmin mainly hydrolyses β-casein to γ-caseins. In raw milk, plasmin occurs predominantly as its inactive precursor plasminogen, typically in a plasminogen-to-plasmin ratio of 9 : 1 to 2 : 1 (Richardson 1983; Dupont et al. 1997; Dupont et al. 1998). Activation of plasminogen is mediated by plasminogen activators, which are not normally inhibited in dairy products unless they are inactivated by heat treatment.

Bovine milk contains measurable levels of plasmin and plasminogen, which vary with lactation stage and processing conditions. Studies have reported that raw milk typically contains approximately 4–5 U/ml of plasmin, while plasminogen-derived activity ranges from about 26 U/ml in early lactation to 45 U/ml in late lactation (Saint-Denis et al. 2001a). Quantitative assays have further shown that plasmin concentrations in pasteurised milk fall within 0.14–0.73 µg/ml, whereas plasminogen levels range between 0.55–2.75 µg/ml (Bastian and Brown 1996).

The concentration and activity of plasmin in milk are markedly influenced by udder health. In mastitic milk, plasmin levels are significantly elevated compared to healthy quarters. Kaartinen et al. (1988) reported an average plasmin concentration of 2.9 µg/ml (range 0.5–12.5 µg/ml) in mastitic milk, while milk from healthy cows showed minimal or no activity. This increase results from enhanced plasminogen activation and leakage of plasma-derived plasmin due to disrup-

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tion of the blood–milk barrier. Similarly, Andrews (1983) demonstrated that bovine milks with high SCC, as observed during mastitis, exhibited accelerated degradation of caseins by plasmin. Such elevated proteolytic activity has negative technological consequences, including impaired protein stability, reduced cheese yield, and deterioration of milk quality (Table 7).

Role in dairy production. Plasmin activity plays a dual role in dairy technology. Beneficially, it contributes to cheese ripening, particularly in cheeses subjected to high post-heating or in those with elevated pH during maturation (e.g. processed cheeses), where plasmin supports the development of characteristic flavor and texture (McSweeney 2013). Conversely, in pasteurised and UHT milk, excessive plasmin activity can cause undesirable proteolysis, leading to gelation, sediment formation, and a reduced shelf life (Ismail and Nielsen 2010).

Although no universal threshold value has been defined for plasmin activity that would ensure technological stability, elevated levels are strongly associated with quality defects. For instance, in cold-stressed UHT milk, plasmin concentrations of approximately 607.9 µg/l (≈0.608 µg/ml) and enzymatic activity of 15.99 U/l (≈0.016 U/ml) were linked to gelation after about 60 days of storage (Liu et al. 2023). These findings highlight that while plasmin is a natural component of milk, its excessive activity or insufficient inactivation during processing can severely compromise the technological quality of long shelf-life dairy products.

From a processing perspective, plasmin, plasminogen, and plasminogen activators are relatively heat-stable at temperatures up to 75 °C, whereas their inhibitors are more heat-labile (Deeth 2021; Leite et al. 2021, 2023). Saint-Denis et al. (2001a) reported *D*-values (time for 90% inactivation) at 60 °C of 59 520 s for plasmin, 57 900 s for plasminogen, and 39 300 s for the activator. At 75 °C, these values decreased markedly to 660 s for plasmin and plasminogen, and 840 s for the activator, while at 85 °C they further declined to approximately 126–129 seconds. Richardson (1983) observed that conventional pasteurisation (72 °C/15 s) reduced plasmin activity by only 17%, confirming its high thermal stability. Prado et al. (2007) further showed that plasmin activators remain stable at <75 °C for 15–30 s; at 74.5 °C/15 s, the plasminogen activator inhibitor decreased by 81%, whereas the plasmin inhibitor decreased by only 36%. More pronounced decreases in plasminogen than in plasmin were observed at ≥85 °C/30 seconds.

Several studies have proposed the use of preheating to achieve more effective plasmin inactivation. Newstead et al. (2006) showed that preheating at 90 °C for 30–60 s followed by sterilisation at 140 °C/4 s resulted in complete plasmin inactivation. Similarly, Rauh et al. (2014a) and Van Asselt et al. (2008) demonstrated that preheating at 80 °C/300 s or 95 °C/180 s significantly reduced plasmin activity (Table 7).

Although proteases in milk are not toxic or harmful to human health, their uncontrolled activity

Table 7. Content of protease (plasmin) in bovine milk and the associated technological or physiological impact

Milk type/ condition (plasmin)	Reported plasmin level/activity	Technological/ physiological impact	References
Colostrum	higher protease and plasmin activity than in normal milk (qualitative increase; no quantitative ratio reported)	faster casein proteolysis; may affect coagulation and early-lactation cheese yield	Dupont et al. (1998); Madsen et al. (2004); McGrath et al. (2016)
Normal milk	plasmin: ~4–5 U/ml; plasminogen: 26–45 U/ml	contributes to cheese ripening	Richardson (1983); Bastian and Brown (1996)
Mastitic milk	2.9 µg/ml on average (0.5–12.5 µg/ml); increased SCC	accelerated casein degradation; reduced cheese yield	Andrews (1983); Kaartinen et al. (1988)
Heated milk	plasmin: 0.14–0.73 µg/ml; plasminogen: 0.55–2.75 µg/ml	highly heat-stable; conventional pasteurisation reduces activity by ~17%; risk of proteolysis during storage	Bastian and Brown (1996)

SCC = somatic cell count

https://doi.org/10.17221/146/202-CJAS

compromises product quality and sensory attributes. Therefore, in long shelf life dairy products (e.g. UHT milk, processed cheeses), strict control of plasmin activity is essential to ensure stability and consumer acceptance.

Analytical methods. A wide range of analytical methods has been developed to quantify the plasmin system in milk. Conventional approaches include colorimetric, fluorometric, and spectrophotometric assays, as well as gel electrophoresis techniques for monitoring casein degradation. Immunochemical approaches, particularly the enzyme-linked ELISA, have also been employed for specific detection and quantification of plasmin, plasminogen, and plasminogen activator inhibitor (Dupont et al. 1997; Saint-Denis et al. 2001b; Bendicho et al. 2002; Rauh et al. 2014b; Khan et al. 2025).

In addition to these classical methods, novel spectroscopic approaches have been introduced. Ozen et al. (2003) described the application of Fourier-transform infrared spectroscopy (FT-IR) combined with multivariate statistical analysis, which enables determination of plasminogen concentration and differentiation between plasmin and plasminogen in protein solutions. This method provides an advanced and non-destructive alternative to conventional biochemical assays, offering both sensitivity and specificity for studying the plasmin system. Complementary to these techniques, acoustic methods have recently been developed for monitoring proteolysis in milk. Increased plasmin activity results in β-casein cleavage and altered gelation behaviour; acoustic biosensors and high-resolution ultrasonic spectroscopy (HR-US) allow real-time detection of such changes with sensitivity comparable to traditional enzymatic assays (Dizon et al. 2020).

CONCLUSION

Native inhibitory substances in bovine milk represent essential components of the mammary gland's defense system and have a significant impact on the technological properties and processability of milk. Although they do not present a direct health hazard, their elevated concentrations, typically associated with inflammatory processes in the udder, may disrupt fermentation, suppress the activity of starter cultures, impair coagulation, and ultimately reduce the quality and yield of dairy products. As summarised in Tables 1–7, bovine milk contains a broad spectrum of bioactive proteins and endogenous enzymes whose concentrations vary according to physiological status, stage of lactation, udder health, and technological treatment. On the other hand, these compounds play indispensable biological roles in neonatal immunity and nutrition; however, their uncontrolled or increased activity represents a technological risk, particularly in fermented and long shelf-life dairy products.

Current trends in dairy farming and analytical methodologies place increasing emphasis on understanding the factors influencing the natural variability of these native inhibitors. Due to their very low concentrations and their close association with immune physiology, progress in this field depends primarily on advances in analytical techniques. Rapid developments in materials engineering, sensor miniaturisation, automation, robotics, and large-scale data processing have substantially enhanced the capacity to detect, quantify, and interpret subtle changes in the concentrations of native inhibitors. In addition to reference methods, routine indirect analytical approaches have also improved, enabling the development of more robust calibration models and the broader application of rapid diagnostic technologies.

The need for analytical progress is defined by two major aspects: (*i*) the utility of native inhibitors as biomarkers of udder health, allowing prediction, early diagnosis, and control of milk secretion disorders; and (*ii*) the ability of native inhibitors to mimic residues of exogenous inhibitory substances (RIS) by suppressing the growth of test microorganisms or influencing fermentation processes, which complicates both residue testing and dairy processing technologies.

The dynamic expansion of knowledge in this field highlights the necessity of continuously structuring, synthesising, and interpreting new information for both the scientific and professional communities. Systematic integration of emerging findings can improve diagnostic strategies for monitoring udder health, enhance routine milk-quality control, and support optimisation of technological procedures throughout the dairy chain.

Future research should aim to elucidate the molecular mechanisms regulating the synthesis and secretion of native inhibitors and their interactions with immune and inflammatory path-

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ways within the mammary gland. The integration of multi-omics approaches, including proteomics, peptidomics, metabolomics, and transcriptomics, holds strong potential for identifying novel biomarkers and characterising dynamic responses during subclinical and clinical mastitis. Another promising avenue involves the development of advanced analytical platforms capable of detecting extremely low concentrations of bioactive proteins and enzymes with greater precision and efficiency. Innovations in biosensor technology, microfluidics, lab-on-a-chip systems, and machine-learning algorithms are expected to significantly strengthen routine monitoring of raw milk quality.

A deeper understanding of the interactions between native inhibitors and dairy fermentation processes will also be essential for optimising the technological performance of milk, developing more robust starter cultures, and preventing defects in long shelf-life dairy products. Finally, translating new scientific insights into practical applications, such as predictive udder-health models, precision-livestock tools, and improved quality-control systems across the dairy sector, will be crucial. This revised and structured synthesis of current knowledge supports such developments and underscores the importance of continuously updating scientific understanding to promote a sustainable, safe, and value-oriented dairy supply chain.

Conflict of interest

The authors declare no conflict of interest.

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Received: October 21, 2025
Accepted: December 10, 2025
Published online: December 30, 2025