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Use of Bioactive Extracts from Plant Matrices in Dairy Cows: Effects on Production Quality and Welfare

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1.1. Natural feed additives for animal health and productivity

The steady growth of the global population, together with the increasing demand for food, has led to a substantial expansion of livestock production worldwide. These changes, closely linked to evolving human lifestyles, have intensified the exposure of farm animals to various diseases, often resulting in a decline in the quality of animal-derived products consumed by humans. In dairy cattle, the implementation of intensive production systems designed to maximize milk yield has been associated with impaired animal health and welfare, reflected in a higher incidence of metabolic disorders. Such challenges emphasize the need to identify and develop alternative feeding strategies that can improve productivity while maintaining animal well-being (Jalal et al., 2023).

For decades, antibiotic growth promoters were widely used in livestock production to enhance growth rate, feed efficiency, and animal survival, while simultaneously reducing morbidity and mortality. However, the misuse of antibiotic growth promoters contributed to the spread of antibiotic-resistant bacteria in livestock populations (Naves Aroeira et al., 2023). To address this concern, the European Union permanently banned the inclusion of antibiotics in animal feed additives as of 1 January 2006 (Tian et al., 2021). Although this measure initially led to a rise in infection rates and a temporary decline in productivity, it significantly reduced the spread of antibiotic resistance and the associated risks to human health.

In response, research has increasingly focused on natural feed additives such as enzymes, probiotics, prebiotics, phytonutrients, and fatty acids, which can support growth and disease prevention without contributing to antimicrobial resistance. Modern livestock systems are also placing greater emphasis on environmental sustainability and animal health, promoting the use of feed formulations enriched with naturally occurring bioactive compounds that exhibit antimicrobial, antioxidant, anti-inflammatory, and antitumor activities (Michalak et al., 2021, Savvidou et al., 2023, Singh et al., 2024).

Plants, whether incorporated directly, used in dried form, or processed into extracts, offer a rich source of bioactive substances that can enhance livestock health and performance (Manuelian et al., 2021). The inclusion of such compounds in feed aligns with the growing consumer demand for safe, natural, and environmentally friendly food products. In this context, agro-industrial by-products have gained attention as a sustainable and cost-effective resource for animal nutrition (Georganas et al., 2023, Shah et al., 2025). Studies involving small and large ruminants, including goats, sheep, cows, and buffalo, have demonstrated that agro-industrial by-products can positively influence rumen fermentation parameters (such as pH, gas production, volatile fatty acids, ammonia, and methane), digestibility, and both the quantity and fatty acid composition of milk (Loy, 2007, Halmemies-Beauchet-Filleau et al., 2018, Correddu et al., 2020).

Recent advances in animal nutrition research have highlighted the relevance of plant-derived polyphenols as key bioactive molecules capable of modulating digestive efficiency, metabolic activity, and the quality of animal-derived products (Gessner et al., 2017, Forte et al., 2025b). Their chemical diversity, biological activity, and potential role in reducing methane emissions make them particularly attractive in the context of sustainable animal production (Jayanegara et al., 2010).

1.2. Chemical complexity of plant bioactive compounds used as livestock feed

Plants are known to produce an extraordinary diversity of bioactive compounds, a characteristic that has been recognized since ancient times. However, interest in these substances has grown significantly in recent decades following the emergence of the nutraceutical concept. The term, a combination of “nutrition” and “pharmaceutical,” was introduced to describe compounds naturally present in foods that are capable of exerting beneficial physiological effects on health (Bajaj, 2024). In animal nutrition, nutraceuticals have become a valuable category of feed additives and can be classified according to their mechanism of action, chemical structure, or source of origin. Their use has increased substantially in recent years due to their potential to promote animal health and productivity. Although the majority of nutraceutical research has focused on humans and non-ruminant species, numerous studies have also demonstrated positive outcomes in ruminant livestock

through the dietary inclusion of probiotics, prebiotics, bioactive lipids, functional peptides, and plant extracts (Ballou et al., 2019).

Among the vast array of plant-derived substances, secondary metabolites, also known as specialized metabolites, have attracted particular attention (Correddu et al., 2020). These compounds are organic molecules synthesized by plants primarily for defense, growth regulation, and stress response (Isah, 2019). They originate from the shikimate, acetate, and mevalonate/deoxyxylulose biosynthetic pathways, which convert primary and intermediate metabolites into structurally diverse secondary products. Because of this biochemical diversity, plants serve as a renewable source of bioactive compounds applicable to animal health and welfare. Within this group, phenols and polyphenols are among the most extensively studied due to their broad range of biological properties and effects on ruminant metabolism. Their inclusion in feed has been shown to influence rumen fermentation dynamics, biohydrogenation processes, milk yield and composition, and even inflammatory status (Jafari et al., 2019, Vasta et al., 2019).

Polyphenols comprise a large and chemically heterogeneous family of compounds characterized by one or more hydroxylated aromatic rings (Correddu et al., 2020). Their carbon skeletons vary considerably, reflecting their biosynthetic origin from the shikimate and/or acetate pathways, or from the integration of both (Formato et al., 2022a). The shikimate pathway leads to the formation of aromatic amino acids and phenylpropanoid derivatives, which in turn serve as precursors of coumarins, lignans, stilbenes, and flavonoids. The latter also partially derive from the acetate/malonate pathway, resulting in a wide structural diversity. Low-molecular-weight phenolic acids, such as protocatechuic, vanillic, and gallic acids, may originate from either biosynthetic route, depending on the substitution pattern of their aromatic ring (Formato et al., 2022a).

In ruminant feeding, the most relevant polyphenolic compounds are tannins and flavonoids. Flavonoids possess a C6–C3–C6 skeleton comprising two aromatic rings linked by a three-carbon bridge, forming a 15-carbon structure that occurs in six major subclasses (Olagaray and Bradford, 2019). Tannins, on the other hand, represent a complex group of polyphenolic polymers divided into

three main categories: hydrolysable tannins, condensed tannins, and phlorotannins. hydrolysable tannins and condensed tannins are typically found in terrestrial plants and have been the primary focus of research in recent years due to their effects on ruminal fermentation parameters, microbial populations, biohydrogenation, and milk yield and composition (Besharati et al., 2022a).

1.3. Metabolic fate of dietary polyphenols in dairy cows: ruminal and post-ruminal transformations

The metabolic fate of dietary polyphenols in dairy cows is a multifaceted process involving extensive bio-transformations, predominantly initiated by the rumen microbiota and subsequently passed through digesta, absorbed in the gastrointestinal tract and deposited in host tissues. In ruminants, the presence of the rumen introduces variables that differ markedly from monogastric species, as microbial bio-transformations precede host absorption and frequently determine the nature of circulating and ended as milk-borne metabolites (Aguiar et al., 2014). As a result, the chemical species that reach the absorptive epithelia—and are later detected in plasma and milk—are often conjugated or microbially derived catabolites rather than the natural dietary forms (Wein et al., 2016, Mena et al., 2019).

The rumen functions as the principal site for the initial transformation of polyphenols, acting as a dynamic bioreactor due to its dense and taxonomically diverse microbial population (Harun et al., 2025). The direction and extent of transformation depend on molecular class/solubility and on the enzymatic repertoire of the resident microbiota; importantly, a fraction of small polyphenols can also be absorbed across the rumen wall before reaching the small intestine (Scollan et al., 2005, Wein et al., 2016). The extent and nature of these microbial-mediated transformations are largely dependent on the polyphenol's chemical structure and solubility, as well as on the composition and enzymatic capabilities of the resident microbiota (Scollan et al., 2005). These early bioconversions in the rumen are pivotal in determining the profile of polyphenol compounds available for absorption in the subsequent sections of the gastrointestinal tract. A primary transformation is the hydrolysis of glycosidic bonds, as most dietary polyphenols are ingested in glycosylated forms. Classical and more

recent studies show rapid ruminal degradation of several dietary flavonoids. In *in vitro*, rutin, quercitrin, naringin and hesperidin are cleaved under anaerobic rumen conditions to water-soluble phenolic derivatives; rutin degrades rapidly unless fresh rumen fluid is replenished, whereas quercitrin degrades more slowly (Simpson et al., 1969). Consistently, incubations of rumen fluid with flavonoid-rich matrices reported sharp declines in myricetin, kaempferol, rutin, luteolin, ferulic and caffeic acids over 24–72 h, indicating fast microbial transformation; propyl gallate was comparatively stable (Kim et al., 2021). For quercetin, disappearance from rumen fluid coincides with the appearance of 3,4-dihydroxyphenylacetic acid and 4-methylcatechol, supporting ring-cleavage pathways (Berger et al., 2015). Phloroglucinol frequently arises as an intermediate from A/B-ring cleavage during degradation of dietary flavonoids (Cheng et al., 1969, Tsai et al., 1976). Rumen microbes possess an array of glycosidases capable of cleaving the glycosidic linkage, thereby liberating the sugar moiety and the more bioactive aglycone (Shivashankara and Acharya, 2010). This step is frequently essential for subsequent microbial degradation or for direct absorption. For instance, isoflavone glycosides from soy or red clover are rapidly deglycosylated to yield daidzein and genistein (Daems et al., 2016).

Further microbial transformations of polyphenolic skeletons proceed via specific metabolic routes:

- Isoflavones, once converted to their aglycones, undergo reductions and ring cleavage, yielding metabolites such as equol, O-desmethylangolensin (O-DMA), and p-ethylphenol (Bešlo et al., 2022). The compound formononetin, abundant in red clover, is first demethylated to daidzein and subsequently metabolized to equol (Daems et al., 2016). *In vitro* work shows diet form and dose rate can modulate equol output by rumen fluid (Dadáková et al., 2020). Earlier rumen studies confirmed hydrolysis of isoflavone glycosides and heterocyclic ring opening in situ, underpinning downstream formation of equol and O-DMA (McSweeney and Mackie, 1997).
- Flavan-3-ols, including monomers like catechin and epicatechin (from tea or grape by-products) as well as proanthocyanidins, are extensively degraded in the rumen. This degradation involves C-ring cleavage and formation of low-molecular-weight metabolites

such as phenyl-γ-valerolactones (PVLs) and phenylvaleric acids (PVAs) (Mena et al., 2019). Experimental evidence demonstrates that intraruminal administration of green tea extract results in near-complete microbial metabolism of catechins, while bypassing the rumen (via intraduodenal administration) yields significantly higher plasma levels of the intact molecules (Wein et al., 2016).

- Flavonols such as quercetin also undergo microbial catabolism in the rumen, resulting in simpler phenolic acids including 3,4-dihydroxyphenylacetic acid and phloroglucinol (Harun et al., 2025). By contrast, anthocyanins in some plant matrices appear relatively resistant to ruminal degradation *in vitro*, implying that absorption is largely intestinal (Hosoda et al., 2009).
- Hydrolyzable tannins are cleaved in the rumen into their constituent sugars and phenolic acids—typically gallic acid from gallotannins and ellagic acid from ellagitannins (Besharati et al., 2022b). These acids are then metabolized into urolithins (e.g., urolithin A, B, C, D, and isourolithin A), although the bioavailability of ellagic acid is inherently low (González-Barrio et al., 2012). Studies in beef cattle consuming oak leaf ellagitannins indicate isourolithin A and urolithin B as predominant metabolites (González-Barrio et al., 2012). Hydrolysable tannins can be degraded by rumen tannase-producing microbes; *Streptococcus gallolyticus* (formerly *S. caprinus*) decarboxylates gallic acid to pyrogallol, while Selenomonas ruminantium K2 releases gallic acid from gallotannins (O'Donovan and Brooker, 2001). More broadly, rumen bacteria and anaerobic fungi expressing tannases cleave galloyl esters and ellagitannin moieties, facilitating formation of gallic/ellagic acid precursors to urolithins (Kim et al., 2021). A schematic representation of these processes is provided in Figure 2.
- Condensed tannins, or proanthocyanidins, are more recalcitrant to microbial degradation. Nonetheless, partial depolymerization or structural modification may occur. Their principal ruminal function is often attributed to their strong protein-binding capacity, which can reduce

ruminal protein degradation and modulate fermentation patterns (Powell et al., 2009) therby increasing the bypass protein availability for host animal absorption.

- Phenolic acids, either directly ingested or released from complex polyphenols, are subject to microbial transformations such as decarboxylation, demethylation, and reduction. For example, ferulic and caffeic acids can be converted into phenylpropionic, phenylacetic, and benzoic acid derivatives(with dihydrocaffeic acid frequently observed as a key reduction product) (Zieniuk, 2023). Protocatechuic acid may be further decarboxylated to catechol in the rumen, although catechol utilisation appears limited (Simpson et al., 1969).

Overall, the metabolic activity of rumen microbiota produces a dramatically altered profile of polyphenol compounds compared to the ingested forms, and this bio-transformation is central to understanding the systemic availability and biological activity of these metabolites. Additionally, dietary polyphenols can modulate ruminal fermentation parameters:

- Certain compounds, notably flavonoids and tannins, are associated with reduced methane emissions, potentially offering both environmental and nutritional benefits (Bešlo et al., 2022, Harun et al., 2025). Mechanistically, tannins can shift microbial consortia and hydrogen sinks, but responses vary with dietary source and dose rate (Jayanegara et al., 2012b, Herremans et al., 2020).
- Polyphenols can alter the volatile fatty acid (VFA) profile, typically decreasing the acetate:propionate ratio, which may have implications for energy metabolism in the host (Wang et al., 2021). Multiple trials with plant polyphenol sources report lower A:P and higher propionate—consistent with modest shifts toward more energy-efficient fermentation—though effects are not universal (Jalal et al., 2025).
- Through their protein-binding activity, tannins reduce protein degradation and consequently lower ruminal ammonia concentrations, improving nitrogen retention if post-ruminal digestion is efficient (Aguiar et al., 2014). Meta-analytic evidence indicates limited or no

average effect of tannins on milk yield/composition at practical doses despite improved N partitioning (Herremans et al., 2020).

Notably, inter-animal variability ("metabotypes") in microbial composition and function can produce substantial differences in the formation of equol (from isoflavones) or urolithins (from ellagitannins), even under identical diets—adding uncertainty to expected metabolite levels in milk (González-Barrio et al., 2012, Dadáková et al., 2020).

Polyphenol aglycones and microbial-derived metabolites that survive or bypass ruminal degradation can either be absorbed across the rumen epithelium or continue into the small intestine. "Intestinal uptake occurs mainly in the duodenum/jejunum via passive diffusion for low-molecular-weight polyphenols and aglycones, with contributions from carrier-mediated routes involving PEPT1 and ABC transporters (e.g., MRP2, BCRP) (Manach et al., 2004, Bohn, 2014). Once absorbed through the intestinal epithelium and transported to the liver via the portal circulation, they undergo Phase I and Phase II metabolic processes (Shivashankara and Acharya, 2010). While Phase I reactions (oxidation, hydrolysis, etc.) play a minor role for most polyphenols, Phase II conjugation reactions—glucuronidation, sulfation, and O-methylation—predominate (Wein et al., 2016). Following conjugation, circulating metabolites bind plasma proteins—predominantly albumin—with affinities that depend on substitution patterns and conjugation type (Boulton et al., 1998, Dangles et al., 2001). These reactions enhance hydrophilicity, thereby facilitating excretion, often at the cost of reducing apparent bioactivity. For instance, urolithins absorbed in the gut circulate mainly as glucuronide and sulfate conjugates, and similarly, tea catechins are extensively conjugated prior to systemic distribution (González-Barrio et al., 2012, Wein et al., 2016).

Furthermore, enterohepatic circulation plays a significant role in prolonging systemic exposure to certain polyphenol metabolites. Conjugates secreted in bile can be hydrolyzed by intestinal microbiota, releasing aglycones that are reabsorbed into the bloodstream (Shivashankara and Acharya, 2010). In the large intestine, a substantial portion of polyphenols—particularly those with high molecular weights such as proanthocyanidins and ellagitannins—are further metabolized by

colonic bacteria. This hindgut fermentation leads to the generation of low-molecular-weight phenolic acids and aromatic catabolites that are often bioavailable (Shivashankara and Acharya, 2010). For instance, colonic fermentation of flavan-3-ols produces phenyl-γ-valerolactones, while ellagic acid is converted into bioactive urolithins (Espín et al., 2013, Mena et al., 2019). Estimates from nutritional pharmacokinetics suggest that a very large fraction of ingested dietary polyphenols reaches the hindgut, underscoring the colon's role as a key site for generating absorbable metabolites (Shivashankara and Acharya, 2010). While ruminal degradation can lower the bioavailability of certain monomeric flavonoids, microbial depolymerisation/deglycosylation enables utilisation and subsequent absorption of polymeric forms otherwise too large for small-intestinal uptake (Berger et al., 2015, Olagaray and Bradford, 2019). Consistent with limited ruminal degradation, anthocyanin-rich forages appear to rely on intestinal absorption, via mechanisms broadly comparable to those described in monogastric animals (Hosoda et al., 2009). In summary, the metabolic journey of dietary polyphenols in dairy cows encompasses a sequence of microbial and enzymatic processes, beginning with extensive degradation and transformation in the rumen and continuing with absorption, conjugation, and further catabolism in post-ruminal compartments. These processes collectively determine the spectrum and concentration of polyphenol-derived metabolites available for mammary uptake and potential transfer to milk. In line with this, bovine plasma and milk typically contain conjugated metabolites (e.g., glucuronides/sulfates of polyphenols) rather than parent aglycones (González-Barrio et al., 2012, Wein et al., 2016). Figure 1 provides a schematic overview of the metabolic pathways and organ-specific transformations involved in the transfer of dietary polyphenols from feed to the mammary gland and milk.

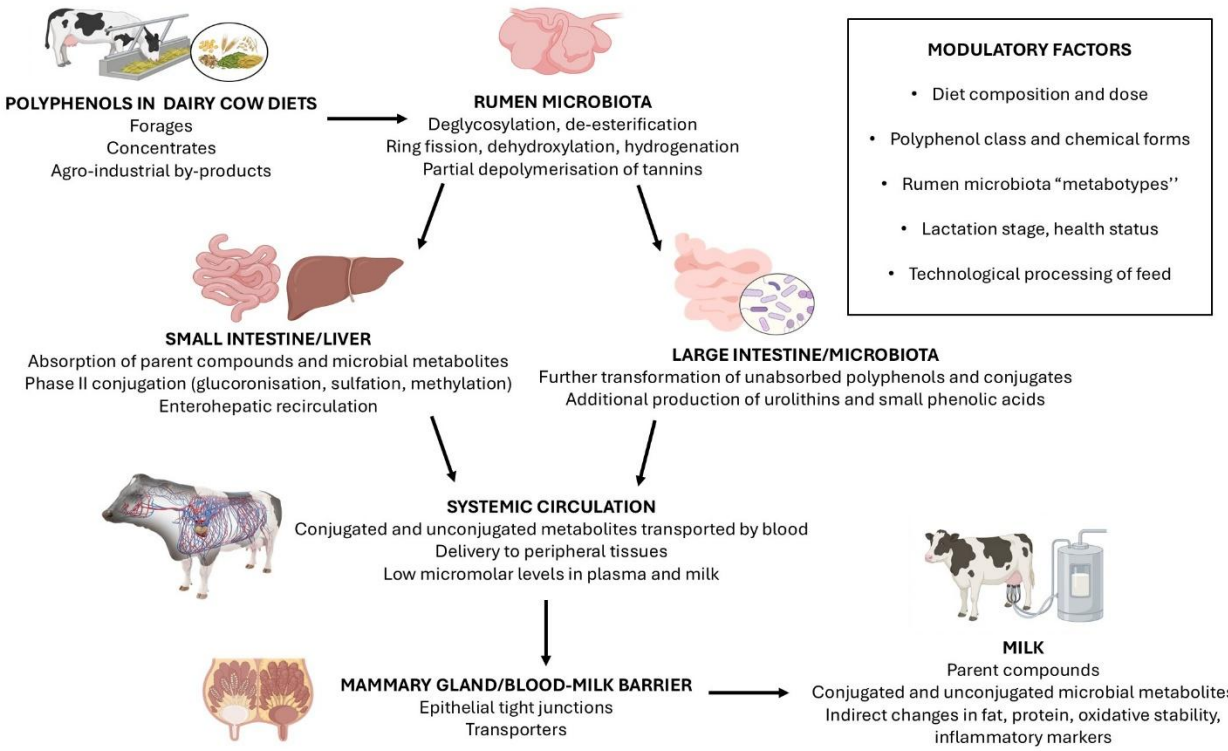

Figure 1. Schematic representation of the metabolic fate of dietary polyphenols from feed ingestion to milk secretion in dairy cows.

1.4. The role of rumen fermentation in polyphenol efficacy

Ruminants possess a highly specialized digestive system that allows them to efficiently utilize fibrous plant materials such as grasses, forages, and agricultural by-products that are otherwise indigestible to monogastric animals. Their stomach is divided into four compartments (rumen, reticulum, omasum, and abomasum) each performing distinct physiological roles. Among these, the reticulo-rumen serves as the primary site for microbial fermentation, hosting a complex symbiotic community of *bacteria*, *protozoa*, *fungi*, and *archaea* that enable the degradation of structural carbohydrates, proteins, and lipids. The digesta then passes to the omasum, where water is reabsorbed and particle size is reduced, and subsequently to the abomasum, the "true stomach," where enzymatic digestion occurs before nutrient absorption in the small intestine. Any unabsorbed material proceeds to the large intestine and is ultimately excreted (Reddy and Hyder, 2023).

The efficiency of this system depends strongly on the chemical and nutritional composition of the feed supplied to the animal. Feed digestibility is determined primarily by the relative proportions of organic and inorganic components within the dry matter (DM) fraction. Carbohydrates constitute the principal component of ruminant feed, while other conventional parameters include moisture, ash, crude protein (CP), ether extract (EE), crude fiber (CF), and nitrogen-free extract (NFE). Modern feed analysis techniques also distinguish between cell contents and cell wall fractions, the latter being quantified as neutral detergent fiber (NDF) after treatment with a neutral detergent solution. NDF represents the sum of cellulose, hemicellulose, and lignin, which are negatively correlated with voluntary feed intake. Within NDF, the acid detergent fiber (ADF) fraction corresponds to cellulose and lignin, while acid detergent lignin (ADL) represents lignin alone. Whereas cell contents are almost completely digestible, the digestibility of cell wall components is highly variable and depends largely on the degree of lignification (McDonald and Warner, 1975).

While chemical analyses of feed provide valuable insights into composition, the fermentation processes within the rumen are highly complex and dynamic. *In vivo* trials remain the most reliable approach to assess digestive dynamics in ruminants; however, in vitro fermentation systems offer simplified and controlled environments that help elucidate microbial and biochemical mechanisms. These in vitro methods provide data on dry matter digestibility, crude protein degradation, gas production, and fermentation kinetics, as well as information on end products such as methane, pH, volatile fatty acids (VFAs), and ammonia concentrations (Vastolo et al., 2021, Formato et al., 2022b, Vastolo et al., 2022).

During rumen fermentation, carbohydrates are first broken down into simple sugars, which are then metabolized by microorganisms to produce VFAs, gases, and microbial biomass. The primary VFAs, acetic, propionic, and butyric acids, serve as major energy sources for the host animal. Pyruvate acts as a central metabolic intermediate, linking glycolysis to the production of these end products, along with the generation of carbon dioxide (CO_2) and methane (CH_4), the latter primarily eliminated through eructation. Imbalances in VFA production, particularly the excessive accumulation of

organic acids, can lead to subacute ruminal acidosis, a metabolic disorder associated with laminitis, diarrhea, rumen wall damage, rumenitis, and liver abscesses in dairy cattle (Plaizier et al., 2012).

A secondary stage of microbial digestion occurs in the large intestine, where additional VFAs are produced and absorbed, while microbial cells are excreted with undigested residues (McDonald and Warner, 1975). The proportions of acetate, propionate, and butyrate are crucial, as they influence key metabolic pathways. Acetate is primarily utilized in lipogenesis, propionate serves as a major precursor for gluconeogenesis, and butyrate is converted to D-3-hydroxybutyrate. The balance between acetate and propionate is particularly significant for maintaining the hydrogen equilibrium in the rumen: acetate formation generates hydrogen, while propionate synthesis consumes it, thereby influencing methanogenesis (Moss et al., 2000).

Other branched-chain volatile fatty acids (BCVAs) are produced from the deamination of amino acids, such as isobutyric acid from valine, valeric acid from proline, 2-methylbutyric acid from isoleucine, and 3-methylbutyric acid from leucine (McDonald and Warner, 1975). Proteins in the feed are hydrolyzed by rumen microorganisms into peptides and amino acids, which are further degraded into organic acids, ammonia, and CO_2. The resulting ammonia is reused by microbes for microbial protein synthesis, serving as an indicator of the efficiency of dietary nitrogen conversion (Firkins et al., 2007). Nitrogen in the rumen can also derive from non-protein sources such as amides, amines, and inorganic nitrogen compounds including nitrates.

The rumen microbiota forms a diverse anaerobic ecosystem comprising *bacteria*, *protozoa*, *archaea*, *fungi*, and viruses (Matthews et al., 2019, Cholewińska et al., 2020). Each group contributes distinct enzymatic and metabolic functions, influencing fermentation efficiency and end-product composition. Therefore, compounds with antimicrobial activity, such as certain plant secondary metabolites, may selectively modulate microbial populations, enhance nutrient digestion, and alter fermentation profiles. Through these mechanisms, plant secondary metabolites, especially polyphenols, can affect VFA production, methane emissions, and microbial composition within the rumen (Ma et al., 2020).

The complex interplay between microbial metabolism, substrate availability, and dietary factors underscores the importance of evaluating polyphenols not only as antioxidant or anti-inflammatory agents but also as modulators of ruminal fermentation. Their impact on fermentation parameters, methane generation, and microbial ecology represents a critical aspect of their potential use in sustainable ruminant nutrition.

1.4.1. Rumen microbiota composition

The rumen hosts a highly diverse and dynamic microbial ecosystem whose composition is strongly influenced by animal species, dietary composition, and feeding frequency. The main bacterial *phyla* commonly identified in ruminants include *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Fibrobacteres*, and, to a lesser extent, *Tenericutes* and *Actinobacteria* (Khafipour et al., 2016). These microbial communities can be classified according to their predominant metabolic functions, encompassing cellulolytic, amylolytic, proteolytic, lipolytic, methanogenic, saccharolytic, tanninolytic, pectinolytic, ureolytic, and acetogenic groups.

Among cellulolytic bacteria, three species are particularly important: *Fibrobacter succinogenes* (Gram-negative), *Ruminococcus flavefaciens* (Gram-positive), and *Ruminococcus albus* (Gram-variable). These microorganisms play a central role in fiber degradation, producing acetate, butyrate, propionate, and carbon dioxide as major fermentation end-products (Koike and Kobayashi, 2009). Pectinolytic *bacteria*, which specialize in the breakdown of pectins and other complex polysaccharides, include *Lachnospira multiparus*, *Prevotella ruminicola*, and *Butyrivibrio fibrisolvens* (Dušková and Marounek, 2001). The activity of *L. multiparus* is particularly relevant, as it converts pectin to oligogalacturonides while generating significant amounts of acetate.

Proteolytic *bacteria*, such as members of the *genera Clostridium*, *Bacillus*, and *Proteobacteria*, hydrolyze proteins into smaller peptides and amino acids. These microorganisms produce proteases and ureases, enzymes capable of degrading urea into carbon dioxide and ammonia, contributing to nitrogen turnover within the rumen. Similarly, lipolytic *bacteria*, including *Anaerovibrio lipolytica*

and *Butyrivibrio fibrisolvens*, catalyze the hydrolysis of triglycerides and phospholipids, releasing free fatty acids for further microbial metabolism.

The rumen also contains a specialized group of methanogenic *archaea*, primarily belonging to the orders *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, *Methanopyrales*, and *Methanobacteriales*. Within these, four genera, *Methanobacterium*, *Methanobrevibacter*, *Methanosphaera*, and *Methanothermobacter*, represent the dominant methanogens in most ruminant species (Patra et al., 2017). These *archaea* play a pivotal role in maintaining hydrogen balance by reducing carbon dioxide (CO_2) to methane (CH_4) using molecular hydrogen (H_2) produced during fermentation. Recent research on methanogenic populations has deepened understanding of how dietary composition and microbial interactions influence methane emissions from livestock (Knapp et al., 2014).

Protozoa represent another major microbial group in the rumen ecosystem. Rumen *protozoa* are traditionally divided into two main functional categories: holotrichs and entodiniomorphs. Holotrichs utilize soluble sugars as their primary energy source, while entodiniomorphs regulate the rate of carbohydrate fermentation, particularly under diets rich in soluble carbohydrates. The latter group contributes to starch regulation by engulfing starch granules, thus moderating the rate of fermentation and preventing metabolic disorders such as acidosis (Formato et al., 2022a).

Although anaerobic *fungi* constitute a smaller fraction of the rumen biomass, their contribution to fiber degradation is substantial. These *fungi* colonize lignocellulosic materials, penetrating plant cell walls through their rhizoidal structures and secreting an array of hydrolytic enzymes, including cellulases, xylanases, esterases, and β-glucosidases. Their activity facilitates the breakdown of recalcitrant plant fibers, particularly when ruminants consume highly lignified forages. Common fungal species identified in the rumen include *Neocallimastix frontalis*, *Neocallimastix hurleyensis*, *Anaeromyces elegans*, *Anaeromyces mucronatus*, *Orpinomyces joyonii*, *Orpinomyces intercalaris*, and *Piromyces communis*. In buffalo, species such as *Orpinomyces bovis*, *Piromyces communis*, and *Cyllamyces aberensis* have also been reported (Agarwal et al., 2015).

The composition and activity of the rumen microbiota exert profound effects on feed degradation efficiency, methane production, biohydrogenation (BH) processes, and the composition of milk and meat. These microbial dynamics are highly responsive to dietary inputs, meaning that the inclusion of plant secondary metabolites such as polyphenols can modulate microbial populations and fermentation outcomes. Numerous studies have shown that polyphenols can selectively inhibit methanogenic *archaea* or *protozoa* while enhancing bacterial species involved in propionate formation or fiber digestion (Chen et al., 2020).

Given these intricate relationships, the evaluation of polyphenols and other non-nutrient phytochemicals in ruminant diets must consider their multifaceted influence on the rumen ecosystem. By altering the balance among microbial groups, polyphenolic compounds can directly or indirectly affect fermentation efficiency, methane emissions, and nutrient utilization, thus contributing to improved feed efficiency and reduced environmental impact.

1.4.2. Effect of polyphenols on rumen fermentation parameters

Methane (CH_4) is one of the most important greenhouse gases, and ruminants are among its major biological sources. Each animal can produce between 250 and 500 liters of methane per day, making cattle a significant contributor to global methane emissions and, consequently, to climate change (Starsmore et al., 2024). From a nutritional standpoint, methane production also represents an energy loss for the animal, as it accounts for up to 6–10% of gross energy intake. Methane arises primarily from microbial fermentation in the rumen (about 87–90%) and, to a lesser extent, in the large intestine (10–13%). During this process, methanogenic *archaea* utilize hydrogen (H_2) and carbon dioxide (CO_2) produced by other microbes to generate CH_4 as the final product of anaerobic respiration (Kuhla and Viereck, 2022).

To mitigate methane emissions from livestock, numerous strategies have been explored, ranging from feed reformulation to the use of natural additives. Among these, the inclusion of plant-derived

compounds, particularly polyphenols and tannins, has emerged as a promising approach due to their dual role in modulating rumen microbiota and improving feed efficiency(Maggiolino et al., 2019).

Polyphenols influence rumen fermentation through direct and indirect mechanisms. Indirectly, they reduce feed degradability by binding to dietary proteins and carbohydrates, thereby limiting substrate availability for methanogens (Finlay et al., 1994, Morgavi et al., 2012). Directly, they exert antimicrobial effects by inhibiting methanogenic *archaea* and altering the activity of microbial enzymes involved in fermentation (Cieslak et al., 2013). Furthermore, polyphenols can shift fermentation pathways by promoting propionate formation, a process that consumes hydrogen, thus reducing the availability of H_2 for methanogenesis (Chen et al., 2020). The chemical structure of individual flavonoids, including the number and position of hydroxyl or methoxy groups, strongly influences their antibacterial activity and overall impact on fermentation (Xie et al., 2015).

Experimental studies have shown that polyphenol-rich extracts from various plants can significantly alter rumen fermentation patterns. For instance, an alcoholic extract of *Portulaca oleracea*, rich in kaempferol, quercetin, apigenin, and luteolin derivatives, was found to increase total volatile fatty acids (VFAs), especially acetate and propionate, while reducing methane production by 9.44% and halving methanogen abundance compared with control treatments (Wang et al., 2013). Similarly, a methanolic extract from *Pinus radiata* bark, containing flavonoids such as luteolin, quercetin, taxifolin, and catechin, reduced CH_4 generation in a dose-dependent manner while increasing VFA production after 24 hours of incubation (Vera et al., 2021).

Extracts from Brazilian spinach (*Alternanthera sissoo*) also demonstrated a marked reduction in protozoal populations and methane output, decreasing CH_4 concentrations from 20.22 mmol/L to 13.48 mmol/L while elevating propionate levels (Sommai et al., 2021). The influence of flavonoids appears to depend on both the aglycone structure and the degree of glycosylation. When several flavonoids were tested at equivalent concentrations, methane emissions followed the descending order: myricetin ≥ kaempferol ≥ quercetin ≥ naringin ≥ rutin ≥ catechin (Oskoueian et al., 2013).

However, not all findings have been consistent. Some studies using single purified compounds have shown limited or no effect on methanogenesis. For example, metabolomic analyses of rumen fluid incubated with catechin suggested that it may act as a hydrogen sink, sequestering up to three H_2 molecules, yet without a significant reduction in methane levels (Becker et al., 2014). Similarly, rutin supplementation at 100 mg/kg in dairy cow diets did not reduce CH_4 emissions (Stoldt et al., 2016), and both rutin and quercetin showed negligible effects in vitro (Berger et al., 2015). Nevertheless, in vivo supplementation with rutin increased total VFAs without altering protozoal abundance (Cui et al., 2015).

The degree of oxidation in the flavonoid nucleus also appears to influence its efficacy. For instance, luteolin-7-O-glucoside was found to suppress methane production more effectively than quercetin, isoquercitrin, or catechin derivatives, exhibiting a comparable effect to tannic acid but without affecting the acetate-to-propionate ratio or protozoal counts (Sinz et al., 2018). Although total VFAs may remain unchanged, shifts in the acetate/propionate balance often occur, favoring propionate formation and consequently reducing CH_4 generation (Seradj et al., 2014).

The observed reduction in total gas and methane during incubation with polyphenol-rich plant extracts can be attributed to several mechanisms:

- Decreased protozoal abundance and methanogen activity, particularly those associated with protozoal surfaces;
- Direct inhibition of methanogenic archaea;
- Alterations in VFA profiles, leading to changes in hydrogen availability;
- Suppression of cellulolytic enzyme activity, which can affect fiber degradation.

In an in vitro study, Kim et al. (2015) reported variable effects on VFA concentration depending on incubation time, while methane output consistently decreased relative to the control. These effects were associated with a >60% reduction in protozoal populations, accompanied by an increase in *Fibrobacter succinogenes* abundance and a decline in *Ruminococcus albus* and *Ruminococcus flavefaciens*.

The chemical composition of plant extracts plays a crucial role in determining their activity. For example, olive leaf extracts (*Olea europaea L.*), containing high levels of oleuropein, hydroxytyrosol, tyrosol, caffeic acid, and luteolin, significantly reduced methane emissions after 12 hours of incubation while maintaining a stable acetate/propionate ratio (Boussaada et al., 2017, Lee et al., 2021). Similar effects were reported for propolis extracts, which increased acetate formation, likely linked to enhanced fatty acid synthesis, while reducing methane output (de Paula et al., 2016).

Condensed and hydrolysable tannins (CTs and HTs) have been identified as particularly potent modulators of methanogenesis, often displaying stronger activity than their monomeric counterparts. Leaves of neem (*Azadirachta indica*), for example, containing high levels of total phenols but moderate CT concentrations, reduced methane emissions by up to 61.5%, likely through direct inhibition of methanogenic archaea (Bhatta et al., 2015). Likewise, extracts from chestnut (*Castanea spp.*), sumac (*Rhus typhina*), mimosa (*Mimosa tenuiflora*), and quebracho (*Schinopsis balansae*) demonstrated significant methanogen inhibition at concentrations around 1 mg/mL (Jayanegara et al., 2015).

The effects of tannins are dose-dependent. Supplementation with quebracho extract at 2–3% of dry matter decreased total VFAs (Dschaak et al., 2011), while a hydroalcoholic extract from *Castanea mollissima* involucres reduced methane yield at only 0.2% inclusion (Wang et al., 2021). Similar anti-methanogenic effects have been reported for valonia oak (*Quercus ithaburensis* subsp. *macrolepis*), Myrobalan plum (*Prunus cerasifera*), green tea (*Camellia sinensis*), and grape seed (*Vitis vinifera*) extracts (Pellikaan et al., 2011, Thanh et al., 2022). Grape by-products, including grape marc, also decreased methane emissions by approximately 20% when used as feed supplements (Moate et al., 2014).

Variability in results can often be attributed to differences in tannin concentration, plant species, plant part used, harvest time, and geographical origin. For instance, sainfoin (*Onobrychis viciifolia*) collected at different phenological stages showed variable methane reduction potential due to fluctuations in its condensed tannin content (Guglielmelli et al., 2011). Likewise, buckwheat

(*Fagopyrum esculentum*) flowers, but not leaves, reduced methane production by 10%, performing similarly to rutin supplementation at 50 mg (Guglielmelli et al., 2011). Seasonal variations also influence tannin content, as observed in *Acacia* species, where *A. mearnsii* exhibited higher nutritional and nutraceutical potential than *A. dealbata*, with methanogenesis inhibition noted at 30 g/kg DM (equivalent to 15 g total tannin/kg) (Denninger et al., 2020, Uushona et al., 2021).

In vivo studies on *A. mearnsii* extracts at inclusion levels up to 100 g tannin extract per cow per day showed moderate reductions in methane compared with the ionophore monensin, suggesting that plant polyphenols may complement rather than replace conventional additives (Junior et al., 2017, Perna et al., 2020). Combinations of *Acacia*, *Punica granatum*, and *Eugenia jambolana* extracts have also shown synergistic effects in lowering methane emissions by suppressing methanogenic and protozoal activity while reducing hydrogen release during fiber fermentation (Singh et al., 2022).

Although the exact mechanism underlying the anti-methanogenic action of tannins remains unclear, their capacity to form complexes with proteins and carbohydrates appears central to their function. Additionally, tannins often coexist with other bioactive compounds, which may act synergistically or antagonistically, depending on extraction methods and chemical composition. For example, tannin-rich extracts from *Vaccinium vitis-idaea* reduced methane emissions by 8.5% and protozoal counts by 35% without affecting total methanogen abundance (Cieslak et al., 2012). When tested using the Rumen Simulation Technique (RUSITEC), the same extract achieved a 21.9% methane reduction (Cieslak et al., 2014).

Other plant sources, such as sage (*Salvia officinalis*), rich in rosmarinic and salvianolic acids, have also been investigated for their effects on rumen fermentation. At inclusion levels of 40–100 mg, sage extract reduced both methane and total VFAs, while lower doses (around 4 mg) selectively modulated protozoal activity (Cieslak et al., 2016). Brown seaweeds (*Undaria pinnatifida*, *Sargassum fusiforme*, and *Sargassum fulvellum*) similarly decreased methane formation after 12–24 hours of incubation, primarily by reducing protozoal and fungal populations, although methanogenic *archaea* were less affected (Choi et al., 2021).

Overall, the available literature suggests that polyphenolic compounds exert variable yet significant effects on rumen fermentation, methane generation, and microbial ecology. These discrepancies often arise from differences in extraction techniques, solvent polarity, and chemical composition of the extracts, all of which can drastically influence bioactivity. For this reason, a precise chemical characterization of polyphenolic preparations is essential to understand their mechanism of action and optimize their use as feed additives in sustainable ruminant production systems.

1.5. Polyphenols and ruminal biohydrogenation: implications for milk lipid profile

Ruminants are unique among mammals for their ability to modify the fatty acid composition of dietary lipids through the combined actions of ruminal microorganisms. Lipids present in feed are primarily composed of triglycerides, phospholipids, and glycolipids, which are subjected to extensive lipolysis and biohydrogenation (BH) during digestion. These transformations substantially alter the lipid profile of animal-derived products such as milk and meat (Lourenço et al., 2010).

After ingestion, dietary fats undergo hydrolysis by microbial lipases and phospholipases, releasing free fatty acids (FFAs) and glycerol. The FFAs are then subjected to biohydrogenation, a process by which unsaturated fatty acids (UFAs) are sequentially hydrogenated to produce more saturated end-products. The main substrates for BH are linoleic acid (C18:2 n-6) and α-linolenic acid (C18:3 n-3), which are abundant in plant-based feeds and forages. The complete hydrogenation of these UFAs results in the formation of stearic acid (C18:0), which is subsequently absorbed in the small intestine and incorporated into animal tissues.

The biohydrogenation process involves multiple enzymatic reactions carried out primarily by bacteria belonging to the *Butyrivibrio genus*, particularly *Butyrivibrio fibrisolvens* and *Butyrivibrio proteoclasticus* (Lourenço et al., 2010). These microorganisms catalyze the isomerization of linoleic and linolenic acids into conjugated intermediates, which are then reduced to monounsaturated and saturated fatty acids. During this pathway, several conjugated linoleic acid (CLA) and conjugated

linolenic acid (CLnA) isomers are formed, including the biologically significant cis-9, trans-11 CLA (rumenic acid) and trans-11 C18:1 (vaccenic acid) (Or-Rashid et al., 2008).

These intermediates are of particular interest due to their recognized nutritional and health-promoting properties. Numerous studies have demonstrated that CLAs and CLnAs exhibit antioxidant, anti-inflammatory, anti-obesity, and anticarcinogenic effects (Nam and Garnsworthy, 2007). Moreover, rumenic and vaccenic acids serve as biomarkers of rumen microbial activity and diet composition, reflecting the degree of unsaturated fatty acid biohydrogenation within the rumen.

The balance between saturation and desaturation processes is strongly influenced by diet composition, especially by the ratio of forage to concentrate, lipid supplementation, and the presence of bioactive compounds such as tannins and flavonoids. These compounds can interfere with the activity of microbial lipases and hydrogenases, leading to partial inhibition of biohydrogenation and an increased outflow of unsaturated fatty acids to the duodenum (Chilliard et al., 2007, Jayanegara et al., 2011).

Polyphenols, particularly tannins, have been shown to modulate biohydrogenation pathways by altering microbial populations involved in lipid metabolism. Their interaction with cell membranes and extracellular enzymes can affect the growth and activity of *Butyrivibrio* species, thereby modifying the proportions of intermediate fatty acids such as C18:1 trans-11 and C18:2 cis-9, trans-11. Studies have indicated that condensed tannins may suppress the final reduction step that converts vaccenic acid to stearic acid, resulting in higher levels of CLA and trans-monounsaturated intermediates in ruminal fluid and milk fat (Lee et al., 2004, Khiaosa-Ard et al., 2009, Jayanegara et al., 2012a).

For example, supplementation with quebracho tannin extract reduced the extent of BH and increased the accumulation of vaccenic acid in rumen contents, suggesting inhibition of *Butyrivibrio proteoclasticus* activity (Cabiddu et al., 2010). Similarly, chestnut tannins, rich in hydrolysable compounds such as gallic and ellagic acids, were found to modify the fatty acid composition of ruminal digesta by increasing the proportion of unsaturated intermediates while lowering stearic acid levels (Halmemies-Beauchet-Filleau et al., 2013).

Polyphenolic effects on lipid metabolism are also linked to their antioxidant capacity, which may protect unsaturated fatty acids from oxidative degradation both in the rumen and in the final animal products. By modulating microbial enzyme systems and oxidative stress, polyphenols help preserve beneficial fatty acid profiles characterized by higher proportions of polyunsaturated fatty acids (PUFAs) and omega-3 series lipids (Vasta et al., 2008).

Additionally, the structure and reactivity of polyphenols determine their specific impact on BH. Flavonoids such as quercetin and naringenin can influence lipase activity and microbial membrane permeability, whereas phenolic acids may act as hydrogen donors or scavengers, thus modifying redox balance in the rumen environment. Synergistic interactions between different phenolic subclasses are also possible, complicating the prediction of their effects under practical feeding conditions (Ishlak et al., 2015).

Several *in vivo* trials have confirmed that dietary polyphenols can improve the fatty acid profile of milk and meat, enhancing their nutritional quality. For instance, feeding dairy cows with grape seed extracts or olive by-products rich in phenolic compounds has been associated with increased concentrations of CLA, vaccenic acid, and omega-3 fatty acids in milk fat (Kronberg et al., 2007, Szczechowiak et al., 2016). Similar outcomes have been observed in sheep and goats fed diets supplemented with tannin-containing plants, where reduced biohydrogenation was accompanied by lower methane emissions and improved oxidative stability of milk fat (Sato et al., 2020).

However, the magnitude and direction of these effects depend on several factors, including the type and concentration of polyphenols, the animal species, and the overall dietary matrix. Excessive tannin intake may reduce nutrient digestibility and microbial activity, potentially leading to lower feed efficiency. Therefore, optimal inclusion levels must be determined to achieve a balance between methane mitigation, enhanced fatty acid quality, and preserved digestive efficiency (Besharati et al., 2022a).

Overall, the modulation of ruminal biohydrogenation by plant polyphenols represents a valuable nutritional strategy for improving the fatty acid composition of animal-derived foods while

simultaneously contributing to environmental sustainability. Understanding the structure/function relationships of these compounds and their interactions with rumen microbiota is essential for developing targeted feed interventions that optimize both animal performance and product quality.

1.6. Effects of polyphenols on milk and meat production

Beyond their well-documented antioxidant and antimicrobial activities, polyphenols can influence several physiological and metabolic processes, including feed intake, nutrient digestibility, milk yield, and milk composition (Lourenço et al., 2008). At appropriate inclusion levels, polyphenols may improve animal performance by enhancing digestive efficiency and modulating rumen fermentation patterns. However, at excessive concentrations, their ability to bind proteins and carbohydrates can decrease nutrient availability, leading to reduced feed efficiency and productivity. Thus, the dose-dependent behavior of polyphenols remains a crucial factor in determining their overall impact on ruminant nutrition (Keshri et al., 2021).

Several studies have demonstrated the beneficial effects of polyphenols on milk yield and composition. For example, supplementation of dairy cow diets with chestnut tannins at 0.15–0.3% of dry matter improved milk protein content, while slightly reducing milk fat and somatic cell count, an indicator of udder health (Xiao et al., 2021). The improved protein fraction is attributed to the protective action of tannins, which form complexes with dietary proteins, reducing ruminal degradation and increasing amino acid flow to the small intestine.

Similarly, grape by-products (skins, seeds, and pomace), rich in condensed tannins and flavonoids, have been shown to enhance milk antioxidant status and modify its fatty acid composition. The inclusion of grape marc in dairy cow diets at 5–10% of dry matter reduced milk saturated fatty acids while increasing monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA), particularly CLA and omega-3 series lipids (Bradford et al., 2015). These effects were also associated with a reduction in methane emissions, suggesting a dual environmental and nutritional benefit.

Olive by-products, such as olive leaves and olive pomace, represent another important source of bioactive polyphenols, particularly oleuropein, hydroxytyrosol, and tyrosol. Their dietary inclusion has been linked to enhanced milk oxidative stability and improved sensory quality (Abuelo et al., 2019). In ewes, supplementation with olive cake increased milk concentrations of unsaturated fatty acids and reduced oxidative stress markers, likely due to the strong antioxidant capacity of the phenolic compounds (Winkler et al., 2015). Similar improvements have been reported in goats, where polyphenol-rich diets contributed to higher milk yield and increased total antioxidant capacity (Olagaray et al., 2019).

In addition to their effects on milk production, polyphenols can improve animal health and welfare by modulating inflammatory and oxidative pathways. The antioxidant activity of polyphenols helps to neutralize reactive oxygen species (ROS) and protect cellular components from oxidative damage. In high-producing dairy cows, oxidative stress is a common challenge associated with metabolic disorders such as ketosis, mastitis, and reproductive inefficiency. Dietary supplementation with polyphenols has been shown to enhance the activity of endogenous antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT), thereby contributing to metabolic stability and immune resilience (Hao et al., 2020).

Polyphenols also exhibit anti-inflammatory properties, which can help mitigate tissue inflammation and improve overall health status. Flavonoids such as quercetin, kaempferol, and catechin can modulate key signaling pathways, including nuclear factor kappa B (NF-κB) and cyclooxygenase (COX) activity, resulting in reduced expression of pro-inflammatory cytokines (Costa et al., 2020). These effects translate into improved reproductive performance, lower incidence of mastitis, and enhanced postpartum recovery in dairy cows.

The incorporation of agro-industrial by-products containing polyphenols into ruminant diets not only promotes animal performance but also supports circular economy principles by reducing feed costs and minimizing waste. By-products such as pomegranate peel, citrus pulp, and carob pods are rich in hydrolysable and condensed tannins, offering both nutraceutical and environmental benefits. For

example, inclusion of pomegranate peel extract in the diets of dairy cows and goats increased total milk yield and milk protein content while reducing lipid peroxidation markers (Chedea et al., 2017, Bonanno et al., 2019). Similarly, citrus pulp, which contains flavanones such as hesperidin and naringin, has been associated with improved digestibility and feed conversion efficiency (Ianni et al., 2019).

Beyond dairy production, polyphenols can influence growth performance and meat quality in beef cattle and small ruminants. The supplementation of chestnut tannins in beef cattle diets improved feed efficiency and increased polyunsaturated fatty acid levels in muscle tissue without negatively affecting growth rate (Lee et al., 2024). The antioxidant activity of tannins and flavonoids also contributes to improved meat color stability and lipid oxidation resistance during storage, enhancing both shelf life and consumer acceptance (Ma et al., 2021).

In small ruminants, particularly goats and sheep, polyphenol-rich feeds such as quebracho, lotus, or sainfoin have shown promising results in enhancing nitrogen utilization and milk composition while maintaining or improving animal productivity (Cohen-Zinder et al., 2017, Lee et al., 2024). These effects are primarily due to reduced protein degradation in the rumen and improved post-ruminal amino acid absorption.

It is important to note that while polyphenols generally enhance animal performance at moderate levels, excessive inclusion may lead to negative outcomes, such as decreased feed intake, lower nutrient digestibility, and interference with mineral absorption.

In summary, polyphenols represent a valuable class of bioactive compounds capable of improving animal performance, milk and meat quality, oxidative stability, and overall health. Their integration into livestock feeding strategies supports not only animal productivity but also the sustainability of animal production systems by reducing environmental impact and enhancing the nutritional value of animal-derived foods.

1.7 Dietary polyphenols supplementation during the *peripartum* period

The transition period, generally defined as the interval from the beginning of the dry-off to three weeks after calving, is one of the most physiologically demanding stages in a dairy cow's life (Pascottini et al., 2020). During this timeframe, the cow undergoes profound metabolic, endocrine, and immune adjustments to shift from a pregnant, non-lactating state to intense milk production. These coordinated changes are essential for initiating lactation but simultaneously expose the animal to substantial metabolic and physiological stress, making this period critical for health, productivity, and reproductive success (Pascottini et al., 2020).

As parturition approaches, energy and nutrient demands increase sharply due to fetal growth and the onset of milk synthesis. However, dry matter intake (DMI) typically declines, leading to a negative energy balance (NEB). To compensate, the cow mobilizes adipose tissue and, to a lesser extent, muscle protein, resulting in elevated plasma concentrations of non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) (Herdt, 2000). While these adaptations are vital to sustain milk production, excessive lipid mobilization can overwhelm hepatic capacity, promoting triglyceride accumulation, fatty liver, and ketosis, conditions that impair liver function and overall metabolic efficiency (Drackley, 1999).

The metabolic reorganization of this period is closely linked to changes in hormonal and nutrient partitioning. The mammary gland becomes the major site of glucose utilization, relying heavily on hepatic gluconeogenesis from propionate, lactate, glycerol, and glucogenic amino acids. Reduced circulating insulin and tissue insulin sensitivity promote lipolysis and redirect nutrients toward the mammary gland. After calving, growth hormone levels increase to support milk synthesis and lipid mobilization, whereas insulin-like growth factor 1 (IGF-1) declines due to hepatic GH resistance, prolonging NEB and delaying reproductive recovery (Trevisi et al., 2012, Chen et al., 2015).

The high metabolic rate around calving also enhances the generation of reactive oxygen species (ROS), which may exceed the cow's antioxidant capacity and lead to oxidative stress. This oxidative imbalance damages cellular structures, impairs hepatic metabolism, and weakens immune cell function. Depletion of antioxidants such as vitamin E, selenium, and enzymes like superoxide

dismutase (SOD) and glutathione peroxidase (GPx) further contributes to oxidative damage and increases susceptibility to periparturient diseases including ketosis, fatty liver, and hypocalcemia (Nakov et al., 2016). Parallel to these metabolic and endocrine adjustments, the immune system experiences a temporary but significant suppression. Both innate and adaptive responses are weakened, characterized by reduced neutrophil activity, impaired lymphocyte proliferation, and altered cytokine production. This immunosuppression arises from a combination of hormonal fluctuations, particularly elevated cortisol, energy deprivation, oxidative stress, and inflammatory signaling. The activation of pro-inflammatory pathways mediated by nuclear factor κB (NF-κB) and cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) represents a physiological response to tissue remodeling and stress at calving (Santos et al., 2019). However, prolonged inflammation can disrupt metabolic regulation and further depress immune function, creating a self-perpetuating cycle of metabolic and inflammatory imbalance (Trevisi et al., 2012).

The interplay among metabolic, endocrine, and immune systems during the transition period underscores the importance of integrated nutritional and management strategies. Beyond correcting energy deficits, attention must be given to controlling inflammation, oxidative stress, and immune competence. Nutritional interventions aimed at enhancing antioxidant capacity, supporting hepatic function, and modulating immune responses are increasingly recognized as key tools to improve transition cow health (Lopreiato et al., 2020). In this context, bioactive compounds naturally present in feeds or supplements offer promising potential. Substances such as polyphenols, may help sustain metabolic equilibrium, mitigate oxidative and inflammatory stress, and enhance overall performance when conventional nutritional management alone is insufficient. Understanding these intricate adaptive mechanisms is therefore essential for developing feeding and management practices that promote metabolic stability, disease resistance, and optimal productivity in dairy cows during this highly sensitive phase.

Polyphenols, classified into several subclasses such as phenolic acids, flavonoids, lignans, and stilbenes, are recognized for their antioxidant, anti-inflammatory, and immunomodulatory properties

(Lopreiato et al., 2020). Their inclusion in dairy cow diets, particularly during the transition period, may mitigate oxidative damage caused by the accumulation of reactive oxygen species (ROS), thus preserving the integrity of cell membranes and improving immune resilience (Benchaar et al., 2007, Santos et al., 2010). From a ruminal perspective, the administration of polyphenols can exert multiple beneficial effects. They can modulate rumen fermentation patterns, helping to stabilize ruminal pH and prevent sub-acute ruminal acidosis by altering microbial activity and reducing lactic acid accumulation (Jouany, 2006). Moreover, certain phenolic compounds have demonstrated the ability to reduce methanogenesis, thereby improving feed efficiency and reducing energy losses (Drong et al., 2016). Their partial resistance to microbial degradation allows some active molecules to reach the small intestine intact, where they may further enhance nutrient absorption and immune responses (Oh et al., 2013).

The form and dosage of administration are crucial determinants of efficacy. Polyphenols can be supplied as essential oils, plant extracts, or purified compounds, obtained from various plant parts such as leaves, peels, or seeds. Essential oils (EO), in particular, exhibit antimicrobial activity through mechanisms including disruption of bacterial membrane structure and fluidity, inhibition of enzymatic activity, and interference with nucleic acid synthesis (Feldberg et al., 1988, Sikkema et al., 1994, Ultee et al., 2002). Despite these properties, the sensitivity of microbial species differs; gram-negative bacteria tend to be less affected, influencing the selectivity of ruminal microbiota modulation (Chao et al., 2000).

Experimental outcomes on the effectiveness of EO and polyphenolic supplementation during the transition period remain somewhat inconsistent. Drong et al. (2016) observed no significant improvement in milk production or energy metabolism with an EO-blend but reported a reduction in dry matter intake, possibly due to dosage effects. Similarly, Benchaar et al. (2006) and Tassoul and Shaver (2009) found that EO mixtures containing thymol, eugenol, vanillin, and limonene reduced feed intake without altering nutrient digestibility or milk yield. Conversely, Oh et al. (2013) demonstrated an immune-stimulatory effect via CD4+ cell activation following supplementation with

garlic and capsicum oleoresin extracts, highlighting the immunomodulatory potential of such compounds.

Among the various polyphenol subtypes, tannins have received particular attention for their antioxidant and antimicrobial properties. Their chemical structure enables the neutralization of free radicals through the stabilization of phenoxyl radicals (Liu et al., 2013). Tannins and saponins may also influence ruminal biohydrogenation, thereby modifying milk fatty acid profiles and enhancing nutritional quality. However, due to their antinutritional potential—notably the reduction of feed intake and digestibility, dosage and chemical type (condensed vs. hydrolysable tannins) must be carefully managed (Mangan, 1988). Benchaar et al. (2006) reported minimal effects of Yucca schidigera saponins and quebracho tannins on milk yield, whereas Şentürk et al. (2015) found that tannin supplementation improved protein metabolism and reduced circulating BHB, suggesting a mitigation of NEB. Likewise, Wang et al. (2017) demonstrated that tea saponins can lower oxidative stress and enhance immune function in a dose-dependent manner.

Overall, current evidence suggests that polyphenols represent a promising nutritional tool to alleviate oxidative stress and improve metabolic adaptation in dairy cows during the periparturient period. Nevertheless, variability in plant sources, extract composition, and administration protocols highlights the need for further standardized research to clarify optimal dosages and delivery methods for practical application in commercial dairy systems.

2. Chapter 2: Scope and objectives of the study

The *peripartum* period is a physiologically demanding window in which dairy cows undergo coordinated endocrine, immunologic, and metabolic reprogramming to support parturition and the onset of lactation (Drackley, 1999). A central feature of this transition is the emergence of negative energy balance with extensive adipose lipolysis, reflected by elevated circulating non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHBA), which are prospectively associated with impaired health, fertility, and performance (McArt et al., 2013, Ospina et al., 2013). Concurrently, the sharp rise in metabolic activity and immune remodeling heighten reactive oxygen species (ROS) generation and the risk of oxidative stress, with downstream perturbations in inflammatory tone, hepatic function, and disease susceptibility (Sordillo and Aitken, 2009, Abuelo et al., 2015). These intertwined immunometabolic pressures highlight the need for supportive strategies that stabilize redox homeostasis and hepatic metabolism while preserving productive performance. Antioxidant-oriented nutrition has therefore become a major focus of transition-cow management. Classical micronutrients such as vitamin E and selenium, integral to membrane protection and glutathione peroxidase activity, can modulate redox balance and immunity, although effects on energy balance and clinical outcomes vary across studies (Sordillo, 2013, Abuelo et al., 2015, Chen et al., 2023). Organic selenium sources may further enhance antioxidant defenses (Surai et al., 2019). Complementary approaches aim to bolster endogenous systems via thiol donors or methyl-group support: N-acetylcysteine attenuates oxidative/inflammatory responses in bovine mammary cells (Bae et al., 2017, Ninković et al., 2024), while rumen-protected methionine and choline support hepatic methylation, lipid export, and redox homeostasis in early lactation (Toledo et al., 2021, Swartz et al., 2023, Lima et al., 2024). In parallel, plant-derived polyphenols are of interest for their capacity to modulate Nrf2/NF-κB signaling and hepatic stress responses; although rumen biotransformation constrains the systemic appearance of many parent compounds, post-ruminal delivery or appropriate matrices can improve bioavailability, and selected preparations have favorably influenced oxidative

and inflammatory biomarkers in specific experimental contexts (Stoldt et al., 2015, Winkler et al., 2015, Wein et al., 2016, Gessner et al., 2017). Together, these lines of evidence suggest that targeted antioxidant strategies may mitigate the oxidative–metabolic strain characteristic of the calving transition and, potentially, translate into improved quality and oxidative stability of mammary secretions. Against this background, we tested the hypothesis that during the *peripartum* the supplementation with a hydrolyzed-lignin phenolic extract (Oxyphenol®) reduces oxidative stress in blood and peripheral blood mononuclear cells and improves the oxidative stability of colostrum and milk, without compromising milk composition, fatty acids, or coagulation properties.

3. Chapter 3: Materials & Methods

3.1. Experimental design

This trial was approved by the Ethics Committee of the Department of Veterinary Medicine, University of Bari (protocol no. 07/2023). The experimental activities were carried out on a commercial dairy farm located in Southern Italy, starting on December 19, 2023, and ending on July 30, 2024. A total of twenty Brown Swiss dairy cows were enrolled and allocated into two experimental groups, balanced according to parity (CON = 2.9 ± 0.7; OXY = 2,8 ± 0.8): a control group (CON, $n = 10$) and a group receiving Oxyphenol® supplementation (OXY, $n = 10$). All animals received the same TMR, with the diet adjusted according to the production phase: one diet from -60 to -21 days relative to calving, a second from -21 to +7 days, and a third from +7 to +60 days postpartum. The ingredients and the determined nutrient content (Association of Official Analytical Chemists (AOAC, 2000) of the three feeding regimen are shown in Table 1. Oxyphenol® was administered to cows in the OXY group once daily throughout the entire dry period and continued until 60 days postpartum, at a dosage of 35 g per head per day. The supplement was orally administered to each head of the OXY group in the self-locking head gate in the feeding front when TMR was unloaded. It was mixed with water to obtain a cream, which was then administered directly in the mouth using a large syringe, as described by Maggiolino et al. (2019). The chemical composition and antioxidant activity of Oxyphenol® are reported in Table 2, as determined according to the methods described by Maggiolino et al. (2020).

3.2. Blood sampling ad analysis

Figure 2 shows a schematic representation of the experimental design and sampling schedule. Blood samples were collected from the coccygeal vein into sterile Vacutainer tubes (Becton Dickinson and Co., Franklin Lakes, New Jersey, United States) at several time points: at the beginning of the dry period (T-60), 30 days into the dry period (T-30), one week before calving (T-7), three days before

calving (T-3), and on the day of calving (T0). *Postpartum* sampling was performed at 3 days (T+3) and 7 days (T+7) post calving, and subsequently every 30 days during lactation (T+30, T+60, T+90, T+130). At each experimental time point three 9-mL EDTA and three 9-mL clot activator tubes were collected from each cow. Blood samples were centrifuged (1,500 × g for 10 min) to separate serum and plasma, which were then aliquoted and stored at –20 °C until subsequent analyses.

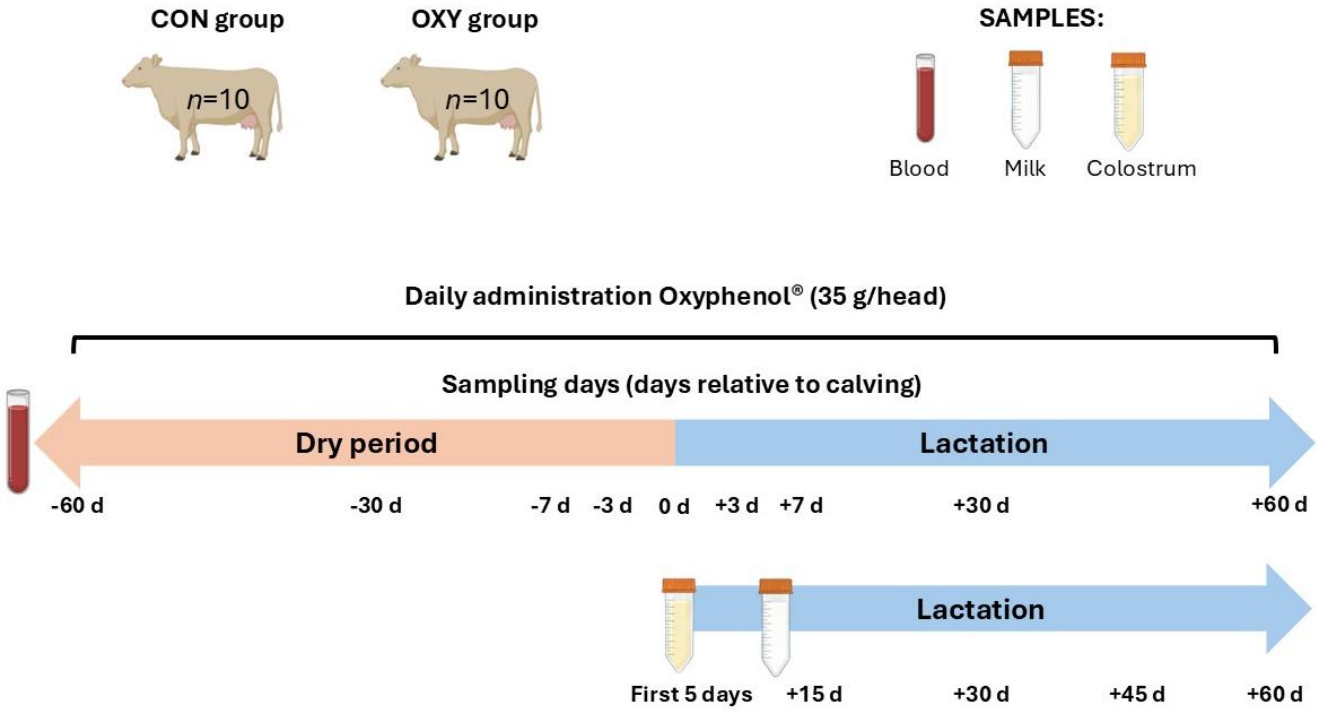

Figure 2. Schematic illustration of the experimental design, experimental groups, and sampling days.

3.2.1. Biochemical profile

Serum biochemical profiles were assessed using an automated clinical chemistry analyzer (CS-300B; Dirui, Changchun, China) (Forte et al., 2025a). The following variables were determined: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), glucose, urea, uric acid, creatinine, total protein, albumin, cholesterol, triglycerides, non-esterified fatty acids (NEFA), calcium, phosphorus, and magnesium, employing commercial kits (Gesan Production Kit, Campobello di Mazara, Trapani, Italy). Globulin concentrations were calculated as the difference between TP and Alb values. Prior to each analytical run, the analyzer was calibrated with standard

solutions supplied with the assay kits (Seracal, Gesan Production Kit, Campobello di Mazara, Trapani, Italy). Accuracy of the measurements was then checked using two control sera (Seracontrol N and Seracontrol P, Gesan Production Kit, Campobello di Mazara, Trapani, Italy). Results were considered acceptable when the observed values differed by less than 3.00% from the reference values provided by the manufacturer (Maggiolino et al., 2025).

3.2.2. Oxidative profile

Plasma samples (0.5 mL) were diluted in 15 mL of deionized distilled water (DDW) within 50-mL test tubes and thoroughly homogenized. From each homogenate, 1 mL was transferred into a glass tube for the determination of thiobarbituric acid reactive substances (TBARS). To this aliquot, 0.05 mL of butylated hydroxytoluene (7.2% in ethanol) and 1.950 mL of a thiobarbituric acid (TBA)/trichloroacetic acid (TCA)/HCl mixture (0.375% TBA, 15% TCA, 0.25 N HCl) were added. The mixture was vortexed and subsequently incubated at 90 °C for 15 min in a thermostatic water bath. Following incubation, samples were cooled to room temperature (15–30 °C) and centrifuged at 2000 × g for 15 min. The absorbance of the resulting supernatant was then recorded at 531 nm, using as blank a solution containing 2 mL of TBA/TCA/HCl mixture and 1 mL of DDW. TBARS concentrations were quantified against a standard calibration curve prepared with 1,1,3,3-tetramethoxypropane, and lipid peroxidation was expressed as milligrams of malondialdehyde (MDA) per milliliter of plasma.

Plasma samples (0.5 mL) were diluted in 20 mL of 0.15 M KCl and kept for 2 min. Two aliquots of the homogenate (50 µL each) were then mixed with 1 mL of 10% trichloroacetic acid (TCA) and centrifuged at 1200 × g for 3 min at 4 °C to prepare for protein carbonyl quantification (Maggiolino et al., 2021). One aliquot, serving as the reference, was treated with 1 mL of 2 M HCl, whereas the other was reacted with 1 mL of 2 M HCl containing 10 mM 2,4-dinitrophenylhydrazine (DNPH). Both tubes were incubated for 1 h at room temperature (15–30 °C), with gentle mixing every 20 min. After incubation, 1 mL of 10% TCA was added to each sample, followed by vortexing (30 s) and

centrifugation at 1200 × g for 3 min at 4 °C. This washing step was repeated three times, carefully discarding the supernatant each time. The resulting pellets were subsequently washed with 1 mL of ethanol:ethyl acetate (1:1, v/v), vortexed, and centrifuged under the same conditions three times before removing the supernatant. Finally, pellets were resuspended in 1 mL of 20 mM sodium phosphate buffer containing 6 M guanidine hydrochloride, shaken, and centrifuged at 1200 × g for 3 min at 4 °C. The absorbance of the DNPH-derivatized samples was read at 360 nm using a Beckman Coulter DU800 spectrophotometer (Beckman Instruments Inc., Brea, CA, USA). Protein carbonyl levels were expressed as nanomoles of carbonyl groups per milligram of protein. Protein concentration was determined by the Biuret method.

The ferric reducing ability of plasma (FRAP) assay was used to evaluate the total antioxidant capacity, following the procedure of Benzie and Strain (1996) with minor adjustments. Briefly, 3 mL of freshly prepared FRAP working solution was obtained by combining 1 mL of 10 mM TPTZ dissolved in 40 mM HCl, 1 mL of 20 mM $FeCl_3$ in distilled water, and 10 mL of 300 mM acetate buffer (pH 3.6). After addition of 100 μL of plasma sample or supernatant, the mixture was incubated at 37 °C for 40 min. The absorbance was then measured at 593 nm, and results were expressed as μmol Trolox equivalents per ml.

3.2.3. Antioxidant enzyme activity

Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined following the principles outlined by Misra (2018). The method relies on the ability of SOD to inhibit the autoxidation of epinephrine, with the inhibitory effect quantified spectrophotometrically at 480 nm (Dinardo et al., 2021). To prevent spurious oxidation caused by trace metal contaminants in the reagents, the assay buffer was supplemented with 10–4 M EDTA to chelate divalent cations. SOD activity was expressed as units per milliliter (U/mL), where one unit corresponds to the amount of enzyme that produces 50% inhibition of epinephrine autoxidation. Glutathione peroxidase (GS-Px; EC 1.11.1.9) activity was assessed following the procedure described by Flohé and Günzler (1984) and further detailed by

Dinardo et al. (2022). The assay evaluates the GS-Px-dependent oxidation of reduced glutathione (GSH) by tert-butyl hydroperoxide. To maintain GSH at a constant level throughout the reaction, exogenous glutathione reductase and NADPH were added, enabling the rapid recycling of oxidized glutathione back to its reduced form. The consumption of NADPH was tracked by monitoring the decrease in absorbance at 340 nm, and enzymatic activity was reported as U/mL. Glutathione S-transferase (GST; EC 2.5.1.18) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate, according to the method of Habig and Jakoby (1981). The assay is based on the conjugation of GSH with CDNB, and the rate of product formation was determined spectrophotometrically by recording the increase in absorbance at 340 nm. The results were expressed as U/mL of enzyme activity.

3.2.4. Determination of reactive oxygen species (ROS) production and cell viability in peripheral blood mononuclear cell (PBMC)

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood following a density gradient centrifugation procedure, as described by Maggiolino et al. (2024). Twenty milliliters of whole blood were diluted 1:1 with cold phosphate-buffered saline (PBS) and gently layered onto 10 mL of Histopaque®-1077 (Sigma–Aldrich, Milan, Italy). Samples were centrifuged at 400 × g for 30 min at 20 °C, and the mononuclear cell layer was carefully collected. The recovered PBMC were washed twice with PBS, and the final pellet was resuspended in calcium- and magnesium-free Iscove's Modified Dulbecco's Medium (IMDM; Sigma–Aldrich, Milan, Italy). Cell concentration was determined using a Bürker hemocytometer, while cell viability was assessed by Trypan Blue exclusion (Sigma–Aldrich, Milan, Italy). For stimulation assays, PBMC suspensions (2×10^5 cells/well in 100 µL) were plated in 96-well U-bottom plates with serum-free IMDM and treated either with concanavalin A (ConA; 5 µg/mL final concentration) or hydrogen peroxide (H_2O_2; 1 mM final concentration). Untreated cells served as negative controls. After 20 h of incubation at 37 °C in 5% CO_2, plates were centrifuged at 400 × g for 10 min at 20 °C. The culture supernatants were further clarified at 10,000 × g, and aliquots were stored at −80 °C until further analysis. Intracellular ROS

generation was quantified using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), as previously reported (Maggiolino et al., 2024). PBMCs (2×10^5 cells/well in 96-well plates) were incubated with 10 μM DCFH-DA in phenol red–free IMDM for 30 min at 37 °C. Subsequently, cells were exposed for 1 h and 30 min to either ConA (5 μg/mL) or H_2O_2 (1 mM). After treatment, the medium was discarded, and cells were rinsed twice with PBS. The fluorescence intensity was measured in phenol red–free IMDM using a spectrofluorometer (excitation 485 nm, emission 525 nm). Control wells followed the same protocol but without oxidant exposure. ROS production was expressed as the percentage change in fluorescence relative to the untreated control. Extracellular ROS levels in PBMC supernatants were assessed using the OxiSelect™ In Vitro ROS Assay Kit (Green Fluorescence) (Cell Biolabs Inc., San Diego, CA, USA), following the manufacturer's protocol. The assay is based on the oxidation of DCFH to the fluorescent product DCF by reactive species. Fluorescence was measured at 480 nm excitation and 530 nm emission using a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Blank values were subtracted from all measurements to remove background signal, and the resulting fluorescence intensity, expressed in arbitrary units, was proportional to the total ROS content of each sample. Cell viability and cytotoxicity of PBMC from the different experimental groups were evaluated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, as previously described by Di Bari et al. (2014). This method relies on the ability of mitochondrial succinate dehydrogenase in metabolically active cells to reduce MTT to insoluble formazan crystals, which are then solubilized and quantified spectrophotometrically. Briefly, after removing the culture medium, cells were washed with PBS and incubated for 2 h at 37 °C and 5% CO_2 with MTT solution (0.5 mg/mL). Plates were centrifuged, the medium discarded, and the formazan crystals dissolved in absolute ethanol. Absorbance was measured at 560 nm and 690 nm using a VersaMax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was expressed as the percentage of the absorbance of treated samples relative to untreated controls (set as 100%).

3.3. Colostrum, transitional milk and milk sampling and analysis

Colostrum and transitional milk were collected once daily during the first five days after calving from each cow enrolled in the trial, and its quality was assessed using Brix refractometry. Milk samples were collected up to 60 days of lactation at 15-day intervals. Milking was performed in a conventional parlor twice daily, at 06:00 and 17:00 h. At the end of each milking, 100 mL of milk per cow was collected, providing a sample representative of the full yield. Immediately after collection, samples were stored at 4 °C, transported to the laboratory, and combined in proportion to the morning and evening yields. The pooled milk was then analyzed within 2 h.

3.3.1. Chemical composition

Moisture, protein, and ash contents of colostrum were determined following the International Organization for Standardization (ISO) protocols (ISO, 1973, 1978, 1998), whereas lipid content was analyzed according to the AOCS Am 5-04 official method (AOAC, 2000). Moisture was quantified by drying samples in an oven at 105 °C for 24 h (Memmert UFP 600, Schwabach, Germany), with results expressed as the weight loss between the initial and final measurements. Protein concentration was assessed using the Kjeldahl procedure: samples were digested with sulfuric acid in the presence of copper sulfate catalyst (Kjeldatherm KB system, Gerhardt, Bonn, Germany), generating ammonium sulfate, which was then distilled under alkaline conditions using a Vapodest 50 Carrousel unit (Gerhardt, Bonn, Germany). Total nitrogen was measured and converted to crude protein by applying the factor 6.25. Ash determination was performed by incinerating the material at 550 °C for 4–5 h in a muffle furnace (Carbolite RWF 1200, Hope Valley, UK), with mineral residue expressed relative to the original sample weight. For lipid content assessment, samples were subjected to acid hydrolysis followed by lipid extraction with petroleum ether at 90 °C for 60 min using an AnkomHCI Hydrolysis System (Macedon, NY, USA). The extracted lipid fraction was then quantified gravimetrically.

Near-infrared spectroscopy was used to determine fat, protein, casein, lactose, citric acid, BHB, short-chain fatty acids (SFA), unsaturated fatty acids (UFA), monounsaturated fatty acids (MUFA), and

polyunsaturated fatty acids (PUFA) in milk (ISO, 2013). Milk coagulation properties, including clotting time, curd-firming rate, and curd firmness, were assessed with a Formagraph (Foss Electric A/S, Hillerød, Denmark) following the protocol described by Dadousis et al. (Dadousis et al., 2016).

3.3.2. Oxidative profile

Lipid oxidation was assessed using the thiobarbituric acid reactive substances (TBARS) assay, as reported by Oancea et al. (2022). Briefly, 0.5 mL of milk was mixed with 1 mL of 20% trichloroacetic acid (TCA) and incubated for 15 min at room temperature. The samples were then centrifuged at 3000 × g for 10 min, and 1 mL of the supernatant was transferred to a clean tube. To this, 0.6 mL of 0.8% thiobarbituric acid (TBA) solution was added. The mixture was incubated at 80 °C for 90 min and subsequently cooled to room temperature (15–30 °C). Absorbance was measured at three different wavelengths to identify degradation products specific to milk oxidation: 450 nm (saturated aldehydes), 495 nm (dienals), and 532 nm (malondialdehyde, MDA), using a Shimadzu IRTracer-100 spectrophotometer. A blank solution containing TBA/TCA/HCl and distilled water was used as reference. Malondialdehyde was quantified using a standard calibration curve prepared with 1,1,3,3-tetramethoxypropane and expressed in µg/l of milk.

Protein oxidation was determined by measuring protein carbonyl content. Two aliquots of milk (50 µL each) were treated with 1 mL of 10% TCA, followed by centrifugation at 1200 × g for 3 min at 4 °C. One aliquot served as a blank and was treated with 1 mL of 2 M HCl, while the second was incubated with 1 mL of 2 M HCl containing 10 mM 2,4-dinitrophenylhydrazine (DNPH). Samples were left at room temperature (15–30 °C) for 1 h with intermittent shaking every 20 min. Subsequently, 1 mL of 10% TCA was added, and the mixtures were vortexed (30 s) and centrifuged three times at 1200 × g for 3 min at 4 °C. The pellets were washed three times with 1 mL ethanol:ethyl acetate (1:1, v/v) under the same centrifugation conditions. Pellets were finally solubilized in 1 mL of 20 mM sodium phosphate buffer containing 6 M guanidine hydrochloride. Carbonyl concentration was determined spectrophotometrically at 360 nm on DNPH-treated samples using a Beckman

Coulter DU800 spectrophotometer and expressed as nmol carbonyls per mg of protein. Protein concentration was measured using the Biuret method, following the procedure of Tokur and Korkmaz (2007).

Antioxidant capacity of milk was evaluated by the Ferric Reducing Antioxidant Power (FRAP) assay. For each sample, 300 μL of milk was added to 4.5 mL of freshly prepared FRAP reagent. After vortex mixing, samples were incubated at 37 °C for 10 min and vortexed again. Subsequently, 4 mL of the reaction mixture was withdrawn with a syringe, filtered through a 0.45 μm pore filter, and the eluate was collected. From this, 250 μL per sample was pipetted in triplicate into the wells of a 96-well microplate. Absorbance was read immediately at 595 nm using a microplate reader. A blank was prepared by substituting milk with 300 μL of distilled water while keeping the reagent volume constant. Final values were corrected by subtracting the blank absorbance. Antioxidant capacity was expressed as nmol ascorbic acid equivalents per mL of milk, using a calibration curve generated with ascorbic acid standards.

3.4. Statistical analysis

Prior to statistical modeling, the data were assessed for normality and homogeneity of variances using the Shapiro–Wilk test. Once the assumptions were verified, data were analyzed through analysis of variance (ANOVA) using the General Linear Model (GLM) procedure in SAS software (version 9.3; SAS Institute Inc., Cary, NC, USA; SAS, 2018). The model employed was structured as follows:

$$y_{ijk} = \mu + \alpha_i + \sigma + T_j + D_k + (T \times D)_{jk} + \varepsilon_{ijkl},$$

where y_{ijk} represents all parameters as dependent variables; μ is the mean; α_i is the dairy cow random effect (1,…, 20), σ is the covariate accounting for the effect of the sampling season/month, T represents the effect of the j^{th} treatment (j = 1, 2), D represents the effect of the k^{th} sampling day (k = 1, ..., 9 for blood parameters; 1, …, 5 for colostrum parameters; 1, …,4 for milk parameters), T × D represents the effect of the binary interaction between the two independent variables (jk = 1,…, 18

for blood parameters; 1, …, 10 for colostrum parameters; 1, …, 8 for milk parameters) and ε_{ijkl} is the error.

Cell viability's data were also analyzed considering only the effect of the treatment, using the following model:

$y_{ijk} = \mu + \alpha_i + \sigma + T_j + \varepsilon_{ijkl}$,

A Bonferroni test was used to carry out pairwise treatment comparisons at each specific time point. When either the effect of sampling day or the treatment × sampling day interaction reached significance, differences among means within a given treatment were assessed using Tukey's test. Statistical significance was considered at $P < 0.05$. Data are reported as least squares means with their corresponding standard errors.

4. Chapter 4: Results

4.1. Blood analyses results

Table 3 presents the fixed effects of treatment, sampling day, and their interaction on the blood biochemical profile of the cows. Sampling day affected total protein, globulin, cholesterol, triglycerides, NEFA, phosphorus, and AST ($P < 0.01$), as well as glucose ($P < 0.05$). Treatment, on the other hand, influenced ALT ($P < 0.05$) and ALP ($P < 0.01$). Total protein levels in the CON treatment were higher on day +30 compared to day -7 ($P < 0.01$) and on day +60 compared to the period from day -60 to day 0 ($P < 0.01$). In OXY treatment total protein increased at day +30 compared to days -7, -3 and 0 ($P < 0.01$). Globulin levels in the CON treatment increased at day +30 and day +60 compared with day -60 and day -7 ($P < 0.01$). In the OXY treatment, globulin concentrations were higher at day +30 compared with day -7 ($P < 0.01$) and day -3 ($P < 0.05$). In both treatments, cholesterol decreased at day -7 compared with day -60 ($P < 0.01$), increased again by day +30 ($P < 0.01$), and reached levels at day +60 that were higher than those observed at day -60 ($P < 0.01$). Triglyceride levels in the CON treatment decreased at day +7 compared with the *prepartum* period ($P < 0.01$). NEFA concentrations increased in both groups on day 0 compared with previous days ($P < 0.01$). In the CON treatment, NEFA levels subsequently decreased at day +7 ($P < 0.05$) and day +30 ($P < 0.01$) relative to day 0, whereas in the OXY treatment, NEFA concentrations declined at day +7 ($P < 0.01$) with a further reduction observed at day +30 ($P < 0.01$). Glucose concentrations in the CON treatment decreased at days +3 and +7 compared with day 0 ($P < 0.05$), while in OXY treatment it decreased at day +7 compared to day 0 ($P < 0.01$). Phosphorus levels in the CON treatment increased at day +30 compared with day +3 ($P < 0.01$), whereas in the OXY treatment, phosphorus increased at day +7 relative to day 0 ($P < 0.01$). At day +3 ALT showed an higher concentration in CON treatment compared to OXY treatment ($P < 0.01$), while AST activity increased at day +60 compared to day -30 ($P < 0.01$).

Table 4 reports the fixed effects of treatment, sampling day, and their interaction on the oxidative profile and antioxidant enzyme activity of plasma. Treatment affected TBARS levels, SOD ($P < 0.01$), and GSPx ($P < 0.05$), whereas sampling day affected TBARS ($P < 0.05$) and SOD ($P < 0.01$). In the CON treatment, TBARS concentrations increased at day +3 compared with day -60 ($P < 0.05$) and were higher than those in the OXY treatment from day +3 to day +30 ($P < 0.01$), as well as at day +60 ($P < 0.05$). Protein carbonyls levels were also higher at day +3 ($P < 0.01$) and at days -3 and +7 ($P < 0.05$) compared with day −60 in the CON treatment. SOD activity in the CON treatment was higher at days -7 and -3 than at day -30 ($P < 0.01$) and further increased at day 0 and day +3 ($P < 0.01$). In contrast, in the OXY treatment, SOD activity was lower at day +60 compared with days -7 and +3 ($P < 0.01$) and was consistently lower than in the CON group at days 0, +3, and +7 ($P < 0.01$). GSPx activity was also higher in the CON treatment than in OXY from day +7 to day +60 ($P < 0.05$). Figure 3 and Figure 4 show the effect of treatment on the cell viability and proliferation of PBMC treated in vitro with ConA and H_2O_2. Compared to CON treatment, OXY treatment showed an higher viability after ConA stimulation at calving day, +3, +30 ($P < 0.05$) and in particular at + 7 days ($P < 0.01$). As regards H_2O_2 stimulation, OXY treatment showed higher cell viability at calving day ($P < 0.05$).

The production of ROS by PBMC is reported in Figure 5. Both treatment and sampling day affected ROS levels ($P < 0.01$), since in both treatment they were higher from -7 days to +3 days compared to the other time points ($P < 0.01$). Moreover, OXY treatment showed lower levels of ROS compared to CON treatment at -3 days ($P < 0.05$), 0 and +3 days ($P < 0.01$).

4.2. Milk analyses results

The fixed effects of treatment, sampling day, and their interaction on the milk production parameters (Table 5) and coagulation properties (Table 6) were evaluated, and none of these parameters were influenced by any of the fixed effects ($P > 0.05$), except for milk yield which was affected by sampling day ($P > 0.01$). In both experimental treatments milk yield increased at 30 and 45 days *postpartum*, compared to day 15 ($P > 0.01$), and further at 60 days ($P > 0.01$.)

Table 7 shows the fixed effects of treatment, sampling day and their interaction on the milk oxidative profile. The treatment affected all the parameters ($P < 0.01$), except for protein carbonyls. Malondialdehydes were higher in CON treatment than in the OXY one at 30 ($P < 0.05$), 45 and 60 days ($P < 0.01$). The FRAP values were higher in CON treatment compared to those of OXY treatment at 15, 30 and 60 days ($P < 0.01$).

The fixed effects of treatment, sampling day, and their interaction on the fatty acids profile of milk, presented in Table 8, showed no significant influence ($P > 0.05$).

4.3. Transitional milk and colostrum analyses results

Table 9 shows the fixed effects of treatment, sampling day, and their interaction on the chemical composition of colostrum. Dry matter, moisture, protein content, and Brix degrees were affected by sampling day ($P < 0.01$). Dry matter decreased in both treatments at 3 days *postpartum* compared to day 1 ($P < 0.05$), and remained lower on days 4 and 5 relative to day 1 ($P < 0.01$). Moisture content increased in both treatments on day 3 compared to day 1 ($P < 0.05$). Additionally, moisture was higher in the CON treatment on day 5 compared to day 1 ($P < 0.01$), while in the OXY treatment it was higher on days 4 and 5 relative to day 1 ($P < 0.01$). Protein content and Brix degrees decreased in both treatments on day 2 compared to day 1 ($P < 0.01$) and remained stable on the following days ($P < 0.01$). In the OXY group, both parameters were lower on day 5 compared to day 2 ($P < 0.05$).

Finally, Table 10 reports the fixed effects of treatment, sampling day, and their interaction on the oxidative profile of colostrum. Malondialdehyde was affected by both treatment ($P < 0.05$) and sampling day ($P < 0.01$), as were protein carbonyls ($P < 0.05$). Malondialdehyde levels in the CON treatment progressively decreased from day 1 to day 3 ($P < 0.01$), and at day 5 they were lower compared to day 3 ($P < 0.01$). In the OXY treatment, MDA concentrations decreased on days 3, 4, and 5 relative to day 1 ($P < 0.05$). Additionally, on day 1, 2 MDA levels were higher in the CON treatment than in the OXY treatment ($P < 0.01$), and the same difference was observed at day 3 ($P < 0.05$). Protein carbonyls in the CON treatment were lower on day 4 compared to day 2 ($P < 0.05$),

while in the OXY treatment, carbonyls levels were reduced on days 4 and 5 relative to day 2 ($P < 0.05$).

5. Chapter 5: Discussion

The comprehensive evaluation of serum biochemical parameters in dairy cows revealed values that were predominantly within the physiological ranges described for the species (Piccione et al., 2012, Coroian et al., 2017). This overall stability suggests that Oxyphenol® did not adversely affect systemic health, as fluctuations in these biomarkers are widely recognized as sensitive indicators of organ functionality and metabolic homeostasis (Puppel and Kuczyńska, 2016). Consistently, studies supplementing transition cows with plant polyphenols reported either neutral or favorable systemic safety profiles while improving redox or hepatic markers, e.g., green tea polyphenols in hyperketonemic cows reduced AST/ALT/GGT, indicating better liver function (Ma et al., 2021), and intraduodenal quercetin lowered postpartum AST and GLDH—enzymes linked to hepatocellular injury (Stoldt et al., 2015). Serum protein concentrations were all within the expected physiological range. Our findings revealed a slight increase in globulin levels during early lactation compared with the late gestation, which was also reflected in the total protein content. Similar trends have been reported by others (Larson and Kendall, 1957, Piccione et al., 2011, Piccione et al., 2012), who suggested that the transient decline in specific globulin fractions during the final month of gestation may be related to their transfer from the bloodstream to the mammary gland, coinciding with colostrum formation. Globulin concentrations then tend to rise again, starting from approximately the fifth week of lactation. This pattern is compatible with immunoglobulin trafficking toward colostrum, followed by recovery as lactation stabilizes, as summarized in recent reviews of periparturient immunity and oxidative status (Ayemele et al., 2021). Protein metabolism–related markers, including urea, uric acid, and creatinine, did not show relevant variations over time, in contrast to biomarkers associated with lipid metabolism. The onset of lactation is characterized by a sharp rise in the demand for key nutrients, such as glucose, amino acids, and fatty acids, to support milk synthesis. In high-yielding dairy cows, this demand frequently exceeds dietary supply, leading to mobilization of body reserves and the development of a negative energy balance (NEB) that may persist for several weeks

after calving (Kessel et al., 2008). A key feature of this metabolic adaptation is the pronounced release of NEFA from adipose tissue, primarily driven by the reduction in dry matter intake observed around calving. The mobilized NEFA serve as an essential energy source, supporting both the rapidly developing mammary gland and the metabolic needs of the fetus. Kumprechtová et al. (2022) evaluated the effect of plant-derived bioactive compounds in the diet of transition dairy cows and observed a reduction in *postpartum* serum NEFA concentrations, suggesting decreased mobilization of adipose tissue and, consequently, a mitigated NEB during the early lactation period. Previous studies have reported that NEFA concentrations often surpass the threshold of 0.6 mmol/L at calving, reflecting these adaptive processes (Vazquez-Anon et al., 1994, Cavestany et al., 2005, Seifi et al., 2007). In line with these findings, both groups in our study exhibited a transient rise in circulating NEFA at calving, followed by a rapid return to physiological values within one week postpartum, consistent with the observations of Vazquez-Anon et al. (1994) and Seifi et al. (2007). Notably, reductions in NEFA with specific flavonoids have also been reported more recently (e.g., naringin during the transition period improved systemic metabolic status and attenuated oxidative stress), reinforcing the plausibility of phenolic-driven mitigation of lipomobilization when bioavailability and dosing are adequate (Li et al., 2024). Epidemiologically, lower NEFA and BHBA around calving are associated with reduced postpartum disease risk and better performance (Ospina et al., 2010, McArt et al., 2013). Parallel to the NEFA dynamics, plasma total cholesterol decreased at calving and increased during early lactation in both groups. This pattern agrees with the well-documented positive correlation between cholesterol concentrations and energy balance in early-lactation cows (Kida, 2002, Reist et al., 2003). The temporary decline observed around calving may indicate a limited capacity to cope with the heightened metabolic demands of this stage, whereas the subsequent rise in cholesterol could be explained by increased milk yield, dietary fat supplementation, or physiological adaptations occurring during early lactation (Vasilenko, 2020). Several studies have reported similar trajectories, with cholesterol levels declining in late gestation and rising markedly during the first months of lactation under standard feeding and management conditions (Seifi et al., 2007, Chalmeh

et al., 2016, Folnožić et al., 2016, García et al., 2017). Mechanistically, the *postpartum* nadir and recovery of cholesterol likely reflect hepatic very-low-density lipoprotein (VLDL) export capacity and the resolution of NEB. The experimental NEB models demonstrate stage-dependent suppression of cholesterol metabolism with recovery as EB improves (Gross et al., 2015). In addition to changes in NEFA and cholesterol, alterations in triglycerides and glucose dynamics were also evident in our study. Serum triglycerides were higher during the dry period and at calving compared with the *postpartum* phase, reflecting reduced mammary uptake during non-lactating stages and increased hepatic synthesis (Seifi et al., 2007). Conversely, glucose levels declined transiently during the first week *postpartum*, likely due to its intensive utilization for lactose synthesis in the mammary gland, before gradually recovering with the improvement of feed intake and energy status, in line with previous reports (Djokovic et al., 2019). Comparable dietary polyphenol interventions (green tea + curcuma) have shown lower hepatic triacylglycerol concentrations and greater energy-corrected milk in early lactation, suggesting that some phenolic mixes can beneficially modulate hepatic lipid handling without compromising intake (Winkler et al., 2015). Regarding serum electrolytes, minerals play a fundamental role in supporting growth, reproduction, and lactation in dairy cows (Samardžija et al., 2011). In our study, electrolyte concentrations remained largely stable, with only a slight and biologically negligible increase in phosphorus observed during lactation compared with calving. Such stability is consistent with well-managed mineral nutrition in the dry/transition diet and aligns with guidance that avoids large periparturient swings in macro-minerals (National Academies of Sciences and Medicine, 2021). For bovine hepatology, however, it is important to note that AST is less liver-specific than glutamate dehydrogenase (GLDH) and GGT; accordingly, elevations in GLDH (and often GGT) track hepatocellular injury and fatty liver more closely in dairy cows. Incorporating GLDH (and/or OCT) in future sampling would therefore increase diagnostic specificity (Kalaitzakis et al., 2010, González et al., 2011, Du et al., 2017). A significant dietary effect was observed on hepatic enzymes, with ALT and ALP showing higher concentrations in CON compared with OXY. Among these, AST is particularly noteworthy, as it is commonly associated with fatty liver diagnosis

(Mohamed, 2014). Similar findings have been previously reported: Ma et al. (2021) demonstrated that supplementation with green tea polyphenols reduced serum concentrations of AST, ALT, and γ-glutamyl transferase, suggesting potential improvements in liver function in cows with hyperketonemia. Consistently, intraduodenal quercetin lowered postpartum AST and GLDH in periparturient cows, offering a mechanistic parallel for phenolic-driven hepatoprotection (Stoldt et al., 2015). Together with evidence that a green tea/curcuma mix modulates hepatic stress-response pathways in early-lactation cows, these data support the biological plausibility that the Oxyphenol® phenolic matrix contributes to the lower ALT/ALP observed in the OXY group (Winkler et al., 2015). Because redox and hepatic lipid handling are tightly interconnected around calving, the enzyme profile described here complements the metabolic results in the previous section (NEFA/cholesterol), strengthening the interpretation that phenolic supplementation can alleviate peri-parturient oxidative-metabolic strain (González et al., 2011, Du et al., 2017, Ma et al., 2021). Phenolics present in Oxyphenol® (e.g., quercetin/rutin-type flavonols, eriodictyol, rosmarinic- and methyl-gallate derivatives) plausibly act through redox-sensitive signaling, activating Nrf2–ARE and tempering NF-κB, thereby upregulating enzymatic defenses (e.g., SOD, GPx, NQO1) and lowering ROS. In bovine systems relevant to the transition period, NEFA and BHBA trigger oxidative injury via ROS–p38–p53/Nrf2 axes, a pathway that polyphenols can counter, reducing oxidative damage and apoptosis (Song et al., 2014, Song et al., 2016). At the hepatic level, phenolics may indirectly enhance PPARα-driven β-oxidation and support VLDL export; in vivo, intraduodenal quercetin lowered postpartum AST and GLDH and tended to reduce liver fat, while a green tea/curcuma mix decreased hepatic triacylglycerol and modulated ER-stress/inflammation pathways with improved production indices (Stoldt et al., 2015, Winkler et al., 2015). Beyond the liver, immune–redox crosstalk provides an additional mechanistic layer: hydrolyzed lignin from *Pinus taeda* modulated bovine PBMC responses ex vivo (↓TNF-α, ↓ROS/RNS, ↑cell viability), aligning with a systemic reduction in oxidative load (Ciliberti et al., 2020). Finally, adipose signaling is critical during the transition; naringin supplementation improved systemic metabolic status and attenuated oxidative stress by modulating

adipose function, offering a relevant comparator for phenolic blends (Li et al., 2024). Taken together, these pathways provide a coherent rationale for the concurrent improvements observed in the OXY group across lipid peroxidation (lower TBARS), metabolic markers, and liver-enzyme readouts. Therefore, the results of the present study further support the hypothesis that polyphenols may exert a beneficial influence on hepatic functionality, although the mechanisms involved remain to be clarified. Overall, as expected, the observed fluctuations in serum biochemical parameters were attributable to the physiological stage of the animals and not to Oxyphenol® supplementation, indicating that the treatment did not interfere with systemic metabolism or organ function. However, the transition period is not only marked by metabolic adaptations but also by profound changes in oxidative balance, which can increase the susceptibility of cows to periparturient disorders (Bernabucci et al., 2005, Sordillo and Aitken, 2009). This makes antioxidant support particularly relevant, as conventional feeding systems largely focus on preventing deficiencies rather than optimizing redox status (Overton and Yasui, 2014). As discussed above for lipid and hepatic indices, the redox trajectory around calving closely interacts with energy balance and liver function, so interpreting oxidative markers alongside NEFA/cholesterol and liver enzymes strengthens causal inference (Bernabucci et al., 2005, Sordillo and Aitken, 2009, Overton and Yasui, 2014). Against this background, the evaluation of oxidative markers in Oxyphenol®-supplemented cows highlights its potential role in mitigating oxidative stress. Specifically, serum levels of lipid peroxidation products were lower in the OXY treatment compared with CON during the *postpartum* period. This effect was most evident at +3 and +7 d *postpartum*, a window that typically shows the peak of circulating MDA/TBARS in dairy cows (Konvičná et al., 2015). Moreover, polyphenols-supplemented cows tended to exhibit reduced protein carbonyls concentrations and higher FRAP values relative to CON, although these differences did not reach statistical significance. Similar decreases in lipid peroxidation and improvements in plasma antioxidant capacity have been reported with plant polyphenol sources in transition cows (e.g., grape seed oil increased FRAP/TAC; pomegranate peel–based supplements modulated oxidative status) (Safari et al., 2018, Akhlaghi et al., 2022, Signor et

al., 2024). These parallels support the biological plausibility that the phenolic mixture in Oxyphenol® can blunt postpartum oxidative pressure. With regard to the antioxidant defense system available to counteract oxidative insults, our analysis of enzymatic activity revealed lower SOD around calving and lower GSPx after calving in OXY than in CON, concomitant with lower TBARS in OXY, indicating less oxidative load and thus less need for enzymatic up-regulation. This pattern is consistent with the canonical response whereby SOD/GSPx rise under greater oxidative pressure and normalize as redox stress subsides (Abuelo et al., 2015, Konvičná et al., 2015, Sayiner et al., 2021). Within the enzymatic antioxidant network, SOD and GSPx represent two pivotal components. SOD acts as the first line of defense against superoxide radicals, neutralizing them before they can accumulate to harmful levels. GSPx, on the other hand, plays a critical role in the detoxification of hydrogen and lipid peroxides, thereby preserving cellular membrane integrity (Sharma et al., 2011). Accordingly, the higher SOD/GSPx activities observed in CON most likely reflect compensatory activation in the face of greater lipid peroxidation, whereas the OXY profile (lower TBARS with lower SOD/GSPx) indicates a mitigated oxidative stimulus rather than impaired defense. Consistently, FRAP showed a numerical rise in OXY at +30 and +60 d, mirroring the postpartum recovery of plasma antioxidant capacity described in transition cows, and aligning mechanistically with the immuno-redox modulation reported for *Pinus taeda* hydrolyzed lignin in bovine PBMCs (↓TNF-α, ↓ROS/RNS, ↑cell viability) (Abuelo et al., 2015, Konvičná et al., 2015, Ciliberti et al., 2020). Finally, this improved redox profile in OXY coincided with lower liver-enzyme activity (ALT/ALP), reinforcing a coherent picture of reduced oxidative-metabolic strain around calving (Bernabucci et al., 2005, Abuelo et al., 2015, Abuelo et al., 2019, Ma et al., 2021). Consistently, the assessment of ROS production by PBMC supported this interpretation. Cells from the CON treatment showed higher intracellular ROS levels compared with those from OXY cows, indicating a greater oxidative burden and, consequently, a higher demand for antioxidant enzyme activity. Conversely, the reduced ROS synthesis observed in PBMC from Oxyphenol®-supplemented animals suggests that the treatment may have mitigated oxidative processes at the cellular level, contributing to a more

balanced redox state and improved cellular response to oxidative stress (Maggiolino et al., 2024). Excessive generation of ROS, when not adequately counterbalanced by antioxidant defenses, can lead to cytotoxic events and oxidative damage to key cellular components such as lipids, proteins, and nucleic acids (Ciampi et al., 2020, Dinardo et al., 2022). Such oxidative imbalance is particularly critical during the peripartum period, when the increased metabolic demand and immune activation make cells more vulnerable to oxidative stress (Mutinati et al., 2014). In our study, the between-group gap in PBMC-derived ROS was most evident from 7 days prepartum to 3 days postpartum (Fig. 3), paralleling the window in which plasma TBARS also diverged (Table 4), thereby reinforcing the systemic–cellular concordance of the OXY effect. This is biologically plausible because periparturient immunosuppression and redox dysregulation are tightly intertwined in dairy cows (Sordillo and Aitken, 2009, Abuelo et al., 2015). In this context, the evaluation of PBMC viability provided additional insights into the potential protective role of Oxyphenol®. During the tight periparturient phase (from 3 days before to 3 days after calving), PBMCs from cows supplemented with Oxyphenol® showed higher cell viability compared with the control group, even following mitogenic stimulation with conA. Although H_2O_2 exposure markedly reduced cell viability in both groups, as expected given its strong pro-oxidant effect jeopardizing catalase activity, PBMC from the OXY treatment maintained significantly higher survival rates at calving. Notably, the OXY–CON differences were most pronounced at calving and persisted to +3 d under ConA stimulation, whereas under H_2O_2 challenge the advantage for OXY was evident at calving (Figs. 1–2). These results are consistent with prior work showing that phenolic supplementation can attenuate immune-cell oxidative responses and sustain ex vivo functionality in cattle; for example, hydrolyzed lignin from *Pinus taeda* reduced ROS and pro-inflammatory readouts while improving PBMC viability (Ciliberti et al., 2020), and naringin improved systemic oxidative status during transition (Li et al., 2024). At the mechanistic level, phenolics abundant in Oxyphenol® (quercetin/rutin-type flavonols, eriodictyol, rosmarinic- and methyl-gallate derivatives) are known to activate Nrf2 and temper NF-κB, limiting mitochondrial and NADPH-oxidase–derived ROS and thereby preserving lymphocyte

proliferation under mitogenic or oxidative stimuli (Song et al., 2014, Song et al., 2016). This cellular protection dovetails with the plasma patterns (lower TBARS with lower SOD/GPx up-regulation) and with the lower ALT/ALP observed in OXY, supporting a unified picture of reduced oxidative–metabolic strain around calving. Finally, because NEFA and BHBA can directly induce ROS-mediated injury in bovine hepatocytes and immune cells, the simultaneous mitigation of lipomobilization markers and PBMC oxidative activity observed here is coherent with an immunometabolic mode of action for polyphenols in transition cows (Sordillo and Aitken, 2009, Song et al., 2014, Abuelo et al., 2015).

Alongside their role in health and oxidative status of the animals, the inclusion of polyphenols in the diet of dairy cows has gained increasing attention for their potential impact on productive performance and milk quality (O'connell and Fox, 2001). Beyond their well-documented antioxidant and anti-inflammatory properties, polyphenols may be transferred into milk, potentially enhancing its nutritional and functional value. However, in contrast to monogastric species, the transfer of polyphenolic compounds from feed to milk in ruminants is considerably more complex (Gessner et al., 2017). Within the rumen, polyphenols undergo extensive biotransformation through microbial metabolism, followed by additional modifications in the liver and other tissues (Wein et al., 2016). Consequently, the compounds ultimately secreted into milk may differ substantially from those originally ingested, both in chemical structure and biological activity (Rocchetti et al., 2022). Consistent with this, targeted metabolomics in ruminants shows the appearance in milk of phase-II conjugates and microbial derivatives (e.g., phenylpropionic/hippuric-type metabolites), rather than the parent flavonoids, with concentrations depending on dose, matrix, and stage of lactation (Gessner et al., 2017, Rocchetti et al., 2022). Understanding these processes is therefore essential to elucidate how dietary polyphenols influence ruminal fermentation, systemic metabolism, and mammary gland function, ultimately shaping milk yield and composition. Nevertheless, a growing body of literature supports the transfer of dietary polyphenols or their metabolites into ruminant milk, or at least improvements in milk quality parameters attributed to polyphenol supplementation, either through

their direct inclusion or via polyphenol-rich by-products (Serra et al., 2021, Formato et al., 2022a). For instance, Cohen-Zinder et al. (2017) observed higher milk yield and antioxidant capacity, along with lower somatic cell counts, in Holstein cows fed *Moringa oleifera* silage (270 g/kg DM) from three weeks before calving until six weeks *postpartum*. Similarly, Delgadillo-Puga et al. (2019) found that the inclusion of *Acacia farnesiana* pods in the diet of goats increased the polyphenol content and antioxidant activity of milk, while reducing its cholesterol concentration. Moreover, Moate et al. (2014) observed an increase in concentrations of MUFAs, PUFAs, and cis-9, trans-11 linoleic acid in milk from cows supplemented with either dried or ensiled grape marc. Other polyphenol-rich feeds (e.g., olive by-products, grape pomace) have likewise improved milk antioxidant capacity and/or oxidative stability, sometimes without altering gross composition, suggesting that redox effects may occur independently of major shifts in fat, protein, or lactose (Castellani et al., 2017, Vargas-Bello-Pérez et al., 2018). In the present study, Oxyphenol® supplementation did not significantly affect milk chemical composition, coagulation properties, or fatty acid profile. This lack of change in rennet coagulation time and curd firmness is plausible given that ruminal biotransformation likely limited direct casein–polyphenol interactions at the udder level (O'connell and Fox, 2001, Malacarne et al., 2014). However, a distinct effect emerged in the oxidative profile, as milk from OXY cows exhibited higher antioxidant potential and lower concentrations of lipid oxidation products. Importantly, these milk outcomes mirrored the systemic findings (lower plasma TBARS and PBMC-derived ROS in OXY), supporting a system-to-milk linkage whereby improved maternal redox status translates into enhanced oxidative stability of milk (Sordillo and Aitken, 2009, Abuelo et al., 2015). Given that the milk fatty-acid profile did not differ between groups, the reduced formation of secondary lipid-oxidation products in OXY is unlikely to be explained by a shift toward less oxidizable lipids; rather, it is more consistent with a combination of (i) carryover of phenolic metabolites with reducing/chelating activity into milk and (ii) improved mammary redox tone via Nrf2-dependent defenses, both of which would limit propagation of lipid peroxidation (Gessner et al., 2017, Rocchetti et al., 2022). This finding is particularly noteworthy, providing compelling evidence in support of the

hypothesis that dietary polyphenols, or their metabolites, may have been transferred into milk. Such an outcome carries important implications not only for human health, due to the possible functional benefits of antioxidant-enriched milk, but also for technological and shelf-life aspects, as enhanced oxidative stability could improve overall product quality and preservation (Cutrim and Cortez, 2018). It should also be noted that the transfer and bioavailability of dietary polyphenols in ruminants depend on several interacting factors, including the specific chemical structure of polyphenols, their inclusion level, the composition of the dietary matrix, and the physiological as well as microbial characteristics of the host (Lee, 2014, Serra et al., 2021). Collectively, these variables determine the extent to which polyphenols are metabolized and ultimately incorporated into milk. In line with this, future work should pair UHPLC-HRMS untargeted/targeted profiling of milk with pharmacokinetic sampling (plasma ↔ milk) across lactation to identify Oxyphenol®-derived metabolites, quantify their carryover ratio, and relate them to oxidative markers and cheesemaking traits (Gessner et al., 2017, Rocchetti et al., 2022). Such an approach would also clarify whether the antioxidant benefits observed here arise primarily from direct phenolic transfer, systemic redox modulation at the mammary level, or both, thereby refining dietary strategies to optimize milk functionality without compromising processing performance.

Although the literature on colostrum is comparatively limited, a growing body of evidence supports the transfer of dietary polyphenols or their metabolites into ruminant secretions, or at least improvements in product oxidative stability with polyphenol-rich feeds (Serra et al., 2021, Formato et al., 2022a). For instance, supplementation with chestnut tannins in dairy cows during the peripartum period has been associated with improvements in colostrum quality (e.g., higher IgG) and antioxidant status (Prodanović et al., 2021). These heterogeneous outcomes align with the upstream constraints imposed by rumen metabolism, matrix effects, and stage of lactation (Rolinec et al., 2021). In our study, the chemical composition of colostrum was not affected by Oxyphenol® supplementation during the *peripartum* period. A decrease in dry matter content was observed, likely due to the natural decline in protein concentrations during the first days after calving. This reduction

in colostral protein is a well-documented physiological process, as the concentration of immunoglobulins (Ig), particularly IgG, decreases markedly with each passing hour after parturition. For this reason, among others, to ensure optimal passive immune transfer, colostrum should be ingested within the first two hours after birth (Ahmann et al., 2021, Röder et al., 2023). To contextualize these targets with standardized cut-points, high-quality first-milking colostrum is commonly defined as IgG ≥ 50 g/L, which corresponds to a Brix threshold of ≥ 22% (McGuirk and Collins, 2004, Godden, 2008, Bielmann et al., 2010, Sockett et al., 2023). In our data, day-1 Brix in OXY averaged slightly above this threshold versus CON (22.85 vs. 21.71), although not statistically different, which is consistent with biological variability and the steep early postpartum decline in IgG (McGuirk and Collins, 2004; Bielmann et al., 2010) (). Interestingly, while Oxyphenol® supplementation did not alter the overall chemical composition of colostrum, it appeared to exert a beneficial influence on its oxidative profile. In particular, cows receiving Oxyphenol® showed reduced levels of lipid oxidation products in colostrum, suggesting that the treatment may have mitigated oxidative processes occurring during the early *postpartum* period. These colostrum findings mirror the systemic patterns (lower plasma TBARS, lower PBMC-derived ROS) and align with the concept that improved maternal redox status can translate into enhanced oxidative stability of secretions. Mechanistically, this could reflect (i) partial carryover of reducing/chelating phenolic metabolites and (ii) Nrf2-linked antioxidant tone at the mammary level limiting peroxidation chain reactions, without requiring changes in gross composition (Celi and Gabai, 2015, Rocchetti et al., 2022). In line with the findings observed in milk, the potential transfer of polyphenolic compounds into colostrum represents a biologically relevant outcome, as it suggests that, beyond ensuring passive immune transfer, colostrum could also convey antioxidant molecules or their metabolites to the newborn, potentially supporting its redox status (De Paris et al., 2020). From a neonatal perspective, attenuating colostrum lipid peroxidation may be advantageous, given the tight coupling between oxidative stress and immune function in the immediate postnatal period; colostrum inherently provides enzymatic (e.g., SOD, GPx) and non-enzymatic antioxidants, and higher-quality colostrum

is associated with better calf health indices (Przybylska et al., 2007, Godden, 2008, Sordillo and Aitken, 2009, Puppel et al., 2019). Future work should pair UHPLC-HRMS profiling of colostrum with plasma↔colostrum pharmacokinetics to identify Oxyphenol®-derived metabolites, estimate carryover ratios, and link them to oxidative markers and passive transfer outcomes.

6. Chapter 6: Conclusions

The present study demonstrated that dietary supplementation with Oxyphenol® during the *peripartum* period did not alter the systemic metabolic profile, milk yield, or chemical composition of colostrum and milk, indicating that the treatment was well tolerated and did not interfere with the physiological adaptations occurring around calving. Concurrently, cows receiving Oxyphenol® showed a consistently improved redox phenotype, lower plasma TBARS with blunted SOD/GPx up-regulation, reduced PBMC-derived ROS with higher peripartum viability, and lower milk/colostrum lipid-oxidation products (with numerically higher FRAP), together with lower ALT/ALP, supporting a reduction in oxidative–metabolic strain. These improvements in metabolic, oxidative, and immune-related parameters suggest that Oxyphenol® supplementation may support enhanced animal welfare during the calving transition. Concurrently, these findings are compatible with partial carryover of phenolic metabolites and/or improved mammary redox tone and suggest that dietary polyphenols, or their metabolites, may reach mammary secretions and contribute to enhanced oxidative stability. From a product perspective, this may translate into technological and shelf-life advantages without compromising processing traits. Future research should adopt an integrated approach: first, by characterizing the polyphenolic and metabolite fingerprints of milk and colostrum using targeted and untargeted UHPLC–HRMS coupled with plasma–to–secretion pharmacokinetics to delineate transfer and biotransformation; second, by including liver-specific enzymes such as GLDH and GGT alongside molecular readouts of Nrf2 and NF-κB to illuminate mechanistic pathways; third, by extending follow-up beyond 60 DIM and validating across multiple herds to test durability and generalizability; and finally, by quantifying downstream consequences for dairy product performance—oxidative stability during storage, cheesemaking behavior, and sensory attributes—as well as neonatal outcomes related to passive transfer and early health.

7. Chapter 7: Tables and Figures

Table 1. Composition and nutritive value of the diets fed to dairy cows during the experimental period.

	From -60 to -21 days relative to calving	From -21 to +7 days relative to calving	From + 7 to +60 days relative to calving
Ingredients (% of DM)			
Grass hay	32	18	-
Alfalfa hay	8	-	-
Triticale silage	25	30	30
Chopped cereal straw	15	8	-
Dried sugar beet pulp	9	6	5
Wheat bran	8	2	-
Mineral–vitamin premix (no anionics)	2	2.8	1.8
Live yeast/buffer	1	0.2	-
Sulla hay	-	8	12
Ground corn	-	9	21
Ground barley	-	6	10
Soybean meal	-	10	10
Grass hay	-	-	5
Rapeseed meal (canola meal)	-	-	4
Rumen-protected fat (Ca salts)	-	-	1.2
Chemical composition (% of DM)			

DM %	48.2	47.8	48.1
Crude protein (CP)	13.2	15.8	17
Neutral detergent fiber (NDF)	44	36	32
Starch	11	19	27
Sugars	6.7	6.6	5
Fat	2.8	3.3	5.5
Net energy for lactation (NEL), MJ/kg DM	5.5	6.1	6.9
Calcium (Ca)	0.60	0.82	0.90
Phosphorus (P)	0.35	0.40	0.45
Magnesium (Mg)	0.35	0.45	0.40

Table 2. Composition and antioxidant activity of *Pinus taeda* hydrolyzed lignin extract (Oxyphenol®) fed to dairy cow of treated group (OXY) for 120 days.

Components (g/kg)	
Vanillin	264
Eriodictyol	34
Quercetin	27
Isorhamnetin	16
Rosmarinic acid	14
Quercetin rhamnoside	139
Methyl gallate rutinoside	423
Epigallocatechin-3-methylgallate	15
Ferulic acid derivatives	67
Antioxidant activity (µmol TE[1] g−1 DW[2])	
TEAC[3]	23.9
ORAC[4]	122.44

[1] TE = Trolox equivalents.

[2] DW = dry weight.

[3] TEAC = Trolox equivalent antioxidant capacity.

[4] ORAC = oxygen radical absorbance capacity.

Table 3. Blood biochemical profile of dairy cows fed with (OXY) or without (CON) Oxyphenol® (*Pinus taeda* hydrolized lignin) from the beginning of the dry period through 60 days into lactation.

Parameter	Group[1]	Days relative to calving									SEM[2]	Analysis of variance[3]		
		-60	-30	-7	-3	0	+3	+7	+30	+60		T	D	T × D
Total protein (g/dl)	CON	6.72^{AB}	7.01	6.35^{A}	6.69^{AB}	6.75^{AB}	7.31	6.81	7.61^{BC}	7.76^{C}	0.27	0.7725	0.0002	0.7743
	OXY	7.04	7.39	6.61^{A}	6.57^{A}	6.64^{A}	6.95	7.11	7.72^{B}	7.32				
Albumin (g/dl)	CON	4.22	3.87	3.79	3.78	3.90	4.19	3.71	3.90	4.10	0.12	0.3116	0.2787	0.0576
	OXY	3.64	4.07	3.97	3.79	3.75	3.84	3.86	3.93	4.05				
Globulin (g/dl)	CON	2.57^{A}	3.15	2.63^{A}	2.77	2.88	3.04	3.20	3.69^{B}	3.70^{B}	0.28	0.3993	0.0043	0.8138
	OXY	3.32	3.38	2.69^{A}	2.78^{a}	3.06	3.11	3.22	3.82^{Bb}	3.28				
Urea (mg/dl)	CON	28.97	25.54	30.57	32.75	29.62	26.00	26.86	27.10	28.70	3.05	0.1263	0.1133	0.7745
	OXY	29.02	29.17	38.37	31.30	32.25	33.42	28.93	26.48	25.64				
Uric acid (mg/dl)	CON	1.45	1.32	1.29	1.20	1.22	1.42	1.63	1.55	1.67	0.19	0.0655	0.3755	0.9918
	OXY	1.27	1.10	1.15	1.16	1.13	1.35	1.25	1.48	1.36				
Creatinine (mg/dl)	CON	1.51	1.61	1.57	1.56	1.61	1.58	1.41	1.36	1.35	0.10	0.5589	0.3845	0.8146
	OXY	1.31	1.50	1.54	1.57	1.65	1.52	1.42	1.29	1.27				

Cholesterol (mg/dl)	CON	119.62^{AB}	93.14^{AC}	75.97^{C}	76.02^{C}	67.31^{C}	82.22^{C}	88.66^{AC}	150.20^{BD}	174.66^{D}	9.95	0.1441	<.0001	0.8392
	OXY	120.95^{A}	103.24^{AB}	73.53^{BC}	72.67^{BC}	58.82^{C}	65.17^{C}	80.35^{BC}	127.42^{A}	165.28^{D}				
Triglycerides (mg/dl)	CON	30.97	34.70^{AB}	35.17^{AB}	36.80^{A}	30.60	23.20	15.82^{C}	21.72	19.72^{BC}	4.59	0.0797	0.0028	0.8168
	OXY	25.92	27.48^{A}	28.64^{A}	29.90^{A}	21.47	18.64^{B}	20.33	18.17^{B}	22.46				
NEFA (mmol/l)	CON	0.40^{A}	0.37^{A}	0.45^{a}	0.60	0.83^{Bb}	0.63	0.44^{a}	0.24^{A}	0.36^{A}	0.09	0.1820	<.0001	0.6454
	OXY	0.37^{A}	0.31^{A}	0.25^{A}	0.38^{A}	0.71^{B}	0.56^{A}	0.34^{A}	0.13^{C}	0.21^{AC}				
Glucose (mg/dl)	CON	57.60^{a}	56.44	56.71	56.28	53.11	41.60^{b}	43.14^{b}	48.66	48.60	6.20	0.6459	0.0153	0.9327
	OXY	59.67^{A}	54.88	51.46	54.45	54.95	50.95	42.75^{B}	50.51	51.33				
Magnesium (mg/dl)	CON	2.56	2.83	2.57	2.85	2.62	3.23	2.50	3.29	3.46	0.30	0.5823	0.0572	0.9214
	OXY	2.52	2.38	2.63	2.73	3.07	2.83	2.63	3.18	3.22				
Phosphorus (mg/dl)	CON	7.06	7.37	6.74	6.91	6.90	6.48^{A}	7.31	8.27^{B}	8.21^{B}	0.44	0.3154	0.0039	0.7090
	OXY	7.12	7.28	6.80	6.64	6.14^{A}	6.52	7.97^{B}	7.13	7.78				
Calcium (mg/dl)	CON	8.50	8.89	8.68	8.79	8.60	7.82	8.50	8.62	8.36	0.33	0.2670	0.2271	0.8068
	OXY	8.67	8.93	8.52	8.42	7.75	8.25	8.25	8.40	7.97				

ALT (U/l)	CON	17.31	17.92	19.71	17.64	19.44	26.04 X	16.42	17.04	21.48	3.12	0.0251	0.5793	0.5937
	OXY	15.70	15.66	16.28	14.60	14.85	14.22 Y	13.17	19.48	20.15				
AST (U/l)	CON	35.75	29.70^{A}	38.11	34.31	40.68	42.95	41.93	53.52	60.86^{B}	7.81	0.4339	0.0038	0.9973
	OXY	28.95	35.11	35.37	29.30	34.67	40.84	35.42	55.42	55.97				
ALP (U/l)	CON	185.31	162.58	189.60	181.60	207.17	176.84	189.86	162.40	174.42	35.38	0.0032	0.9993	0.9911
	OXY	114.07	134.06	125.06	114.67	131.20	130.46	133.97	140.40	155.53				
Bilirubin (mg/dl)	CON	0.49	0.46	0.43	0.48	0.55	0.55	0.44	0.42	0.41	0.05	0.4588	0.0500	0.9954
	OXY	0.49	0.42	0.41	0.45	0.59	0.56	0.37	0.39	0.39				

X, Y – Means with different superscripts differ significantly ($P < 0.01$) due to treatment.

A, B, C, D – Means with different superscripts differ significantly ($P < 0.01$) due to sampling day.

a, b – Means with different superscripts differ significantly ($P < 0.05$) due to sampling day.

[1] CON = control treatment, OXY = Oxyphenol® treatment (*Pinus taeda* hydrolized lignin).

[2] SEM = standard error of the means.

[3] T = treatment, D = sampling day, T × D = binary interaction between treatment and sampling day.

Table 4. Oxidative profile and antioxidant enzyme activity of plasma of dairy cows fed with (OXY) or without (CON) Oxyphenol® (*Pinus taeda* hydrolized lignin) from the beginning of the dry period through 60 days into lactation.

Parameter	Group	Days relative to calving									SEM	Analysis of variance		
		-60	-30	-7	-3	0	+3	+7	+30	+60		T	D	T × D
TBARS [MDA] µM	CON	1.40^{a}	1.38	1.32	1.72	2.31	3.84^{bX}	3.36^{X}	3.19^{X}	2.78^{x}	0.41	0.0022	0.0332	0.1970
	OXY	1.62	1.54	1.33	2.19	1.88	1.94^{Y}	1.98^{Y}	1.49^{Y}	1.42^{y}				
Protein carbonyls (nmol/ml)	CON	77.20	78.06	72.70	69.09	69.43	73.01	74.11	83.08	80.57	6.31	0.0822	0.2026	0.8990
	OXY	80.11^{Aa}	71.63	71.05	64.88^{b}	68.24	61.56^{B}	66.67^{b}	72.90	76.32				
FRAP (nmol ascorbic ac. eq./ml)	CON	92.51	86.41	93.57	89.35	80.18	99.06	96.57	99.94	110.18	6.95	0.1583	0.0910	0.9339
	OXY	88.60	84.23	86.11	85.99	86.89	102.17	105.19	115.96	118.29				
SOD (U/ml)	CON	26.65^{A}	27.86	37.00^{B}	39.64^{B}	46.00^{CX}	48.60^{CX}	41.45^{X}	36.93^{B}	28.28	3.88	0.0058	0.0011	0.1432
	OXY	29.90	29.63	32.58^{A}	30.25	28.30^{Y}	31.50^{AY}	30.08^{Y}	28.50	21.06^{B}				
GSPx (U/ml)	CON	3.76	2.99	2.96	3.47	4.23	4.78	3.70^{x}	4.38^{x}	3.98^{x}	0.63	0.0236	0.1153	0.2267
	OXY	3.75	3.14	3.23	3.32	3.88	3.70	3.28^{y}	3.25^{y}	3.02^{y}				
GST (U/ml)	CON	0.24	0.33	0.38	0.33	0.45	0.35	0.39	0.35	0.23	0.04	0.0546	0.3228	0.5182
	OXY	0.26	0.36	0.24	0.21	0.28	0.26	0.24	0.22	0.21				

X, Y – Means with different superscripts differ significantly ($P < 0.01$) due to treatment.

x, y – Means with different superscripts differ significantly ($P < 0.05$) due to treatment.

A, B, C – Means with different superscripts differ significantly ($P < 0.01$) due to sampling day.

a, b – Means with different superscripts differ significantly ($P < 0.05$) due to sampling day.

[1] CON = control treatment, OXY = Oxyphenol® treatment (*Pinus taeda* hydrolized lignin).

[2] SEM = standard error of the means.

[3] T = treatment, D = sampling day, T × D = binary interaction between treatment and sampling day.

Table 5. Milk production from dairy cows fed with (OXY) or without (CON) Oxyphenol® (*Pinus taeda* hydrolized lignin) from the beginning of the dry period through 60 days into lactation.

Parameter	Group	Days *postpartum*				SEM[1]	Analysis of variance		
		15	30	45	60		T	D	T × D
Milk yield (kg/d)	CON	29.83^{A}	35.72^{Ba}	38.56^{Bb}	41.10^{C}	7.24	0.3941	0.0001	0.2842
	OXY	30.22^{A}	36.48^{Ba}	39.45^{Bb}	42.23^{C}				
Fat (g/100g)	CON	4.21	3.66	3.69	3.30	0.63	0.6995	0.6264	0.2950
	OXY	4.81	3.82	4.87	4.53				
Protein (g/100g)	CON	3.58	3.47	3.36	3.59	0.08	0.2354	0.1091	0.2962
	OXY	3.71	3.51	3.55	3.49				
Casein (g/100g)	CON	2.86	2.75	2.68	2.84	0.06	0.3623	0.0785	0.4964
	OXY	2.94	2.77	2.80	2.77				
Whey proteins (g/100g)	CON	0.71	0.72	0.67	0.75	0.02	0.1296	0.6046	0.1556
	OXY	0.77	0.73	0.74	0.72				
Lactose (g/100g)	CON	4.74	4.82	4.70	4.86	0.07	0.2886	0.6783	0.3059
	OXY	4.82	4.90	4.86	4.75				
Urea (mg/dl)	CON	28.35	24.73	26.23	32.33	2.80	0.5549	0.6300	0.3208
	OXY	28.90	28.08	31.67	27.71				
BHB (mM)	CON	0.01	0.01	0.01	0.01	0.00	0.3091	0.9517	0.9415
	OXY	0.02	0.02	0.01	0.02				
pH	CON	6.55	6.61	6.58	6.62	0.03	0.5228	0.6488	0.4955
	OXY	6.66	6.68	6.69	6.63				
Fat-free dry matter (g/100g)	CON	9.06	9.01	8.75	9.16	0.14	0.2166	0.4231	0.2033
	OXY	9.27	9.15	9.12	8.94				
Citric acid (g/100 g)	CON	0.10	0.09	0.10	0.10	0.00	0.4411	0.7980	0.7146
	OXY	0.08	0.10	0.09	0.10				

Somatic cell score	CON	3.26	3.97	4.00	4.08	0.65	0.8309	0.7541	0.8097
	OXY	3.86	3.87	3.84	3.85				
Total bacterial load (UFC/ml)	CON	11665.81	12245.8	8841.03	16961.78	0.70	0.2897	0.1963	0.9619
	OXY	12311.37	13967.93	9944.03	14053.61				

A, B,C – Means with different superscripts differ significantly ($P < 0.01$) due to sampling day.

a, b – Means with different superscripts differ significantly ($P < 0.05$) due to sampling day.

[1] CON = control treatment, OXY = Oxyphenol® treatment (*Pinus taeda* hydrolized lignin).

[2] SEM = standard error of the means.

[3] T = treatment, D = sampling day, T × D = binary interaction between treatment and sampling day.

Table 6. Coagulation properties of milk from dairy cows fed with (OXY) or without (CON) Oxyphenol® (*Pinus taeda* hydrolized lignin) from the beginning of the dry period through 60 days into lactation.

Parameter	Group	Days *postpartum*				SEM[1]	Analysis of variance		
		15	30	45	60		T	D	T × D
a30[4] (mm)	CON	26.25	26.43	26.37	25.96	2.89	0.3612	0.4007	0.3308
	OXY	26.35	26.14	26.50	26.92				
k20[5] (min)	CON	10.18	11.12	10.75	10.89	0.66	0.5819	0.5140	0.3267
	OXY	11.64	11.57	11.21	11.42				
RCT[6] (min)	CON	14.12	14.18	14.81	15.52	2.39	0.3424	0.2076	0.3017
	OXY	14.50	15.47	15.78	15.00				

[1] CON = control treatment, OXY = Oxyphenol® treatment (*Pinus taeda* hydrolized lignin).

[2] SEM = standard error of the means.

[3] T = treatment, D = sampling day, T × D = binary interaction between treatment and sampling day.

[4] a30 = Curd firmness 30 min after rennet addition, [5] k20 = time to reach a curd firmness of 20 mm,

[6] RCT = rennet coagulation time.

Table 7. Oxidative profile of milk from dairy cows fed with (OXY) or without (CON) Oxyphenol® (*Pinus taeda* hydrolized lignin) from the beginning of the dry period through 60 days into lactation.

Parameter	Group	Days *postpartum*				SEM[1]	Analysis of variance		
		15	30	45	60		T	D	T × D
Malondihadehyde (µg/l)	CON	69.51	68.05^{x}	70.21^{X}	70.89^{X}	0.00	0.0012	0.3560	0.1036
	OXY	63.62	61.42^{y}	59.33^{Y}	57.05^{Y}				
Protein carbonyls (nm/mg proteins)	CON	38.40	40.03	40.26	40.77	2.15	0.0599	0.8949	0.3073
	OXY	39.77	37.84	38.81	37.58				
FRAP (nmol ascorbic acid eq./1 ml milk)	CON	176.23^{x}	170.43^{x}	167.07	159.82^{X}	20.66	0.0050	0.0714	0.7932
	OXY	190.29^{y}	184.58^{y}	179.51	177.07^{Y}				

X, Y – Means with different superscripts differ significantly ($P < 0.01$) due to treatment.

x, y – Means with different superscripts differ significantly ($P < 0.05$) due to treatment.

[1] CON = control treatment, OXY = Oxyphenol® treatment (*Pinus taeda* hydrolized lignin).

[2] SEM = standard error of the means.

[3] T = treatment, D = sampling day, T × D = binary interaction between treatment and sampling day.

Table 8. Fatty acids profile of milk from dairy cows (expressed in % on milk) fed with (OXY) or without (CON) Oxyphenol® (*Pinus taeda* hydrolized lignin) from the beginning of the dry period through 60 days into lactation.

Parameter	Group	Days *post-partum*				SEM[1]	Analysis of variance		
		15	30	45	60		T	D	T × D
SFA (%)	CON	3.11	2.42	3.18	2.13	0.43	0.8422	0.5992	0.3271
	OXY	2.74	2.35	2.49	3.01				
UFA (%)	CON	1.46	1.20	1.44	1.00	0.18	0.5225	0.6133	0.3421
	OXY	1.25	1.13	1.10	1.29				
TFA (%)	CON	0.09	0.06	0.08	0.01	0.02	0.7096	0.2968	0.5340
	OXY	0.08	0.04	0.03	0.05				
MUFA (%)	CON	1.36	1.06	1.33	0.86	0.20	0.4376	0.6165	0.2411
	OXY	1.16	0.99	0.80	1.19				
PUFA (%)	CON	0.15	0.12	0.15	0.09	0.02	0.5867	0.4262	0.4661
	OXY	0.14	0.11	0.11	0.12				
SCFA (%)	CON	0.68	0.53	0.70	0.43	0.09	0.6398	0.5438	0.3123
	OXY	0.58	0.51	0.51	0.60				
MCFA (%)	CON	1.63	1.31	1.69	1.29	0.18	0.9802	0.4381	0.3452
	OXY	1.51	1.28	1.45	1.71				
LCFA (%)	CON	1.72	1.36	1.69	1.09	0.26	0.4136	0.4941	0.3860
	OXY	1.44	1.03	1.25	1.50				
C14:0 (%)	CON	0.43	0.33	0.45	0.33	0.05	0.8624	0.4804	0.3536
	OXY	0.38	0.32	0.37	0.44				
C16:0 (%)	CON	1.10	0.83	1.14	0.74	0.16	0.9971	0.5861	0.2480
	OXY	1.00	0.82	0.86	1.13				
C18:0 (%)	CON	0.46	0.36	0.42	0.26	0.07	0.8381	0.4003	0.5833
	OXY	0.43	0.35	0.32	0.37				
C18:1 (%)	CON	1.42	1.11	1.37	0.90	0.20	0.4528	0.5226	0.3528
	OXY	1.22	0.97	0.95	1.22				

[1] CON = control treatment, OXY = Oxyphenol® treatment (*Pinus taeda* hydrolized lignin).

[2] SEM = standard error of the means.

[3] T = treatment, D = sampling day, T × D = binary interaction between treatment and sampling day.

Table 9. Chemical composition of colostrum and transitional milk from dairy cows fed with (OXY) or without (CON) Oxyphenol® (*Pinus taeda* hydrolized lignin) from the beginning of the dry period through 60 days into lactation.

Parameter	Group	Days *postpartum*					SEM [1]	Analysis of variance		
		1	2	3	4	5		T	D	T × D
Dry matter (%)	CON	18.95^{A}_{a}	17.12	16.36_{b}	16.30_{B}	15.38^{B}	0.83	0.6354	0.0003	0.9406
	OXY	19.52^{A}_{a}	18.02	16.34_{b}	15.85_{B}	15.62^{B}				
Moisture (%)	CON	81.05^{A}_{a}	82.87	83.63_{b}	83.75_{b}	84.61^{B}	0.83	0.6216	0.0002	0.9466
	OXY	80.47^{A}_{a}	81.97	83.65_{b}	84.14_{B}	84.37^{B}				
Protein (g/100 g)	CON	8.99^{A}	5.17^{B}	4.53^{B}	3.90^{B}	4.40^{B}	0.87	0.3002	<.0001	0.7008
	OXY	9.44^{A}	7.25^{a}	4.69^{B}	4.50^{B}	4.00^{Bb}				
Fat (g/100 g)	CON	5.65	5.79	6.26	5.65	4.89	1.03	0.3480	0.9187	0.9797
	OXY	4.87	4.77	5.62	4.66	5.11				
Ash (g/100 g)	CON	0.83	0.80	0.78	0.82	0.84	0.06	0.6328	0.9356	0.9582
	OXY	0.85	0.86	0.80	0.85	0.80				
Brix (°)	CON	21.71^{A}	13.87^{B}	12.37_{B}	12.06_{B}	11.62^{B}	1.28	0.2319	<.0001	0.8899
	OXY	22.85^{A}	16.16^{B}_{a}	13.78_{B}	12.35_{B}	11.42^{B}_{b}				

A, B – Means with different superscripts differ significantly ($P < 0.01$) due to sampling day.

a, b – Means with different superscripts differ significantly ($P < 0.05$) due to sampling day.

[1] CON = control treatment, OXY = Oxyphenol® treatment (*Pinus taeda* hydrolized lignin).

[2] SEM = standard error of the means.

[3] T = treatment, D = sampling day, T × D = binary interaction between treatment and sampling day.

Table 10. Oxidative profile of colostrum and transitional milk from dairy cows fed with (OXY) or without (CON) Oxyphenol® (*Pinus taeda* hydrolized lignin) from the beginning of the dry period through 60 days into lactation.

Parameter	Group	Days *postpartum*					SEM[1]	Analysis of variance		
		1	2	3	4	5		T	D	T × D
Malondihadehyde (µg/l)	CON	344.94^{AX}	267.36^{BX}	162.32^{Cx}	125.32^{CD}	87.13^{D}	0.04	0.0001	<.0001	0.1125
	OXY	228.72^{aY}	139.94^{Y}	112.79^{by}	100.26^{b}	79.37^{b}				
Protein carbonyls (nm/mg proteins)	CON	37.94	36.00^{a}	39.41	41.22^{b}	41.02	1.72	0.0134	0.0103	0.9947
	OXY	34.70	33.12^{a}	36.53	38.12^{b}	39.21^{b}				
FRAP (nmol ascorbic ac. eq./1 ml milk)	CON	209.88	211.39	227.18	203.01	208.8	17.99	0.1546	0.6288	0.9195
	OXY	282.74	273.24	286.74	256.39	253.16				

x, y – Means with different superscripts differ significantly ($P < 0.05$) due to treatment.

A, B, C – Means with different superscripts differ significantly ($P < 0.01$) due to sampling day.

a, b – Means with different superscripts differ significantly ($P < 0.05$) due to sampling day.

[1] CON = control treatment, OXY = Oxyphenol® treatment (*Pinus taeda* hydrolized lignin).

[2] SEM = standard error of the means.

[3] T = treatment, D = sampling day, T × D = binary interaction between treatment and sampling day.

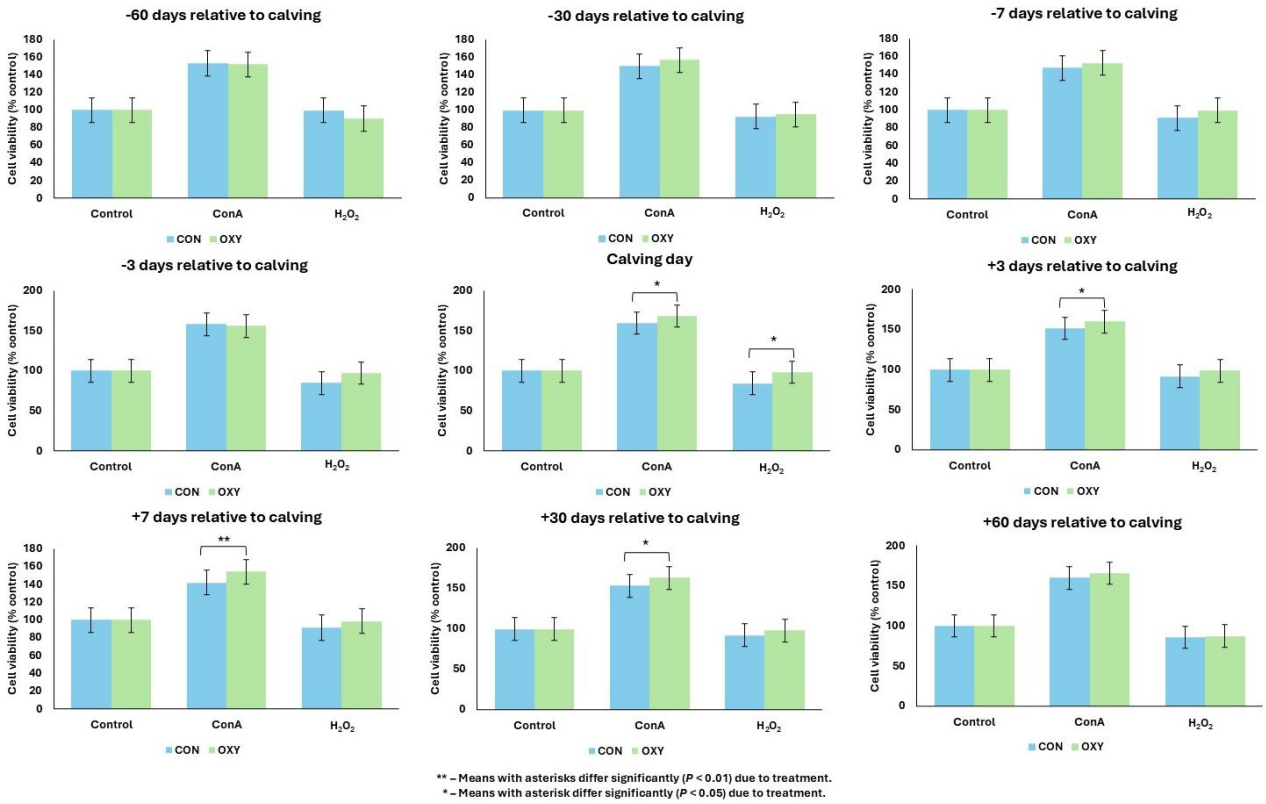

Figure 3. Cell viability and proliferation of peripheral blood mononuclear cells (PBMC) treated *in vitro* with concanavalin A and hydrogen peroxide of dairy cows fed with (OXY) or without (CON) Oxyphenol® (*Pinus taeda* hydrolized lignin) from the beginning of the dry period through 60 days into lactation.

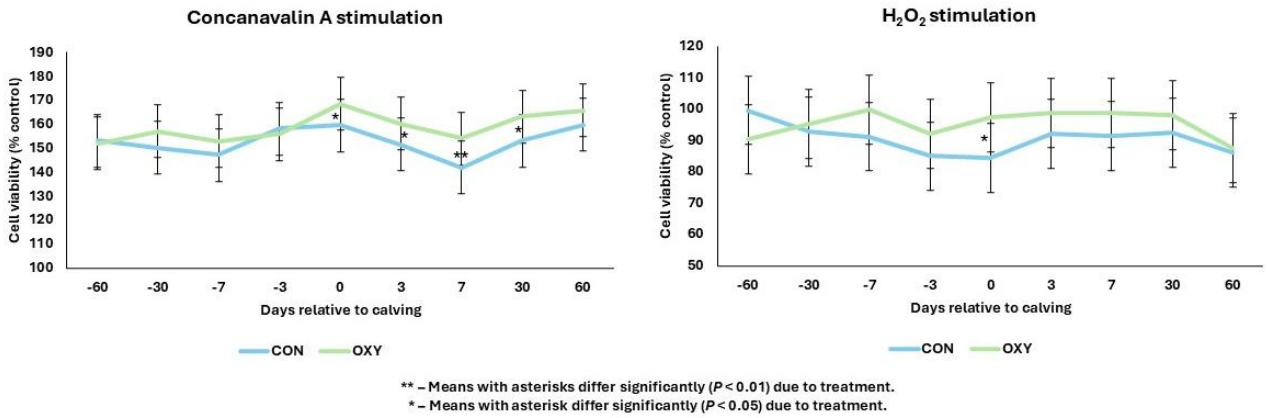

Figure 4. Cell viability and proliferation of peripheral blood mononuclear cells (PBMC) treated in vitro with concanavalin A and hydrogen peroxide of dairy cows fed with (OXY) or without (CON) Oxyphenol® (*Pinus taeda* hydrolized lignin) from the beginning of the dry period through 60 days into lactation.

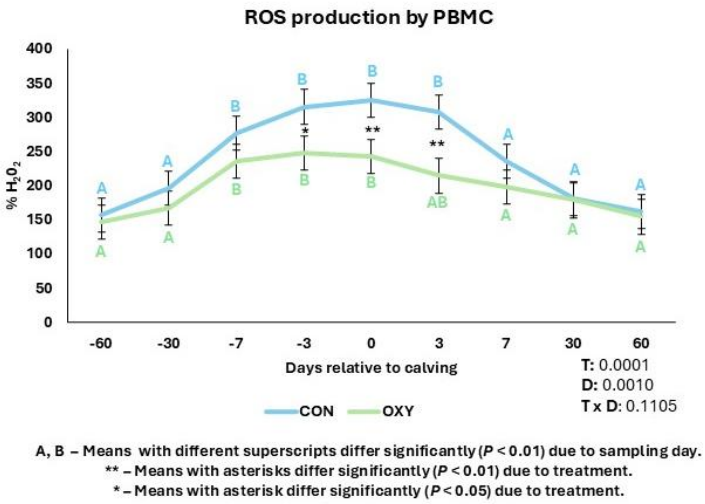

Figure 5. Reactive oxygen species (ROS) production by peripheral blood mononuclear cells (PBMC) of dairy cows fed with (OXY) or without (CON) Oxyphenol® (*Pinus taeda* hydrolized lignin) from the beginning of the dry period through 60 days into lactation.

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