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Development of strategies for the adaptation of the livestock sector to the new climate regime with machine learning and artificial intelligence methods

Settore scientifico-disciplinare (s.s.d. AGRI-09/B - Nutrizione e alimentazione animale)

PhD student:

Dott.ssa Chiara Evangelista

Course Coordinator

Chiar.mo Prof. Andrea Vannini

Co-Supervisor

Dott. Marco Milanese

Supervisor

Prof. Giovanni Chillemi

Thesis Co-Supervisor's

Prof. Umberto Bernabucci

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Riassunto

La presente tesi di dottorato, realizzata nell'ambito del Programma Green (Azione IV.5 D.M. 1061/2021) e finanziata dal programma FSE-REACT-EU, PON "Ricerca e Innovazione" 2014-2020, intende sviluppare soluzioni innovative finalizzate a migliorare la sostenibilità e a ridurre l'impatto ambientale nel settore zootecnico, con particolare riferimento all'allevamento di bufale da latte. Tale ricerca si colloca in un contesto globale in cui la conservazione degli ecosistemi, la tutela della biodiversità e la mitigazione dei cambiamenti climatici costituiscono priorità strategiche, soprattutto considerando il ruolo fondamentale dei ruminanti nella produzione alimentare e nelle emissioni di gas serra, in particolare il metano prodotto dalla fermentazione enterica.

Il lavoro si articola in diversi capitoli. In primo luogo, si effettua una revisione dello stato dell'arte sulle emissioni enteriche di metano, mediante un'analisi bibliometrica basata su tecniche di *machine learning*, finalizzata a mappare l'evoluzione della ricerca, la distribuzione geografica degli studi e a individuare le principali tematiche affrontate, evidenziando le attuali lacune conoscitive. Successivamente, l'attenzione si sposta sullo sviluppo e l'applicazione della spettroscopia nel vicino infrarosso (NIRS) come strumento per la gestione di precisione dell'alimentazione. In particolare, sono stati impiegati un sistema da banco e uno portatile per l'analisi della composizione chimica e del profilo degli acidi grassi del TMR (*Total Mixed Ration*) destinato alle bufale; inoltre, sono state applicate tecniche di *machine learning* per selezionare le lunghezze d'onda chiave nella predizione della frazione fibrosa, con l'obiettivo di rendere disponibili dispositivi più economici e accessibili agli allevatori. Infine, è stato valutato uno strumento NIRS portatile commerciale nella determinazione dei fattori che influenzano l'omogeneità di distribuzione del TMR.

Il successivo ambito di studio si concentra sulle strategie nutrizionali finalizzate all'ottimizzazione delle prestazioni produttive degli animali, con particolare attenzione all'uso di antiossidanti naturali e alla supplementazione di micronutrienti (zinco e selenio) per valutare la capacità di tali interventi di sostenere la produzione e la qualità del latte delle bufale durante i mesi estivi. Inoltre, nell'ambito dell'alimentazione di precisione, è stato condotto, per la prima volta nella bufala da latte, uno studio sulla caratterizzazione fisica della dieta e sui suoi effetti sulla produzione, sulla qualità del latte e sulla digeribilità dei nutrienti, impiegando il *Penn State Particle Separator*. Un ulteriore studio pionieristico ha analizzato il microbiota fecale delle bufale, esplorando questo metodo non invasivo per identificare i fattori dietetici che influenzano la composizione microbica delle feci.

L'obiettivo principale di questa tesi è stato quello di testare soluzioni applicabili alla nutrizione dei ruminanti, finalizzate a migliorare il benessere animale e l'efficienza del settore zootecnico, riducendo contestualmente l'impatto ambientale. Pur concentrandosi sull'allevamento di bufale, le tecnologie e gli

approcci sviluppati presentano potenzialità di applicazione anche ad altre specie ruminanti, contribuendo così a una maggiore sostenibilità e innovazione nel settore agroalimentare.

Abstract

This doctoral thesis, conducted within the framework of the Green Program (Action IV.5 D.M. 1061/2021) and funded by the FSE-REACT-EU program, PON "Research and Innovation" 2014-2020, aims to develop innovative solutions to improve sustainability and reduce the environmental impact in the livestock sector, with particular reference to dairy buffalo farming. This research is set within a global context where the conservation of ecosystems, the protection of biodiversity, and the mitigation of climate change are strategic priorities, especially considering the fundamental role of ruminants in food production and greenhouse gas emissions, particularly methane produced by enteric fermentation.

The work is structured into several chapters. First, a review of the state of the art on enteric methane emissions is conducted through a bibliometric analysis based on machine learning techniques, aimed at mapping the evolution of research, the geographical distribution of studies, and identifying the main topics addressed, highlighting current knowledge gaps. The focus then shifts to the development and application of Near Infrared Spectroscopy (NIRS) as a tool for precision feeding management. Specifically, a benchtop system and a portable device were used to analyse the chemical composition and fatty acid profile of the Total Mixed Ration (TMR) for buffalo; machine learning techniques were also applied to select key wavelengths for predicting the fiber fraction, with the aim of making more affordable and accessible devices available to farmers. Finally, a commercial portable NIRS device was evaluated in determining the factors that influence the homogeneity of TMR distribution.

The next area of study focuses on nutritional strategies aimed at optimizing animal production performance, with particular attention to the use of natural antioxidants and supplementation of micronutrients (zinc and selenium) to assess their ability to support buffalo milk production and quality during the summer months. Furthermore, within the scope of precision feeding, a pioneering study was conducted for the first time in dairy buffalo, focusing on the physical characterization of the diet and its effects on milk production, quality, and nutrient digestibility, using the Penn State Particle Separator. An additional pioneering study analysed the faecal microbiota of buffalo, exploring this non-invasive method to identify dietary factors influencing the microbial composition of the faeces.

The main objective of this thesis was to test solutions applicable to ruminant nutrition, aimed at improving animal welfare and the efficiency of the livestock sector, while simultaneously reducing environmental impact. Although focused on buffalo farming, the technologies and approaches developed have potential applications for other ruminant species, thus contributing to greater sustainability and innovation in the agri-food sector



Chapter 1

Chiara Evangelista

Chapter 1

General introduction

Positioning of the Research within the Green Program (Azione IV.5 D.M. 1061/2021)

The doctoral research was carried out within the FSE-REACT-EU financial program, PON "Research and Innovation" 2014-2020 (D.M. 1061/2021), Action IV.5 "PhD Programs on Green Topics." The main objectives of the Green Program focus on ecosystem conservation, biodiversity, reducing the impacts of climate change, and promoting sustainable development. The entire thesis work, as well as the activities carried out, aimed to study, investigate, and implement practices primarily focused on increasing sustainability and, indirectly, reducing environmental impact, all within the livestock sector.

Background

The livelihoods of farmers and other participants in the agrifood chain, as well as global nutrition and food security, depend heavily on ruminants (Kotsampasi et al., 2024). Ruminant animals, including cattle, sheep, goats, and buffalo, constitute the majority of the world's livestock production and provide more than half of the protein derived from the livestock industry, primarily in the form of milk and meat (Kotsampasi et al., 2024). According to FAO data from 2023 (FAOSTAT, 2025), cattle are the most prevalent species, followed by sheep, goats, and buffalo (Figure 1). In Europe, the country with the highest number of buffalo is Italy, with approximately 416,000 buffalo raised, followed by Bulgaria with 20,940, Romania with 16,900, and Germany with 12,150 (data from 2023, FAOSTAT). Buffalo farming in Italy is therefore a significant sector of livestock farming.

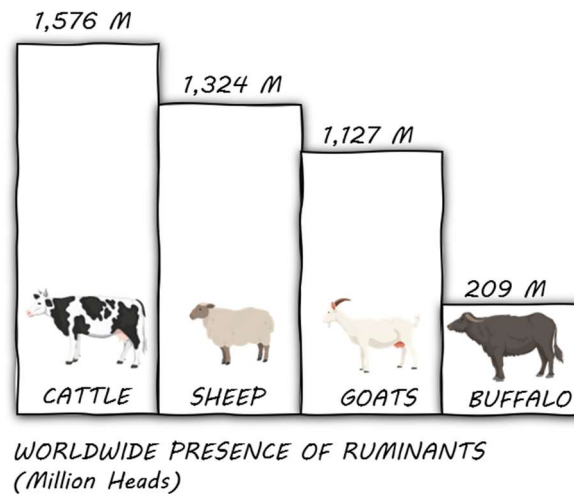


Figure 1. Worldwide population of ruminants (Cattle, sheep, goats and buffalo) million heads, in 2023 (FAOSTAT, 2025).

Agricultural activities, particularly livestock farming, are increasingly threatened by climate change, which manifests in rising temperatures, altered rainfall patterns, and the increased frequency of extreme weather events such as heatwaves and droughts. These climatic shifts not only compromise animal health but also reduce overall productivity, leading to diminished animal welfare and jeopardizing the sustainability of livestock farming. In turn, this affects the ability to produce sufficient and quality livestock feed to meet the growing global demand for animal products (Bernabucci, 2019). The livestock sector itself is a significant contributor to climate change, primarily through the emission of greenhouse gases (GHGs) such as methane (CH_4), carbon dioxide (CO_2), nitrous oxide (N_2O), and ammonia (NH_3). Of these, CH_4 is particularly impactful as its global warming potential is approximately 25 times greater than that of CO_2 (Cassandro et al., 2013). Methane is mainly produced during enteric fermentation in ruminants and through manure management. In fact, the livestock sector is responsible for approximately 14.5% of total anthropogenic GHG emissions, with enteric CH_4 emissions accounting for 39% of these CH_4 emissions linked to animal production (Grossi et al., 2019).

Several factors influence CH_4 production in animals, including the species, level of production, diet composition, carbohydrate types, lipid saturation, and feed intake (Johnson and Johnson, 1995). Environmental conditions, such as temperature, and genetic factors, such as feed conversion efficiency, also play crucial roles in CH_4 emissions (McAllister et al., 1996). Additionally, CH_4 produced by livestock results in significant energy losses—up to 12% of gross energy intake in ruminants is lost as methane (Tseten et al., 2022).

The environmental impact of CH₄ and other GHGs is expected to grow as global demand for animal products increases. According to the World Population Prospects 2022, the global human population is projected to reach 9.7 billion by 2050, escalating pressure on the global food system and amplifying the environmental challenges posed by livestock farming. Climate change further complicates the situation, as livestock farming is vulnerable to the negative impacts of heat stress (HS) and diseases, which can reduce growth rates, milk and egg production, and reproductive performance, while also affecting feed availability and quality.

Livestock farming, therefore, plays a dual role in climate change: it represents a significant source of GHG emissions within the agricultural sector, and it is also highly vulnerable to the adverse impacts of a changing climate. The continued growth of the livestock sector will exacerbate the challenges associated with both mitigating and adapting to climate change. Consequently, it is essential to explore sustainable practices, including improved feeding strategies, genetic selection for heat-tolerant breeds, and the adoption of more efficient land management practices to minimize both the sector's environmental footprint and its vulnerability to climate change. To achieve our objectives, it is crucial to understand the current body of knowledge available in the literature. This understanding requires efficient and comprehensive access to all relevant studies. However, as the volume of scientific literature on this topic continues to expand, traditional systematic reviews can become both resource-intensive and time-consuming. In this context, the adoption of innovative tools and machine learning (ML) algorithms has become essential for efficiently identifying, analysing, and synthesizing the vast amount of existing research. ML, a branch of artificial intelligence focused on developing algorithms for forecasting, inference, and clustering (Trapanese et al., 2024), offers powerful techniques capable of managing large datasets, such as those derived from bibliographic research on a given topic. In the livestock sector, these methods are particularly well-suited to handle non-linear, complex, noisy, and imprecise data, such as those encountered in dairy production - which encompasses aspects of production, reproduction, and animal welfare - as well as sensor data (Trapanese et al., 2024).

Enteric Methane Emissions in Livestock: A Machine Learning-Based Bibliometric Analysis

Systematic literature reviews are essential for assessing the current state of knowledge on specific topics and guiding future research directions (O'Connor and Sargeant, 2015). However, they can be resource-intensive and time-consuming, requiring the identification, evaluation, and synthesis

of numerous studies. As scientific literature grows rapidly - at an annual rate of 8–9% in recent years (Bornmann and Mutz, 2015) - efficiently obtaining relevant information becomes increasingly challenging (Cesari et al., 2024).

To address this challenge, text mining (TM) and topic analysis (TA) have emerged as powerful unsupervised techniques. These approaches automate the identification of recurring themes and hidden patterns within large datasets (Wang et al., 2016). By summarizing data visually and revealing relationships between concepts, TM and TA enable the creation of structured maps that provide deeper insights into the literature (Abutridy, 2000). Data mining, which involves analysing large datasets to uncover correlations and trends, leverages mathematical and statistical methods (Garcia, 2013). In livestock studies, these tools have increasingly been used to identify key themes and suggest future research directions, contributing to the development of more sustainable and efficient livestock systems (Garcia, 2013).

Enteric CH₄ emissions are a significant concern within the livestock sector; however, no study has yet applied TM or TA to investigate this issue. Therefore, the aim of **Chapter 2** is to conduct a review using TM and TA based on machine learning techniques to examine the evolution and geographical distribution of research, identify the most studied topics, and highlight existing knowledge gaps regarding enteric CH₄ emissions from ruminants.

Advancements in Near Infrared (NIR) Spectroscopy for precision feeding management in dairy farming

A significant portion of GHG emissions in livestock farming stems from the production of animal feed, with feed production and processing contributing approximately 45% to total emissions (Grossi et al., 2019). As a result, optimizing feed formulation and distribution can play a crucial role in reducing both GHG emissions and farm costs. This is the core objective of Precision Feeding (PF), an integral component of Precision Livestock Farming (PLF). In dairy farming, where feed costs account for over half of milk production expenses (Evangelista et al., 2021), the importance of minimizing inefficiencies cannot be overstated. Inaccurate feed rations can not only lead to economic losses but can also cause digestive problems, poor milk quality, reduced reproductive performance, and increased waste. Additionally, optimizing feeding practices contributes to a decrease in environmental impact and the need for pharmaceutical interventions, thereby enhancing overall farm sustainability (Evangelista et al., 2021).

Near-Infrared Spectroscopy (NIRS) is a well-established technique, with its application in the livestock sector becoming increasingly prominent over the past 50 years. Pioneered by Norris in 1979 for forage analysis, NIRS marked a significant advancement in the ability to analyse and assess raw materials on farms. Over time, the method has been increasingly used to optimize feeding practices, providing real-time data on the quality and performance of feed ingredients, thus ensuring that the final product meets desired nutritional standards.

One of the most notable advancements in NIRS technology is the development and widespread adoption of portable devices. These portable NIRS systems have become more compact, user-friendly, and capable of providing real-time analysis, even under field conditions. With improvements in battery life, wireless connectivity, and integration with mobile platforms, these devices have become more accessible, even in remote locations (Hossain et al., 2024). While portable NIRS devices initially lagged behind benchtop models in terms of accuracy and spectral range, recent technological advancements - including improved calibration methods and enhanced data processing algorithms - have significantly narrowed this gap (Hossain et al., 2024). Additionally, NIRS systems can be used manually on farms to assess the quality and composition of raw materials and the Total Mixed Ration (TMR) (Evangelista et al., 2021). For example, understanding the actual dry matter content of forages - especially silage - is critical, as short-term moisture fluctuations can significantly impact ration composition (Weiss, 2012). Such variations can negatively affect milk health and yield, leading to issues such as abomasum displacement, subclinical acidosis, and fluctuations in milk production (Sova et al., 2014). Moreover, variability in the dry matter content of TMR can influence animal preference, resulting in imbalanced intake and decreased production efficiency (Leonardi & Armentano, 2003; DeVries et al., 2007). Piccioli-Cappelli et al. (2019) demonstrated that using portable NIRS systems on mixer wagons (MWs) can result in more consistent rations, reducing dry matter usage by 1.35% and increasing milk production by 2.4%. NIRS is increasingly being adopted for more detailed analyses especially in the dairy sector, where it is possible to predict a wide range of components from macronutrients to micronutrients (Giannuzzi et al., 2024). The aim of **Chapter 3** is to explore the potential of NIRS to predict components of the ration. The first study presented in **subchapter 3.1** is titled "Assessment of the Predictive Performance of a Benchtop and Portable NIR Instrument for Analysing the Chemical Composition and Fatty Acid Profile of Dairy Buffalo's Total Mixed Ration". This study evaluates the chemical composition of rations and fatty acid content using two types of instruments: a benchtop and a portable NIR device. Additionally, it focuses on assessing the adequacy of the portable NIR device in accurately predicting data compared to the benchtop model.

However, a significant limitation to the widespread adoption of portable instruments remains their relatively high cost. Factors influencing cost reduction include the spectral range (Milanesi et al., 2023), which directly affects the price. To lower the cost of portable instruments, it is crucial to identify a set of key wavelengths (Wvs) that can accurately estimate the parameters being studied. Therefore, the objective of the second study, presented in **subchapter 3.2** is to identify the most relevant wavelengths (Wvs) for the characterization of fiber in various feeds through the application of Machine Learning (ML) techniques. ML algorithms provide flexible, general-purpose approaches that can automatically learn complex relationships and patterns from both homogeneous and heterogeneous data, enabling the identification of hidden or difficult-to-detect relationships. The second objective was to evaluate the potential of using the selected Wvs (either partially or entirely) in in silico NIRS models.

Currently, there are several portable NIRS devices on the market capable of predicting the chemical composition of TMR, both in terms of its chemical and physical aspects (Evangelista et al., 2021). These devices typically cost between 15,000 and 20,000 euros. One such device, called PoliSPEC^{NIR} (ITPhotonic, Italy), has patented applications capable of assessing the homogeneity of the mixed feed. Proper mixing is essential because if the ration is not uniform, cows may select more palatable components, leading to an unbalanced intake that reduces rumen efficiency and production consistency (Serva et al., 2016). Therefore, ensuring the nutritional consistency and distribution of TMR is an economically valuable practice for farm management. Several factors, such as the type of mixer wagon, mixing time, fill level, loading sequence, and raw material composition, influence mixing homogeneity, which in turn impacts the efficiency and quality of TMR (Buckmaster, 2009; Costa et al., 2019; Marchesini et al., 2020; Moallem & Lifshitz, 2020; Tangorra et al., 2022). This aspect is addressed in the final part of Chapter 3, (**subchapter 3.3**) where the use of PoliSPEC^{NIR} spectrophotometer for analysing TMR homogeneity is presented, investigating the possible factors that could influence TMR homogeneity in dairy buffalo farms.

Nutritional strategies in precision feeding for optimizing dairy animal performance

Environmental sustainability is a cornerstone of the PF paradigm. By reducing feed waste and minimizing nutrient excretion, PF offers an effective strategy to lessen the environmental impact of livestock production (Sonea et al., 2023). Dietary nutrition and management are critical to

ruminant performance, as they profoundly influence rumen function, reproductive status, and overall animal welfare (Kotsampasi et al., 2024).

Improper nutrition can act as a stressor, and stress, in turn, has been shown to markedly reduce milk production, thereby compromising the sustainability of dairy farming. These declines in productivity are frequently linked to deficiencies in essential minerals, which must be addressed through dietary supplementation to maintain metabolic balance and physiological well-being (Kotsampasi et al., 2024). Ensuring an adequate intake of these nutrients is therefore vital for sustaining both performance and animal welfare.

A well-balanced diet is essential to provide livestock with sufficient proteins, energy, vitamins, and minerals, key elements for maintaining health and maximizing productivity (Akintan et al., 2025). It is also important to consider the transfer of these nutrient into milk, as this can enhance the presence of beneficial compounds that humans can incorporate into their diets. Thus, although the relationship is complex, there is a strong link between the quality of animal-derived products and the diets provided to the animals (Khan et al., 2017). Today, it is common practice to manipulate and enrich animal diets, aiming to deliver specific nutritional substances through the consumption of animal-derived products.

Vitamins and minerals (both macro and microelements) are fundamental to animal nutrition, playing pivotal roles in metabolism, growth, production, and reproduction (Khan et al., 2014). Although required in relatively small amounts, careful management of their intake is essential because an excess or imbalance can adversely affect health (Conte et al., 2018). These nutrients influence productivity and reproductive performance either directly or indirectly, and extensive research has demonstrated their importance in preventing and managing nutritional deficiencies and related disorders (Kotsampasi et al., 2024). Certain vitamins support immune function and help mitigate stress, while oxidative stress, resulting from an overproduction of free radicals in the absence of adequate antioxidants, can further impair health and performance.

Key micronutrient antioxidants, such as vitamin E, beta-carotene, and vitamin C, along with minerals like copper, zinc, iron, selenium, silicon, and manganese, work synergistically to combat oxidative stress (Khan et al., 2014). Micronutrient deficiencies can compromise physiological functions and exacerbate stress responses. To counter these issues, feed additives and supplements have been extensively studied for their ability to enhance nutrient absorption in the small intestine, thereby improving animal performance during periods of stress (Conte et al., 2018; An et al., 2023). Supplementing dairy animals with essential minerals, vitamins, and antioxidants has been

shown to mitigate oxidative stress by neutralizing free radicals, leading to increased resilience, improved health, and enhanced productivity (Conte et al., 2018; Lakhani et al., 2021; Evangelista et al., 2022). Among these micronutrients, zinc and selenium are particularly important for immune function and metabolic regulation, directly influencing both milk yield and quality. **Chapter 4** discusses issues related to the effects of the diet - covering both micronutrients and the chemical-physical characteristics of the diet - on productive performance and nutrient digestibility. Specifically, **Subchapter 4.1** investigates the effects of natural antioxidants, along with zinc and selenium supplementation, on the milk production and quality of Italian Mediterranean lactating buffaloes. This research provides valuable insights into effective nutritional strategies aimed at optimizing production during stressful periods, particularly in the summer months when heat stress negatively impacts performance.

Another aspect that remains relatively understudied in the feeding of dairy buffaloes is the physical characteristics of the diet, particularly particle size. This parameter has a significant impact on dry matter intake (DMI), milk yield and composition, and overall animal health (Schadt et al., 2012).

The effect of particle size on dry matter digestibility (DMD) continues to be a subject of debate. While some studies suggest that an increase in particle size can enhance DMD (Kononoff & Heinrichs, 2003a; Yang & Beauchemin, 2005), others have observed the opposite effect (Kononoff & Heinrichs, 2003b), and still others report no significant impact at all (Krause et al., 2002; Yang & Beauchemin, 2006). These conflicting findings underscore the complex interactions between particle size and various dietary and management factors. The physical structure of feed is a critical determinant of digestion kinetics, affecting rumen mat consistency, rumination activity, salivary secretion, rumen pH stability, and fiber digestibility (Teimouri Yansari et al., 2004). Smaller feed particles, by providing a larger surface area for microbial fermentation, can reduce rumen retention time and enhance rumen turnover, potentially increasing DMI and overall productivity. Conversely, reducing particle size in dairy diets has been linked to decreases in milk fat concentration, diminished chewing activity, and lower rumen pH (Grant et al., 1990; Beauchemin et al., 1994). Thus, optimizing performance requires a balanced formulation that carefully considers both the neutral detergent fiber (NDF) content and the particle size of the diet.

In practice, tools such as the Penn State Particle Separator (PSPS) are widely employed to assess particle size in TMR and forages, providing an essential metric for evaluating diet quality (Lammers et al., 1996). Research indicates that when more than 50% of feed particles are retained on the larger sieves (sieves 1 and 2), rumen fill may become a limiting factor for feed intake. This limitation can reduce the production of volatile fatty acids (VFA), compromise energy supply, and

ultimately lead to lower milk production (Armentano, 2010). Hence, the physical characteristics of the diet, notably particle size, not only govern feed intake but also affect rumination, rumen pH stability, and overall digestive efficiency, thereby influencing milk composition and animal health (Allen, 1997).

Although extensive research has been conducted on particle size distribution and physically effective NDF (peNDF) in relation to feed intake, digestibility, and lactation performance in dairy cow (Righi et al., 2007; Heinrichs, 2013; Caccamo et al., 2014; Fustini et al., 2016; Haselmann et al., 2019; Simoni et al., 2021), a significant knowledge gap remains with respect to dairy buffaloes.

Subchapter 4.2 addresses this gap by presenting a pioneering study on the physical characteristics of dairy buffalo rations and their impact on milk quality and digestibility. This research marks the first application of the PSPS to dairy buffalo diets, offering novel insights into how particle size influences the milk yield, milk quality, and apparent digestibility in this species.

The final **subchapter, 4.3**, presents a pioneering study on the characterization of the faecal microbiota of Italian Mediterranean dairy buffaloes. This investigation was conducted concurrently with previous research on the physical characterization of buffalo diets, with both sets of samples collected simultaneously. The study was initiated to develop alternative, non-invasive methods for gaining insights into rumen function.

There is a growing need for less invasive sampling techniques to correlate microbial populations with functional traits (Mott et al., 2022) and to better understand the factors influencing microbial diversity and composition. Consequently, the faecal microbiome has emerged as a valuable focus of study. According to some authors (Callaway et al., 2010; Andrade et al., 2020), the rumen and faeces share a bacterial "core microbiome" at the family and/or genus level, which plays a crucial role in the gastrointestinal ecosystem. This overlap underscores the strong interconnection and interdependence between these microbial communities (Andrade et al., 2020).

Research on dairy cows and goats has shown that the faecal microbiota is highly responsive to dietary changes and is primarily shaped by the animal's diet (Callaway et al., 2010). The objectives of this study were to investigate the faecal microbiota and determine which dietary component most significantly influences its composition.

Objective of the thesis

The main objective of this thesis, in compliance with PhD programs focusing on green topics aimed at ecosystem conservation, biodiversity, climate change impact reduction, and the promotion of sustainable development, was to test potentially applicable solutions in the field of ruminant nutrition and feed management. These solutions aim to improve aspects related to both animal welfare and the reduction of environmental impact, while also increasing the overall efficiency of the livestock sector.

Specifically, the thesis focused on a small sector within dairy buffalo farming. However, the technologies, tools, and studies applied have the potential to be used for other ruminant species as well. The choice of the buffalo species is justified by its growing importance in Italy (Evangelista et al., 2024). On one hand, genetic advancements have made significant progress in the sector, leading to increasingly productive phenotypes. On the other hand, however, this progress has not been accompanied by advancements in management, particularly in nutrition.

Currently, farms tend to follow the nutritional model used for dairy cows due to the lack of in-depth studies on the current buffalo genotype being bred. However, the nutritional needs of buffaloes do not align with those of cows. For this reason, it is necessary to further investigate nutritional aspects, considering that diet plays a crucial role in both animal welfare and the expression of the animal's productive potential. Moreover, the development and implementation of precision feeding allow for promising results in this sector, which remains largely unexplored to date.

Thesis structure

The outline of the thesis is presented in Figure 2. **Chapter 1** provides general information about the topics addressed in the thesis, introducing the impact of the livestock sector and strategies for its mitigation. The livestock sector plays a dual role in climate change: on the one hand, it is a victim of its effects, and on the other, it contributes to the increase in GHGs, particularly enteric methane. This compound is the central focus of a state-of-the-art review on enteric methane emissions from the livestock sector, presented in **Chapter 2**. The review employs a ML approach to highlight the development of scientific research, using Scopus as the database. Among the strategies for mitigating climate change, an important aspect is the application of precision livestock farming, with particular emphasis on precision feeding. This topic is the focus of

Chapter 3, which discusses studies on NIR spectroscopy, specifically its application in feeding management practices within the livestock sector. The goal is to enable “precise and real-time” knowledge of ration composition directly at the farm level. The chapter begins (**Subchapter 3.1**) by assessing the predictive capacity of both a benchtop NIR instrument and a portable device for analysing the chemical composition and fatty acid profile of dairy buffalo TMR. In this study, ML techniques, such as LASSO (Least Absolute Shrinkage and Selection Operator) regression, were applied to enhance model accuracy and interpretability. In the **Subchapter 3.2**, the discussion will shift to ML-based wavelength selection for NIR spectroscopy, focusing on its application for a low-cost portable instrument designed for livestock feed management. The objective is to obtain a narrow range of wavelengths that can be applied in increasingly affordable and user-friendly NIR instruments. The final section of Chapter 3, **Subchapter 3.3** will evaluate the key factors influencing the preparation of a proper ration for dairy buffaloes using a commercially available portable NIR spectrometer. The identification of factors that influence the proper formulation of a ration is a critical aspect to monitor, with the ultimate goal of improving the accuracy of ration preparation. The formulation of the ration must closely align with the theoretical model developed by the nutritionist, ensuring that the nutritional requirements of each animal are met optimally. Proper nutrition is essential not only for enhancing productive performance but also for the well-being of the animals. These aspects are further explored in **Chapter 4**, which examines the use of antioxidant supplementation containing selenium and zinc to enhance buffalo performance (**Subchapter 4.1**). In **Subchapter 4.2**, a study is presented on the physical characterization of dairy buffalo rations using the Penn State Particle Separator, originally designed for evaluating dairy cow diets and applied to buffaloes for the first time. Additionally, the interactions between the physical characteristics of the diet and milk production, milk quality, and digestibility are discussed. Finally, in **Subchapter 4.3**, the buffalo faecal microbiome is explored for the first time in relation to different diet compositions. Furthermore, the potential of the faecal microbiome to provide insights into diet digestibility is investigated. **Chapter 5** presents the Summary and Conclusion, where the key findings of the research are discussed, along with their implications and recommendations for future studies or practical applications. **Chapter 6** summarizes all the activities related to the PhD program, including seminar presentations, conference participations, and courses, as well as providing a list of all publications directly and indirectly related to the program.

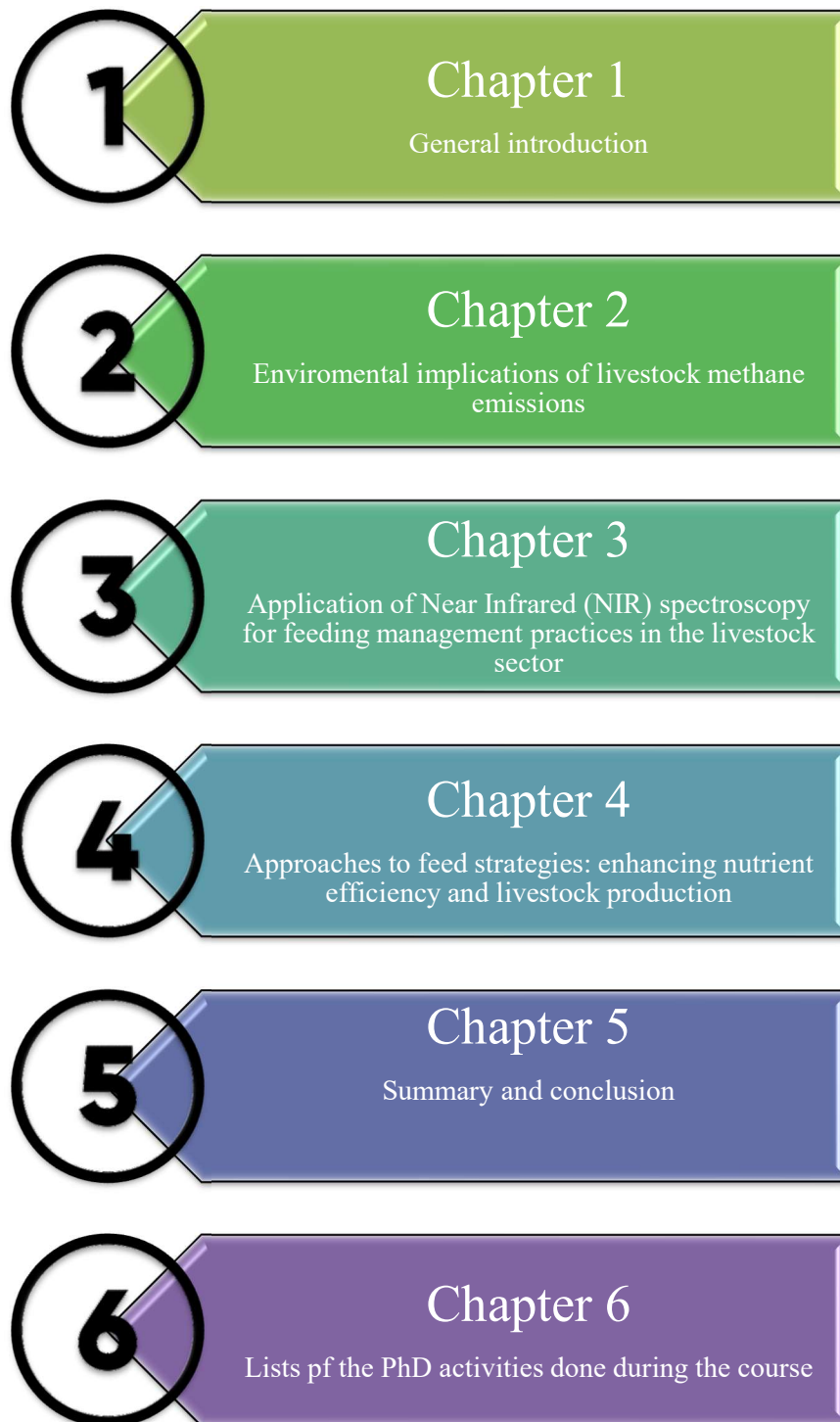


Figure 2. Structure of the thesis.

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Chapter 2

Chapter 2

Environmental implications of livestock methane emissions

The enteric methane emission in livestock sector: bibliometric research from 1986 to 2024 with text mining and topic analysis approach by machine learning algorithms

Evangelista Chiara¹, Milanesi Marco¹, Pietrucci Daniele¹, Chillemi Giovanni¹,
Bernabucci Umberto²

¹ Department for Innovation in Biological Agro-Food and Forest Systems, University of Tuscia,
Italy

² Department of Agriculture and Forests Sciences, University of Tuscia, Italy

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ABSTRACT

Methane (CH₄) from livestock, particularly enteric CH₄ emission (EME), is one contributor to greenhouse gas emissions and climate change. This review analysed 1,294 scientific abstracts on EME in ruminants from 1986 to May 2024, using Scopus® data. Descriptive statistics, text mining, and topic analysis were performed. Publications on EME have risen significantly since 2005, with the Journal of Dairy Science being the most frequent publisher. Most studies (82.1%) were original research, with Northern Hemisphere countries leading in publication numbers. The most frequent terms were “milk,” “cow,” and “diet,” while key research topics included green-house gas emissions from livestock, diet composition, and prediction models. Despite progress, some areas like CH₄ emission from animals need further investigation.

Keywords: Ruminants, Enteric Methane, Text Mining, Topic Analysis, Machine Learning.

INTRODUCTION

Methane (CH₄), together with carbon dioxide (CO₂) and nitrous oxide (N₂O), are the main greenhouse gases (GHGs) produced by the livestock sector. Greenhouse gases concentration contributes to climate change and are responsible for the increase in temperature and water scarcity (Zobeidi et al., 2022). Compared to pre-industrial levels, global temperature has increased by 1.5 °C (Pörtner et al., 2021) and changes in the natural hydrological cycle of water are occurring, such as: the increase of intense precipitation; variations in the quantity and seasonal distribution of precipitation; increase of sea level in coastal communities; increased evapotranspiration, and decreased soil moisture (Zobeidi et al., 2022). According to FAO (FAO, 2023) livestock agrifood systems are responsible for 12% of all anthropogenic GHG emissions, corresponding to 6.2 Gt CO₂ equivalent emissions (data referring to 2015). Total CH₄ produced by the livestock sector represents 54%, 31% is CO₂, 15% is N₂O (data referring to 2015, FAO, (2023)). Methane seems to have the most impact on the GHGs produced by the livestock sector, since it represents a major share of the GHGs and the effect on global warming of CH₄ is approximately 28 times that of CO₂ (Grossi et al., 2019). Emission of GHGs from the livestock sector can occur both directly (enteric fermentation and manure) and indirectly (through processing, fertilization, feed production, etc.) (Grossi et al., 2019). Methane produced directly by manure represents 7.8% of the total CH₄ deriving from the livestock sector, while the CH₄ deriving from ruminal fermentations, called enteric CH₄, is equal to 46% of the total CH₄ emissions (FAO, 2023). Significant variations in emission level and/or intensity are noted among different species, geographical areas, and production systems (Gerber et al., 2013). According to the FAO report (2023), of the 6 species of

livestock animals responsible for producing GHGs, 62% is the contribution of cattle, 8% buffaloes, 4% goats, 3% sheep, and 23% is by monogastric (pigs and poultry) (Figure 1). Considering the production system, 2/3 of the GHGs emission is associated with meat production (67%), followed by milk production (30%) and eggs (3%). A distinction must be made in ruminants, the greater production of CH₄ is derived from ruminal fermentation, whereas in monogastric systems, the main contributors are feed production, land use change, and manure management.

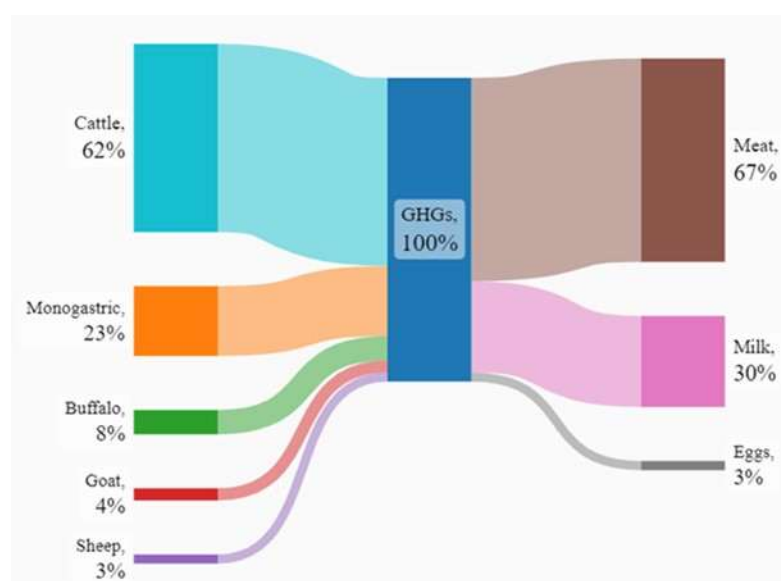


Figure 1. Sankey diagram of GHG' emission sources in 2015 by species and products expressed as a percentage (Based on GLEAM 3. FAO, 2023). Monogastric is composed by Pigs 14% and Chicken 9%.

The demand for animal products (meat, milk, and eggs) is projected to grow by 20% by 2050 compared to 2020 levels due to the world's population growth and increasing prosperity (FAO, 2023). This increased demand leads to an inevitable increase in GHGs emissions from the livestock sector to satisfy the growing demand for proteins. Implementing sustainable methods is essential for achieving reduced emissions and lessening the negative effects of livestock systems on the environment. Methane emission has a shorter average lifespan in the atmosphere than CO₂ (~12 years versus hundreds of years, respectively) which makes it an attractive improvement target for short-term gains in global warming abatement (Balcombe et al., 2018). Enteric CH₄ is produced during enteric fermentation in ruminants by anaerobic microorganisms collectively known as methanogens in the *Archaea* domain and the phylum *Euryarchaeota* (Hook et al., 2010). The CH₄ produced by animals is affected by many factors: the species, the production level and direction, the diet composition, the type of carbohydrates present, the level of ingestion, the degree of lipids saturation, some environmental factors such as temperature and genetic factors such as feed

conversion efficiency (McAllister et al., 1996). Searching for feasible mitigation strategies is essential to reduce CH₄ emissions increasing feed efficiency, so that CH₄ emissions from ruminants not only aggravate the global GHG effect but also can cause energy losses in livestock, accounting for 3.9–10.7% of ingested metabolic energy (Appuhamy et al., 2016), and representing one of the most important inefficiencies and economic losses in ruminant production systems (Palangi and Lackner, 2022).

Recognizing the importance of this issue, the FAO has been actively studying methane and mitigation strategies since 2005, when it began developing the Global Livestock Environmental Assessment Model (GLEAM). The first version was released in 2010, and GLEAM has since been continuously updated to enhance the accuracy of GHG emission estimates across species, regions, and production systems. In addition to the FAO, numerous researchers around the world are actively investigating innovative approaches to reduce methane emissions. Simultaneously, the Intergovernmental Panel on Climate Change (IPCC) has played a crucial role by providing global guidelines for estimating emissions. In 2006, the IPCC published the 2006 Guidelines for National Greenhouse Gas Inventories, which established standardized methods for calculating emissions from agriculture, including livestock. These guidelines are continuously updated, with the most recent update occurring in 2019, to incorporate advancements in estimating methane from enteric fermentation and other agricultural sources (FAO, 2023).

The data mining approach, also known as text mining (TM), is useful for extracting multiple information from large text databases (VijayGaikwad et al., 2014). Finding the most relevant words in a text and identifying significant patterns in text data are the aim of TM analysis. Text mining employs methods from computational statistics and machine learning (Wang et al., 2016). Another text analysis is the topic analysis (TA), that is a technique for identifying hidden textual patterns and the structure of significant themes within record collections using probabilistic models (Wang et al., 2016). The usefulness of quantitative analysis is to organize knowledge on a specific subject/topic/discipline and identify the main trends in a specific sector (Marino et al., 2023). This is because scientific literature contains potentially new knowledge (Wang et al., 2016). Recently, in many sectors, it has been observed a growing interest in this kind of text analysis. This is largely attributable to the advancement of recent analysis techniques (*e.g.*, R and Python software) and the availability of platforms that provide reference data (*e.g.*, Scopus, Web of Science). Even in the livestock sector, several papers have recently been published on several topics from precision livestock farming (Marino et al., 2023), automated milking systems (Gislon et al., 2024), to the wellbeing of several species, including beef cattle (Nalon et al., 2021), horses (Benedetti et al.,

2023) and buffaloes (Trapanese et al., 2024) utilizing techniques such as text mining and topic analysis.

Enteric CH₄ emission is a hot topic in the livestock sector, but no TM and TA study has been done on it to date. For this reason, the goal of this review was to use TM and TA techniques to describe literature's evolution and geographical distribution, identifying the most investigated research topics, and highlighting the knowledge gaps regarding enteric CH₄ emission from ruminants.

MATERIAL AND METHODS

Dataset

A bibliometric study of documents related to enteric methane emission (EME) in ruminants using a bibliometric database of Elsevier©, namely Scopus®, has been carried out. The topics were searched using keywords. Enteric methane (EM) AND another keyword among "emission"; "ruminants"; "cow"; "buffalo"; "sheep OR goat"; "additive"; "microbiome OR microbiota" were used in the research. The selected dates ranged from 1986 to May 2024. Several filters were added, including the selection of publications from scientific fields like agricultural and biological sciences, environmental sciences, biochemistry, genetics and molecular, veterinary, engineering, earth, and planetary sciences. Under these circumstances, 3,624 records were generated. Each search was exported as a Comma Separated Values file (Microsoft Excel®, v16.0, Redmond, WA, United States), then the files were merged into a single file containing 3,624 lines. Table 1 shows the total number of documents for each pair of keywords. The data was arranged in a tabular manner using the Excel spreadsheet, with each record being showed in a row and its contents arranged in columns. The columns contained information about title, author, year of publication, affiliation, type of record (*e.g.*, article or review), source of publication (*i.e.*, name of the journal), language, and abstract. Screenings were conducted on this single file to eliminate documents that were not in English, those in which the abstract was absent, those that misspelled "erratum", and finally, those in which the author was absent. After this, duplicate documents were eliminated. The articles dealing with rats, humans, manure, and soil were not included. In vitro studies have been included due to their significant importance.

Table 1. Bibliographic search strings for the text mining analysis on the enteric methane emission in the livestock sector carried out on titles, abstracts, and keywords of peer-reviewed literature in English published between 1986 and May 2024.

Search words	Original n. of records
Enteric Methane AND Emission	1,711
Enteric Methane AND Ruminants	742
Enteric Methane AND Cow	612
Enteric Methane AND Sheep OR Goat	242
Enteric Methane AND Additive	221
Enteric Methane AND Buffalo	68
Enteric Methane AND Microbiome OR Microbiota	28
Total	3,624

Finally, a manual screening of 1,850 articles was performed. The screening process involved reading each abstract carefully to evaluate the relevance of the study to our review's scope. The criteria for inclusion were: i) the article had to focus on EME from ruminants, ii) the study needed to present experimental or empirical data related to EME measurement, mitigation, or related environmental impacts, or be a review of published data, and iii) the article had to focus on ruminant species (cattle, sheep, goats, etc.). Articles were excluded if they did not focus on EME or were unrelated to methane emissions in livestock, addressed monogastric species (pigs, poultry) without reference to ruminants. Where abstracts did not provide enough detail, the full article was reviewed to ensure proper inclusion or exclusion. Details regarding the exact number of records downloaded from each research string, the eligibility procedure and initial screening are displayed in the flow diagram (Figure 2).

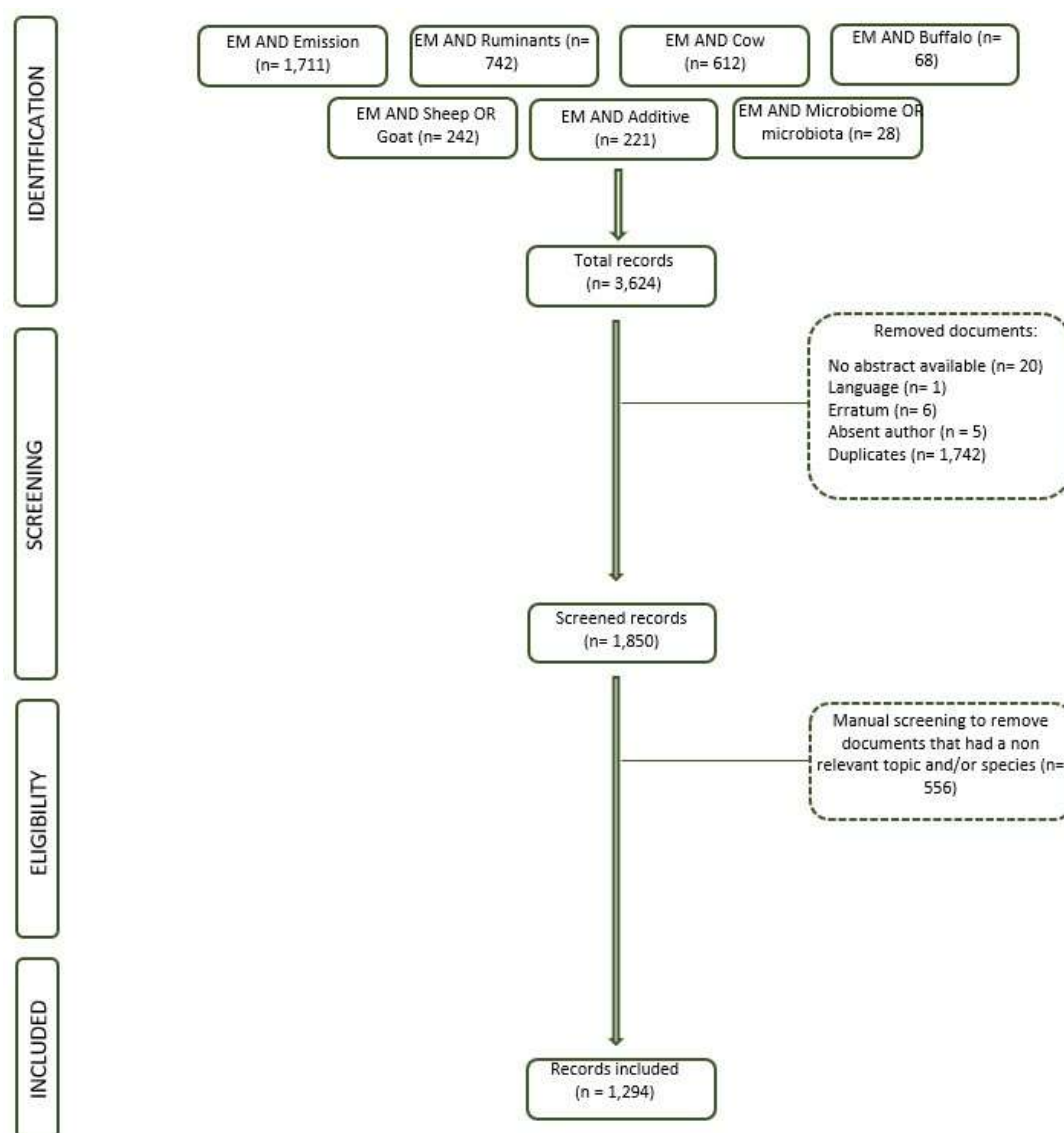


Figure 2. Flow chart illustrating the process of searching for and selecting scholarly literature on the EME. The dashed lines indicate the quantity of excluded records and the rationale behind their removal from the study.

A total of 1,294 abstracts were selected, and descriptive statistics were performed on these articles regarding the year of publication, country, and journal of publication using Excel Pivot tables and graphics. Each record was associated with the nationality of the first author, and this information was displayed on a world map.

Text mining

The TM technique transforms text into numerical data by emphasizing word frequency distributions, allowing for the identification of primary terms and their correlations within the data corpus. Text mining analysis was performed to identify the primary terms in the data corpus and

their correlations. Text mining analysis was performed with R studio environment (R core Team, <https://www.r-project.org>) using a combination of functions in the package's "tm" (v. 0.7.11) (Feinerer et al., 2008), "snowball" (v. 0.7.1) (Bouchet-Valat et al., 2024), "ggplot2" (v. 3.4.4) (Wickham, 2024), "dplyr" (v. 1.1.4) (Wickham et al., 2024), and "tidyverse" (v. 2.0.0) (Wickham et al., 2019), (information about the packages used is provided in the supplementary materials).

The abstracts of the 1,294 selected documents were compiled into an Excel file. This file contained two columns: one with the document's progressive ID and another labelled "text", which contained the abstracts. Text mining was then performed using this dataset. Before TM analysis, the three stages of pre-processing test are done, tokenization, filtering, and stemming (Sebastiani, 2002). Tokenization involves splitting text into individual words or phrases and converting these words to their base forms. This prevents counting the same word more than once when it appears in various grammatical forms (Adamakopoulou et al., 2023). Tokenization and filtering phases were conducted as follows:

- Text was converted to lowercase;
- Remove strange symbols and punctuations ("@", "/", "*");
- Remove number and extra white spaces;
- English common language words such as articles prepositions and conjunctions are removed (e.g. "the", "a", "and", "on", "at", etc.) as they provide little information about the contents of the corpus;
- Remove stop words: "emission", "enteric", "methane", "buffalo", "cow", "sheep", "goat", "ruminants", "cattle", "additive", "microbiome", "microbiota".

In the final phase, text stemming was performed to reduce words to their root forms, thereby standardizing word representation and enhancing the accuracy of word frequency and association analysis. The words were then organized into a document-term matrix (DTM), with terms arranged in columns and documents in rows. The term frequency-inverse document frequency approach (TF-IDF) was used to give terms a relative weight (Salton and Buckley, 1988). This displays a term's frequency that has been adjusted for its total usage, highlighting the word's significance across the collection of texts. The words with the greatest relevance ($TF-IDF \geq 9.5$) were represented as histogram, and a cloud of the most relevant words was created using the website <https://www.wordclouds.com/>. Finally, the association between words with $TF-IDF \geq 9.5$ were explored. The frequency of co-occurrence of word pairs was measured, considering a correlation of 1 when two words always appeared together. Associations with a coefficient of correlation greater than 0.40 were considered significant.

Topic analysis

Topic analysis is a methodology used to uncover semantic connections hidden within documents. For the TA of our abstract corpus, we employed latent Dirichlet allocation (LDA), a widely recognized approach for topic modelling analysis (Blei, 2003). Using a Bayesian probabilistic technique, words that frequently co-occur in the documents to identify thematic areas were analysed. The words used in the TA were drawn from the titles, keywords and abstracts of the 1,294 scientific literature records. The LDA function was used with the Gibbs sampling option of the “topic models” package in R (Grün and Hornik, 2011). Since the “optimal” number is typically unknown, multiple trials with varying numbers of topics have been done (e.g., 5,7,9). The perplexity index and log-likelihood harmonic means were used to determine the appropriate number of topics. Nine topics were selected at the end of the procedure because they produced the most logical outcomes, with more than 5% of the papers in each topic. Each topic was represented as an individual bar histogram with the probability of the first 10 words inside each topic (based on beta values). Once the right number of topics was found, we proceeded with topic labelling based on the first 10 words and evaluating the types of articles that fell back into each topic. Excel spreadsheet was used to provide descriptive statistics of the number of papers and the initial year of publication for each topic.

RESULTS

Descriptive statistics

The distribution of the initial bibliographic search results by string on titles, abstracts, and keywords is reported in table 1. Most articles were related to the string “enteric methane emission” (47%), followed by “enteric methane ruminants” (20%), “enteric methane cow” (17%), “enteric methane sheep and goat” (7%), “enteric methane additive” (6%), “enteric methane buffalo” (2%) and, lastly, “enteric methane microbiome or microbiota” (1%). After eliminating overlapping records and manually removing unnecessary ones, 1,294 records were retained for further examination. The trend in the number of publications per year from 1986 to May 2024 is reported in Figure 3. It can be seen how publications on the subject began to grow from 2005 onwards with an exponential increase ($R^2 = 0.72$). The literature search was conducted in May 2024, so the number of publications related to this year is not complete.

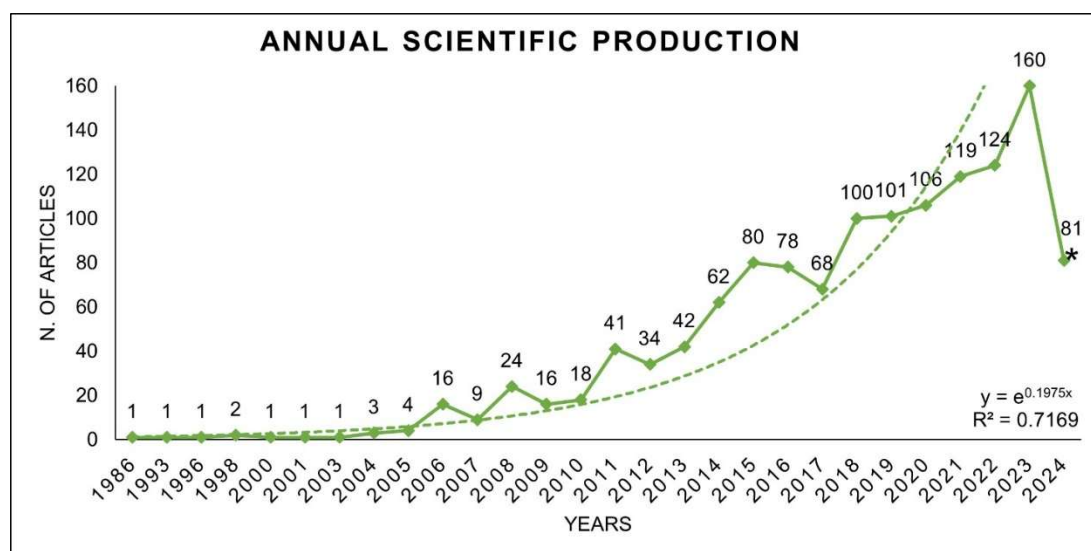


Figure 3. Records from 1986 to 2024 of peer-reviewed scientific literature (n = 1,294) pertaining to EME in livestock sector. * The asterisk on the year 2024 indicates that results for that year are related to the period from January to May.

Figure 4 shows the first 10 journals in which articles relating to EME were published. In terms of percentages, the “Journal of Dairy Science, JDS” published 12.7% of total articles, followed by “Animal Feed Science and Technology, AFST” (7%), “Journal of Animal Science, JAS” (6.3%), “Animals” (6.1%), “Animal Production Science, APS” (5.9%), “Animal” (4.3%), “Livestock Science, LS” (2.3%), “Tropical Animal Health and Production, TAHP” (1.8%), “Canadian Journal of Animal Science, CJAS” (1.6%) and lastly “Frontiers in Veterinary Science, FVS” (1.5%). About the type of documents, the most published on the subject are original articles (82.1%) followed by reviews (10.5%), and conference papers (4.9%), the rest is book chapter and short survey (2.5%). The most cited article (932 citations) was published in 2000 and concerned "Methane production by ruminants: Its contribution to global warming" (Moss et al., 2000) followed by a review published in by Beauchemin et al. (2008) with 755 citations. The average number of citations of the 1,294 documents is 24.9.

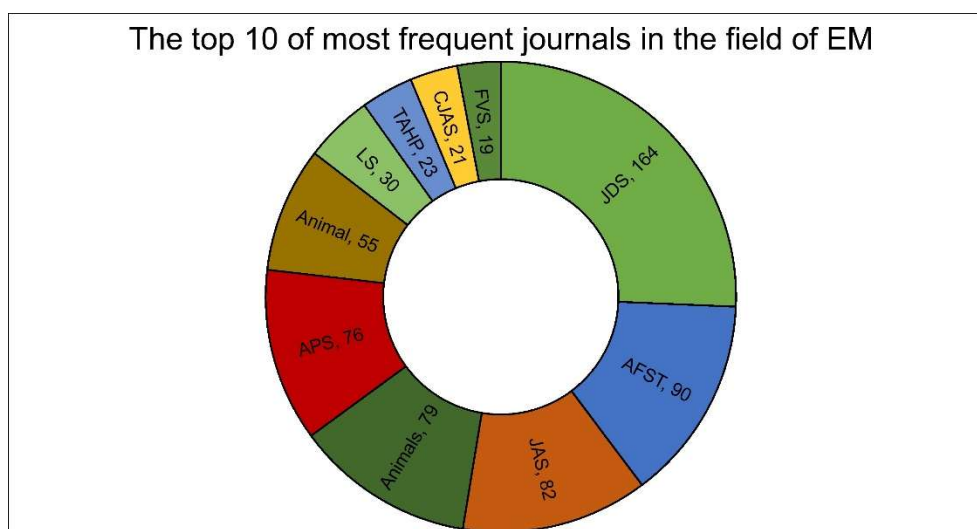


Figure 4. The top 10 of most frequent journals in the field of EME from 1980 to 2024 with the relative number of articles. JDS: Journal of Dairy Science; AFST: Animal Feed Science and Technology; JAS: Journal of Animal Science; APS: Animal Production Science; LS: Livestock Science; TAHP: Tropical Animal Health Production; CJAS: Canadian Journal of Animal Science; FVS: Frontiers in Veterinary Science.

Figure 5 illustrates how the 1,294 scientific papers were distributed throughout the continent according to the first author's affiliation country. The number (and percentage) of publications by continent was 369 (28.5%) from Europe, 271 (20.9%) from North America, 253 (19.6%) from Asia, 182 (14.1%) from South America, 163 (12.6%) from Oceania and 56 (4.3%) from Africa.

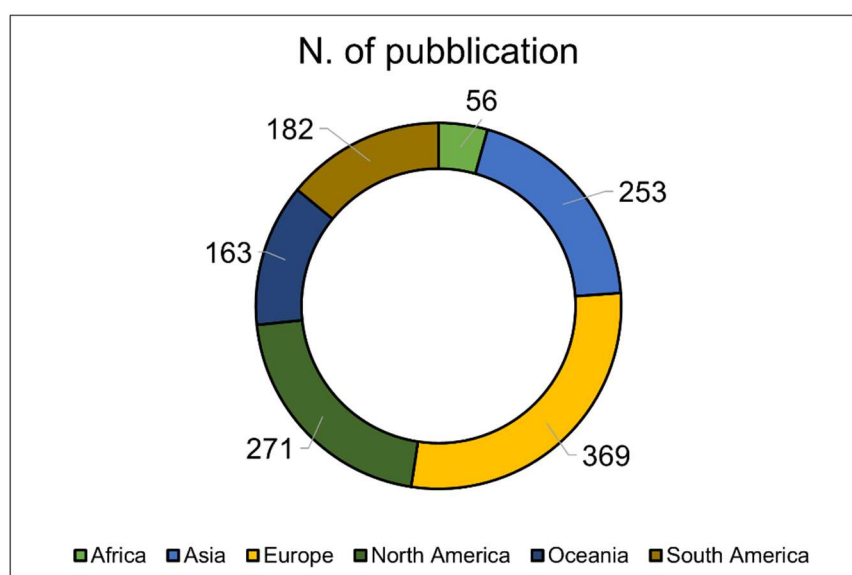


Figure 5. Pie graph depicting the number of scientific publications per continent.

Figure 6 shows the geographical map indicating the number of publications per country (the darker colour indicates a greater number of publications). The first countries with the highest number of publications were then explored for each continent. Concerning Europe, the countries with a greater number of publications were: United Kingdom (50), France (46) and Netherlands (41). For

North America the first 2 were: United States (153) and Canada (117). India (101), China (57) and Indonesia (25) represented the top three states with the highest number of publications in Asia. For South America the top three states were Brazil (82), Mexico (53) and Colombia (22). Australia (113) represents the state with the highest number of publications in Oceania followed by New Zealand (50). Finally, as far as Africa is concerned, the greatest number of publications comes from South Africa (25), followed by Kenya (10) and Ethiopia (4).

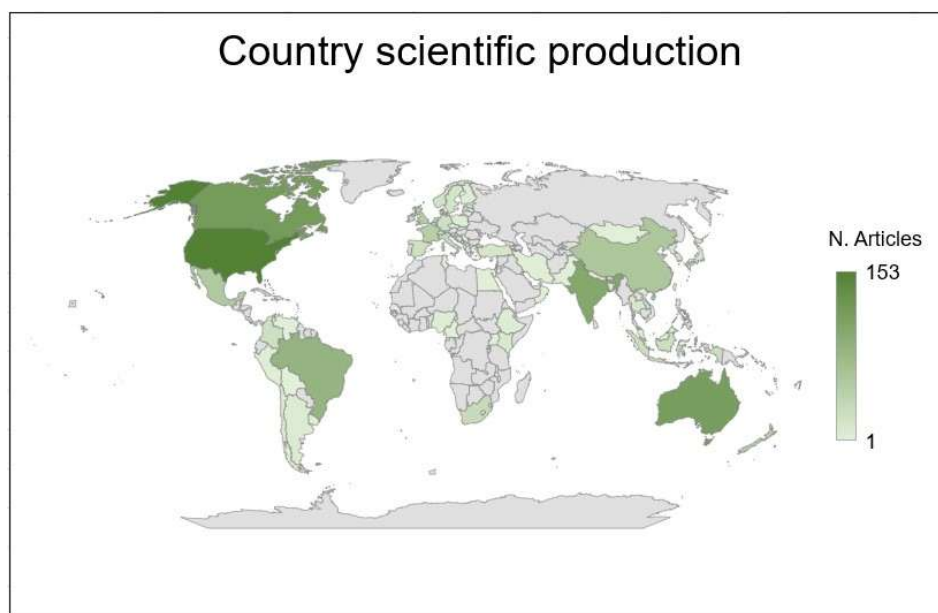


Figure 6. World map with the representation of the number of publications for each country (based on the nationality of the first author).

Text mining

To determine which words in the data corpus were the most frequent, TM analysis was carried out. Following the data pre-processing, 1,288 root terms were kept from the 1,294 documents that were chosen after sparseness was reduced (*i.e.*, "rare words" were excluded). "Rare words" are words which appeared only once or a few times in the entire corpus. These words have been excluded during data pre-processing to avoid noise and improve the efficiency and effectiveness of the analysis. The results of the most frequent root words with a weight over 9.5 of TF-IDF are reported in Figure 7. The weights of the terms found (calculated as TF-IDF) ranged from 18.57 to 0.50. The word "milk" achieved the greatest value (TF-IDF of 18.57). The other words with the highest TF-IDF were "cow" (16.57), "diet" (13.76), "dmi" (12.10), "supplement" (11.81), "rumen" (11.54), "predict" (11.48), "model" (11.16), "day" (11.07), "digest" (10.49), "dairi" (10.43), "nitrat" (10.35), "graze" (10.25), "concentr" (10.09), "measur" (9.97), "yield" (9.95), "energi" (9.76), "increas" (9.75), "anim" (9.72), "emiss" (9.67), "intak" (9.64), "feed" (9.63), "treatment" (9.51). A

word cloud with the most frequent terms is in Figure 8 in which the size of the font is proportional to the TF-IDF of each term. Table 2 shows the correlations between the most relevant terms ($r \geq 0.40$) and the other terms in the matrix. The terms “anim”, “concentr”, “dairi”, “day”, “diet”, “dmi”, “emiss”, “feed”, “intak”, “milk”, “nitrat”, “rumen”, “supplement”, and “yield” did not show any correlation.

Table 2. Relationships between the most pertinent terms (TF-IDF ≥ 9.5) and the other terms in the corpus of 1,294 records.

Words (TF-IDF ≥ 9.5)	Associated words (grade of correlation ≥ 0.4)
Cow	Lactat (0.47)
Model	Error (0.46)
Digest	Nutrient (0.42)
Energi	Gross (0.46); Metaboliz (0.41)
Graze	Pastur (0.43)
Increas	Linear (0.45)
Measur	Chamber (0.41)
Predict	Error (0.50); Equat (0.47); Extant (0.42)

The correlation grade is expressed in parentheses. The minimum grade of correlation was set ≥ 0.4 . Because the text stemming method reduced the words to their origins, words can be partially cut.

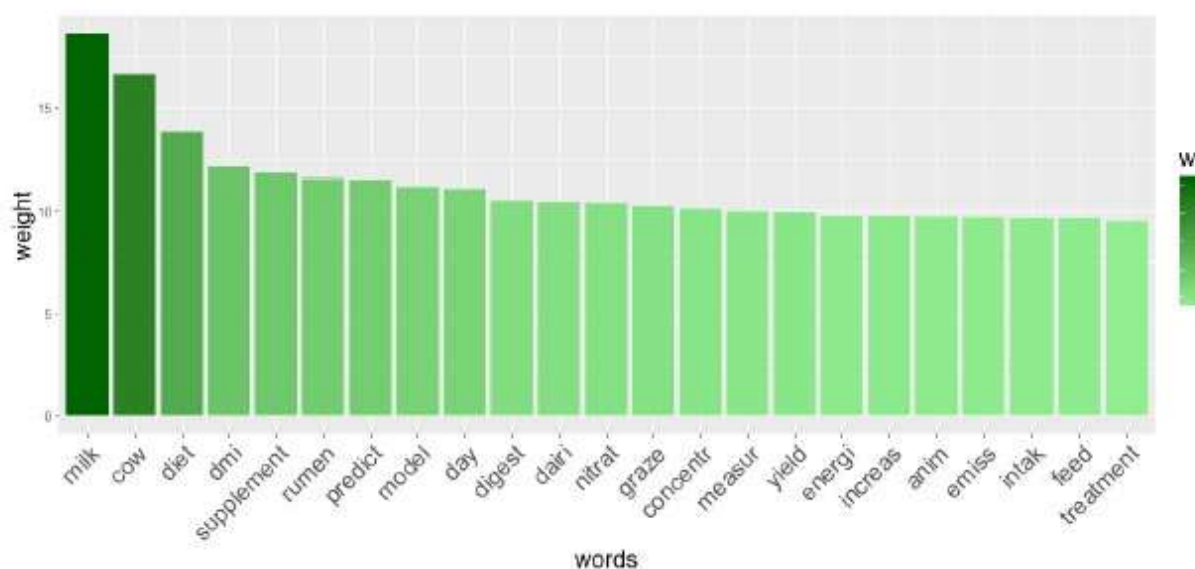
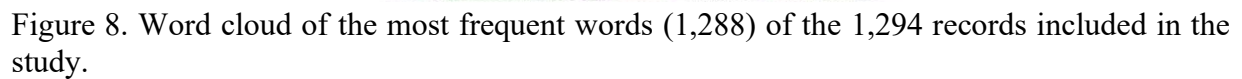


Figure 7. Histogram representation of the most relevant words (stems) in the database (TF-IDF ≥ 9.5).



The first 10 words of the nine topics are displayed in Figure 9. Table 3 shows the list of the 9 topics, their nomenclature, the number of articles published (and relative percentage) and the year of first publication for each topic. The topic that showed the most documents is topic 9 “Greenhouse gas emission from livestock” (18.08%) followed by topic 6 “Diet composition” (13.45%), topic 8 “Prediction model” (11.28%), topic 4 “Supplement and additive” (10.97%), topic 5 “Ruminal fermentation” (10.20%), topic 3 “In vivo measurement system” (9.81%), topic 2 “Extensive farming system” (9.27%), topic 7 “Dairy production” (9.20%) and finally the topic that showed the lowest percentage of published documents is topic 1 “Methane emission-animal” (7.73%).

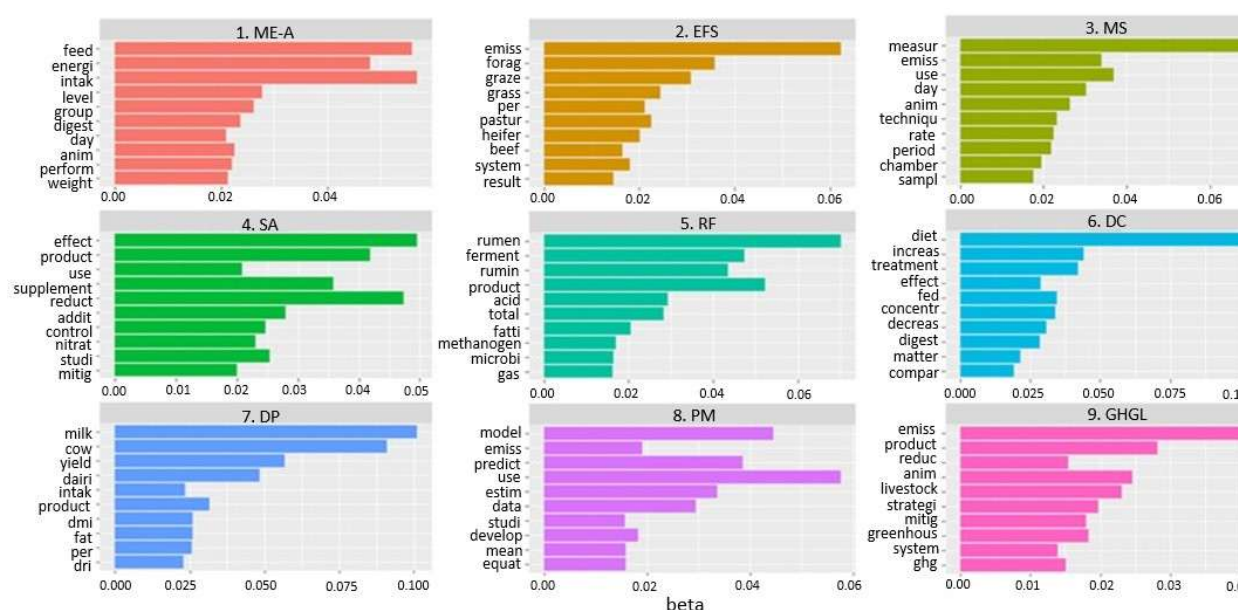


Figure 9. Histograms showing the most relevant terms for each of the 9 topics in the Latent Dirichlet Allocation (LDA). The top 10 terms with the highest beta value are shown. beta = probability that a word corresponds to a certain topic.

Table 3. Topics obtained from Latent Dirichlet Allocation (LDA) analysis.

Topic	Label of topic	Acronyms	N. of records per topic (%)	Year of first publication
1	Methane emission - animal	ME-A	100 (7.73%)	1998
2	Extensive Farming System	EFS	120 (9.27%)	2005
3	In vivo Measurement System	MS	127 (9.81%)	2001
4	Supplement and additive	SA	142 (10.97%)	2006
5	Ruminal fermentation	RF	132 (10.20%)	2006
6	Diet composition	DC	174 (13.45%)	2005
7	Dairy production	DP	119 (9.20%)	2005
8	Prediction model	PM	146 (11.28%)	2005
9	Greenhouse gas emission from livestock	GHGL	234 (18.08%)	1986

DISCUSSION

This study aimed at evaluating the evolution of EME from the livestock sector through a machine learning approach with TM and TA analysis methods over the years from 1986 to May 2024. The number of publications relating to EME in the livestock sector is growing. The first published article is from Germany, dated to 1986 and published by Crutzen et al. (1986) who aimed to provide an overview of the global CH₄ production by people, domestic ruminants, and wild

ruminants. Those authors concluded that CH₄ production by domestic and wild animals contributed to about 15-25% of the total tropospheric CH₄. Until 2004, publications were fluctuating and limited. Starting from 2005, there was an increase in publications until 2019 in which the number of publications exceeds 100 articles/year. The rising number of publications on EME reflects the growing interest in this potent GHG due to its significant climate-altering effects, with a notable increase in attention since 2005. That year saw the entry into force of the Kyoto Protocol signed in 1997 by 40 approximately developed countries. The protocol sets binding targets for reducing GHGs for industrialized countries, also referred to as developed countries. The main objective of the Kyoto Protocol was to limit the increase in global average temperature to less than 2 °C above pre-industrial levels. Furthermore, the Kyoto Protocol paved the way for international negotiations and agreements on climate change, including the annual Conference of the Parties (COP) under the United Nations Framework Convention on Climate Change (UNFCCC). The COP serves as the main decision-making body for global climate policy, where countries come together to negotiate and adopt decisions and agreements to address climate change. Another significant milestone in efforts to curb GHG emissions was the 2015 Paris Agreement. This agreement outlined the goal of limiting the increase in global average temperature to well below 2 °C and aiming to keep it within 1.5 °C. The heightened focus of the scientific community on addressing climate change and reducing GHG emissions, particularly from EME, is evident in the surge of publications following 2005, with a peak coinciding with the adoption of the Paris Agreement in 2015.

The largest number of publications was produced by the Journal of Dairy Science (JDS), followed by Animal Feed Science and Technology (AFST), Journal of Animal Science (JAS), and Animals. These journals' research scopes are in the livestock sector, in particular relating to dairy science (in the case of JDS), feeds as in the case of AFST, and livestock much more generally in the case of the last two journals (JAS and Animals). These journals reflect the most common terms discovered with TM, namely “milk”, “cow”, and “diet” which will be discussed later.

The geographical distribution of publications based on the first author showed that the countries with the highest number of published articles correspond to the northern hemisphere, where there is the greatest production of anthropogenic GHGs (Della Rosa et al., 2022). Additionally, in these areas, there is a high number of farms, and there is a greater contribution of funding for research in the livestock sector (Della Rosa et al., 2022). According to FAO (2023), regions with high milk production include Europe, South Asia and North America, whereas meat production is concentrated in East Asia, Europe, and North and South America. According with findings

reported by Della Rosa et al. (2022) studies on EME measurement techniques were conducted predominantly in Europe (32%), followed by Oceania (23%), North America (20%), Asia (15%), South America (9%), and Africa (1%). Additionally, Della Rosa et al. (2022) highlighted that most studies focused on cattle (64%) and sheep (22%), with only 7% involving goats, 5% involving buffalo, and 2% involving other ruminants such as alpaca, bison, llama, and deer (Table 4). Their review findings partially align with our own, indicating a similar distribution of focus in terms of geographical regions studied.

Table 4. Continental animal-CH₄ measurement studies published from 1994 to 2018 adapted to Della Rosa et al. (2022).

Continent	Studies (%)	Species ¹	EME-Technique ²
Europe	32	Cattle, goat, sheep, other	RC, SF ₆ , GF and other
Oceania	23	Cattle, sheep and other	RC, SF ₆ , GF and other
North America	20	Cattle, goat, sheep and other	RC, SF ₆ and GF
Asia	15	Buffalo, cattle, goat, sheep and other	RC, SF ₆ , GF and other
South America	9	Cattle, goat and sheep	RC, SF ₆ and other
Africa	1	Cattle and goat	SF ₆ and other

¹other includes other species: alpaca, llama, deer, bison; RC: respiration chamber; SF₆: sulfur hexafluoride; GF: GreenFeed; ²other includes other techniques: face mask, sniffer, laser methane detector and portable accumulation chamber.

Text mining

From TM analysis, the most frequent words were “milk,” followed by “cow,” “diet” and “dmi” with TF-IDF higher than 12. It is not surprising that the word “milk” followed by “cow” are the most common ones in our survey on the EME from the livestock sector. Looking at the world map of the countries in which most of the articles were published, the same countries are the largest breeders and producers of cow milk according to FAO data (2023). The word “cow” is directly linked to the EME as, among all the livestock species, cattle represent the largest producers of GHGs (62%). As discussed before, the major component of CH₄ is given by enteric fermentation compared with monogastric animals. Methane production can be expressed in 3 different ways: i) CH₄ production in liters or grams per day, ii) CH₄ yield, defined as liters or grams of CH₄ per kilogram of dry matter intake (DMI), iii) CH₄ intensity, defined as liters or grams of CH₄ per kilogram of milk/meat produced (Negussie et al., 2017). Therefore, the word “milk” could be related to the expression of the intensity of EME.

The word “diet” represents the ration provided to animals. Diet plays an important role in modulating the EME in ruminants. Indeed, among the top important terms highlighted by the TM,

terms related to nutrition have emerged, such as “diet”, “supplement”, “digest”, “graze”, and “concentr”. Several studies agree that diet modulation remains the most straightforward and inexpensive approach to lessen EME (Haque, 2018; van Gastelen et al., 2019; Beauchemin et al., 2022). Methane output can be lowered by 10% to 40%, depending on the type of feeding strategy intervention (Benchaar et al., 2001). Numerous feeding strategies for mitigating CH₄ are currently in use. These strategies can be summarized in three broad categories (adapted to Haque, 2018): i) dietary supplementation of feed additives that either directly block methanogens or change the metabolic pathways, resulting in the reduction of the substrate for methanogenesis; ii) improving the quality of the forage and modifying the forage-concentrate ratio and iii) improving feeding management. The word “dmi” (DMI – dry matter intake), is a very important acronym in the context of EME, since there is a strong correlation between DMI and EME (Appuhamy et al., 2016; Bielak et al., 2016; Niu et al., 2018; Benaouda et al., 2019). Notably multiple EME prediction models are based on DMI. Some studies (Millis et al., 2003; Ellis et al., 2007) demonstrated that DMI predicts CH₄ production (g/d) with a coefficient of determination (R^2) of 0.60 and 0.64, respectively. Furthermore, as previously discussed, DMI is used as an expression of CH₄ yield (g of CH₄/kg of DMI). It has been observed that this is a precise method to evaluate how effective a mitigation strategy is, regardless of possible changes in feed intake, given that feed intake is the main factor driving CH₄ production (Beauchemin et al., 2022). To confirm the above, following the word “dmi,” “predict” and “model” are found. Even though the remaining words have a lower weight according to the TF-IDF analysis (< 9.5), they are all noteworthy. By studying key factors such as cow diet, DMI, supplement usage, and grazing management, it is possible to identify effective strategies to reduce GHGs without compromising milk production or cattle health. Utilizing mathematical models and predictive methods allows to anticipate the impact of these strategies and develop tailored solutions to mitigate the increases of CH₄ emissions. Furthermore, improving the energy efficiency of cattle metabolism can decrease overall GHG from the dairy and meat industries.

Topic analysis

From the TA several expected topics emerged, ranging from ruminal fermentation (RF) to CH₄ measurement systems (MS), to diet composition (DC) up to a topic representing extensive farming system (EFS). We were also expecting a topic for meat production (just like the one that emerged for dairy production), but this could be hidden in the other topics. For example, it has been found the term “beef” in topic 2. The topic that generically encompasses the subjects dealing with GHGs

is the one that was born before the others (1986) and is the one with the highest number of articles published (18.08%) in the present survey. Topic 9 was generically defined as “greenhouse gas from livestock, GHGL” as the most important terms that emerged from the TA reflect this topic (“livestock”, “strategi”, “greenhouse”, “ghg”, “mitig”). Furthermore, compared with the other 8, in this topic, there is a large majority of reviews (32% of documents) probably because it is very generic. This broad topic includes studies that, indirectly, quantify EME, and have, as their general objective, the estimation of life cycle assessment (LCA) of livestock supply chains. Initially, the studies on LCA focused on primary sector, while only few included additional post-agricultural components (*e.g.* transport) in the analysis (Thoma et al., 2013). Life cycle assessments of several farming systems have shown that on-farm emissions are the largest contributor to the carbon footprint of dairy or beef supply chains (Thoma et al., 2013; Vitali et al., 2018; Rossi et al., 2024). The carbon footprint, an indicator calculated with a LCA approach, evaluates the GHGs associated with the life cycle of a product (Vitali et al., 2018). A cradle to grave LCA study on organically farmed beef Vitali et al. (2018) reported that the greatest production of GHGs (75-89%) along the food chain is associated with farms and that the EME was the greatest source of GHG arising directly from agricultural activities (47%), with a certain variability due to the different types of farming systems (conventional, organic), and/or methodological approaches adopted. Thoma et al. (2013) reported that even if the substantial majority of GHG derives from enteric CH₄, the impacts of the entire chain and supply chain can be reduced.

The Topic 2 contains terms related to extensive farming systems (*e.g.* “graze”, “forag”, “pasture” and “grass”), hence it has been defined as “extensive farming system, EFS”. This topic encompasses more than 9% of the publications considered in this review, representing the 5th most important topic identified in TA. Extensive farming systems likely warranted a separate topic because they exhibit distinct characteristics that differentiate them from other farming systems. Systems based on pasture constitute a substantial contributor to GHG. Although grazing systems are widely utilized, there are difficulties in measuring and reducing the CH₄ emissions from these systems (Soder and Brito, 2023). Furthermore, grazing systems are one of the most significant habitats for CH₄ exchange. Their CH₄ budget comprises two main sources: soil bacterial populations that, depending on the soil's physical and biological conditions, can either produce or consume CH₄, and ruminants on pasture that generate CH₄ during the digestion of grass. Such systems are also considered inefficient, associated with low animal performance, which tends to increase the emission rate per unit of product (CH₄/kg of milk/meat) (Hristov et al., 2013; Mapfumo et al., 2018). These issues are more pronounced in developing and poor countries, such as African countries, which currently stand as some of the largest GHG emitters (Mapfumo et al.,

2018). In these regions, pasture is scarce, and what is available is often of poor quality, resulting in digestive inefficiency and, consequently, increased emissions (Schuman et al., 2002). On the other hand, EFS are considered a potential GHGs mitigation practice (Schuman et al., 2002). Although extensive systems are recognized as carbon sinks, and silvopastoral systems can be effective in protecting animals from extreme weather conditions (Agethen et al., 2024) it has been observed that proper pasture management (*e.g.* adjusted grazing intensity, fire management, legume or grass sowing, pasture fertilization) leads to soil carbon sequestration, averaging 1.76 t CO₂ ha⁻¹ per year (Conant et al., 2017). Agro-ecological factors, as well as historical and current farming practices, significantly impact soil carbon sequestration rates (Godde et al., 2020). The complexity of interactions between soils, vegetation, grazing animals, and human interventions makes it challenging to categorize them in farming management categories typically evaluated in scientific literature, thus assessing the sequestration potential of grazing practices remains one of the major challenges (Godde et al., 2020).

Topic 3 was one of the expected ones. Among the most important terms, the terms "measur", "animal", "techniqu", "chamber" entirely fall within in "in *vivo* measurement systems, MS". More than 9% of the selected articles are included into this topic. The trend of publications has experienced some fluctuations over the years. The variations in the number of publications over time could be linked to the introduction of new measurement systems. For example, in 2010 the commercial GreenFeed (GF) system (C-Lock Inc., Rapid City, South Dakota, USA) was introduced. Since 2015, studies utilizing the GF system have been observed, but its adoption began around 2016, evidenced by a peak of publications during that year. In 2016, within topic 3, over 45% of the studies were conducted using the GF system. It is obvious that after the introduction of a new measurement system many studies seem to verify the actual efficiency of the instrument. Several techniques have been developed to measure CH₄ emissions from individual animals, despite the UNFCCC (1997) stating that "comparable methodologies" should be used for compiling the GHG inventory to make national results comparable in a consistent manner (Della Rosa et al., 2022). Every technique has an impact on the variability of the EME, methodology may add unpredictability to national inventories and assessments of CH₄ emissions (Hammond et al., 2016; Della Rosa et al., 2021).

To date, several methods are available to measure CH₄ emissions from ruminants (Storm et al., 2012; Bekele et al., 2022). None of them is ideal, and each has pros and cons. The best approach will rely on the goal, the tools, the expertise, the time, and the resources available to help manufacturers and researchers develop and track effective CH₄ mitigation techniques (Storm et

al., 2012; Bekele et al., 2022; Nejad et al., 2024). Although the number of techniques available for EME measurements has increased during the last decade, respiration chambers (RC), sulphur hexafluoride (SF₆) tracer and GF automated emissions monitoring system are the most used. Della Rosa et al. (2021) reviewed 397 studies published between 1995-2018 and reported that the majority of EME measurements were performed with RC (55%), followed by SF₆ (38%) and lastly GF (7%). The remaining 6%, mostly from Europe, comprised 2% face mask (FM), 2% sniffer (SNF), 1% laser methane detector (LMD) and, 1% portable accumulation chamber (PAC) (from Oceania).

There is uncertainty in all measurement techniques due to random components such as changes in animal diets, management practices and environmental conditions. Current methods still have the potential to over- or under- estimate the reference level of EME in ruminants due to their random factors (Nejad et al., 2024). Furthermore, a source of variability is attributed to different measurement procedures, which could be reduced using similar settings and protocols within each technique (Della Rosa et al., 2021). The measurement of EME directly on the animal, although with varying degrees of accuracy, is an expensive and burdensome method (Pickering et al., 2015; Negussie et al., 2017) and, therefore, it is not feasible for large-scale routine measurements, essential requirement for genetic selection (Negussie et al., 2017). Given the need for cheaper, more rapid methods to be used on a large scale, empirical methods for estimating EME have become widespread (Ellis et al., 2010). Therefore, identifying proxies (*e.g.* indirect indicators or traits) that are related to CH₄ emissions, but which are easy and relatively cheap to record on a large scale is a much-needed alternative (Negussie et al., 2017).

In topic 8, terms such as “model”, “predict”, “data”, “equat”, “estim” were highlighted, therefore all terms related to EME estimation models using proxies. For these reasons this topic was called “Prediction model, PM”. Since CH₄ is the result of feed rumen fermentation (the production of CH₄ is related to feed intake and their fermentability; Vlaeminck et al., 2006; Hristov et al., 2016), most predictive equations are based on DMI or metabolizable energy (ME) and gross energy (GE) intakes, and amount of NDF/ADF ingested (Vlaeminck et al., 2006). Several milk fatty acids (MFA) have been suggested as potentially useful for EME prediction (Dijkstra et al., 2011; van Gastelen et al., 2016; Bougouin et al., 2019). However, predictive models based on MFA were developed using a narrow range of diets and limited data (Jonker et al., 2017). Milk fatty acids show a potentially effective proxy since fatty acids have common biochemical pathways in the rumen with CH₄ (Jonker et al., 2017). In particular, odd-chain and branched-chain fatty acids, have a strong relationship with the molar proportions of individual volatile fatty acids (VFAs) in the

rumen (Dijkstra et al., 2011; Bougouin et al., 2019). The fatty acids with greater proxy activity are: C14:0 iso, C15:0 iso and C17:0 anteiso were positively related with CH₄ production (Dijkstra et al., 2011; van Gastelen et al., 2016). Recently, in a meta-analysis Bougouin et al. (2019) observed that the prediction equations considering only MFA showed a higher mean square error in estimating CH₄ emissions expressed as production, yield and intensity (65.1 g/day, 2.8 g/kg of DMI and 2.9 g/kg of milk, respectively) compared with more complex equations. The complex equations included DMI, NDF, etheral extract, days in milk and body weight, for which the mean square error in EME estimation was reduced (46.6 g/day, respectively 2.6 g/kg of DMI and 2.7 g/kg of milk, respectively for production, yield and CH₄ intensity). Jonker et al. (2017) reported that DMI has an effect of over 90% in the prediction of CH₄ emission.

Rumen size and feed retention time, according to Goopy et al. (2013) are factors that affect the CH₄ yield as a more rapid passage of the material from the rumen translates into a reduction in the time available to ferment the substrate. It was observed by Nkrumah et al. (2006) that the duration of feeding and the presence of animals in the feeder were related to CH₄ production, suggesting the influence of feeding behaviour on circadian patterns of CH₄ production (Jonker et al., 2014). Other authors (Watt et al., 2015) studied rumination time in dairy cows as a possible proxy for EME and observed that cows with high rumination activity produced more milk, consumed more concentrate, and produced more CH₄ than cows with low rumination time. Identifying easily measurable proxies would allow for large-scale data, as with the advent of precision livestock farming it is now possible to physiologically monitor (feeding time, rumination time, etc.) of various parameters 24 hours a day (Lovarelli et al., 2020).

Topic 5 has been defined as "Ruminal Fermentation, RF" because, looking at the words highlighted by the beta probability of the LDA analysis the most important words are "rumen" followed, in order of importance, by "ferment", "rumin", "acid" etc.; all words associated with RF. This topic encompasses numerous articles focused on *in vitro* trials. Such trials play a pivotal role in studying EME by offering a controlled, efficient, and targeted experimental approach to comprehensively understand and address EME.

The majority of methanogenesis takes place in the rumen, and a lesser proportion (~13%) of CH₄ is produced in the cecum and colon (Murray et al., 1976). The microorganisms present in the rumen degrade the feed, producing short-chain fatty acids (acetic, butyric, and propionic acid), CO₂, and metabolic hydrogen (H₂). Short-chain fatty acids (SCFA) are absorbed through the rumen wall to provide energy. Methanogens, belonging to the Archaea domain, utilize H₂ and CO₂ to produce CH₄ (Key and Tallard, 2011). In this process, CO₂ is the carbon source and H₂ is the main electron

donor. Four moles of H_2 can produce 1 mole of CH_4 (Pereira et al., 2022). Methanogenesis is the main biochemical pathway to remove metabolic H_2 to maintain a very low concentration of H_2 in the rumen and to ensure proper feed digestion (Moss et al., 2000). The concentration of H_2 affects the feed degradation rate. If the concentration of H_2 increases, the rate of feed degradation decreases. Methane emissions can be reduced by inhibiting H_2 formation from fermentation or by promoting alternative H_2 pathways (Pereira et al., 2022). While methanogenesis is predominantly driven by methanogenic bacteria, the correlation between EME and the concentration of these bacteria appears ambiguous in several studies (Negussie et al., 2017). Some investigations have uncovered significant positive relationships, others have found no correlation between the concentration of methanogens and methanogenesis (Wallace et al., 2015). Additionally, Bouchard et al. (2013) have reported a reduction in methanogens without a significant decrease in CH_4 production. Methanogens are the dominant organisms in the rumen, there are 10^6 - 10^8 cells/ml of methanogenic Archaea per ml of rumen fluid (Tseten et al., 2022). Other microorganisms, such as protozoa, indirectly influence CH_4 emissions by using substrates (starch, cellulose, hemicellulose, pectin, and soluble sugar) to produce SCFA and H_2 , which is then converted into CH_4 by methanogens (van Wyngaard et al., 2018).

Methanogens are anaerobic organisms that thrive in environments with an optimal pH ranging from 6.0 to 7.5 (Pereira et al., 2022) and are known to be sensitive to low pH. Van Kessel et al. (1996) using ruminal fluid from fistulated cows fed two types of diets (high forage or high concentrate), demonstrated that methanogenesis is pH-dependent, with complete inhibition of CH_4 production at pH values below 6.0, and highlighted those volatile fatty acids, such as acetic acid, can directly affect the activity of methanogens at low pH levels. Despite this sensitivity to low pH, methanogens may survive episodes of low ruminal pH through changes in community structure or sequestration in protected microenvironments within biofilms or protozoa (Hünerberg et al., 2015) allowing them to persist and continue CH_4 production under otherwise inhibitory conditions. Although ruminal methanogens are inhibited by a pH below 6.0 in vitro Van Kessel et al. (1996), CH_4 production rates in vivo do not decrease when ruminal pH drops to levels associated with subacute or acute ruminal acidosis (5.2-5.5) in beef cattle (Hünerberg et al., 2015). This suggests that there are additional factors, such as increased propionate formation or passage rate, that contribute to lower EME in cattle fed high-grain diets compared to high-forage diets. Therefore, reducing ruminal pH alone is not considered an effective CH_4 mitigation strategy (Beauchemin et al., 2008).

Diet is a pivotal factor on EME studies, indeed, one of the emergent topics (Topic 6) was precisely defined as "diet composition, DC". The keywords that define this topic were "diet", "fed", "concentr", "digest", and "matter". This topic represents the second most important in the TA (with a percentage of articles exceeding 13%). Manipulating the diet has the potential to reduce EME and consequently increase the feed efficiency of animals, as CH₄ production results in the loss of GE intake (Johnson and Johnson, 1995). Some studies (Grainger et al., 2011; van Gastelen et al., 2022) demonstrated that the basal diet has significant effects on EME. Additionally, as will be discussed later, the composition of the basal diet has the potential to enhance the efficiency of certain supplements in reducing EME, showing additive effects (Bodas et al., 2012; Kebreab et al., 2023). The term "concentr", which is the root of concentrate, has emerged as a decidedly important term in this topic. Increasing the proportion of concentrates is associated with the increase of the starch content in the diet.

The ability of starch to reduce CH₄ emissions has long been recognized. Starch fermentation produces propionic acid, providing an alternative H₂ sink to methanogenesis. Additionally, starch fermentation can lower ruminal pH, which can reduce the populations of protozoa and methanogens (van Gastelen et al., 2022). However, starch is rapidly fermented in the rumen, leading to high concentrations of VFAs and/or lactate, which increase the risk of acute or subacute ruminal acidosis, proved to be an unsustainable method in attempting to reduce EME in ruminants (Haque, 2018). The type of cereal and its processing technique can affect the fermentation level and EME. In terms of cereal sources, both absolute CH₄ production and CH₄ yield appear to follow the order of wheat and maize flakes < maize < barley, with the ranking strongly dependent on the composition and extent of grain processing (Beauchemin et al., 2005). Processing methods of cereals (applying different combinations of heat, moisture, time, and mechanical actions) can alter starch fermentation and ruminal pH. Hales et al. (2012) observed a 17% reduction in CH₄ yield with a steam-flaked maize-based diet compared to a dry-rolled maize-based diet in beef cattle. Herrera-Saldana et al. (1990) increasing the processing degree (i.e., smaller particle sizes) of cereal grains increased the rate of ruminal degradation of the grain and decreased ruminal pH. The inclusion of concentrated feeds in the diet of ruminants (> 40% of the diet) has the potential to reduce CH₄ intensity (Hristov et al., 2013).

Forages represent a very important share in ruminant feeding, and their quality has been shown to have a high potential for modulating CH₄ emissions (van Gastelen et al., 2019). Different types of forage can influence CH₄ emission due to differences in their chemical composition. High-quality forage, such young plants, has more readily fermentable carbohydrates and less NDF, which

increases digestibility and transit rate, and can modify the fermentation pathway and minimize the formation of CH₄ (Haque, 2018; Sun et al., 2022). In contrast, more mature forages result in a greater loss of CH₄ primarily due to an increase in the C:N ratio, which reduces digestibility (Milich, 1999). The improvement of organic matter digestibility (%) and the increase of protein content (g/kg DM) of grass silage was negatively associated with CH₄ yield ($R^2 = 0.74$ and $R^2 = 0.36$, respectively). In contrast, the NDF content (g/kg DM) showed a positive relationship with CH₄ yield both in grass silage ($R^2 = 0.44$) and in maize silage ($R^2 = 0.60$) diets (van Gastelen et al., 2019).

Applying current precision feeding techniques, it is possible to know the chemical composition of the raw materials and total mixed ration in real time (Evangelista et al., 2021). This could be a rapid and advantageous method in predicting CH₄ yield. Furthermore, it has been shown that forage particle size can affect CH₄ emission per kg of DMI. For example, chopping or pelleting forage can reduce EME per kg of DMI, as smaller particles are less degraded in the rumen (Boadi et al., 2004). The meta-analysis by Arndt et al. (2022) reported that mitigation strategies such as decreasing grass maturity and reducing the dietary forage-to-concentrate ratio could decrease CH₄ intensity by 12% on average (range 9 to 17%).

Dietary lipid supplementation lowers CH₄ emissions. This could include lower levels of methanogen activity, lower populations of protozoa, and lower fermentation of ruminal organic matter (Johnson and Johnson, 1995). Eugène et al. (2008) in their meta-analysis, reported that adding lipids to dairy cows' diet decreased CH₄ production by 0.305 g/kg of DMI for every 1% increase of ether extract, and this was primarily due to the reduced DMI. Furthermore, unsaturated fatty acid-rich lipids are subjected to biohydrogenation in the rumen, lowering the amount of hydrogen available for the generation of methane (Beauchemin et al., 2008). It is generally recommended not to exceed 6-7% of lipids on DM basis of the diet, since greater levels can cause a depression in DMI, thus negating the benefits derived from the increased energy density of the diet (Knapp et al., 2014).

Della Rosa et al. (2022) reported that in most studies on EME measurement systems, 72% tested supplements and/or additives. The topic 4 ("supplement and additive, SA") is characterized by the terms: "supplement", "addit", "nitrat", and "reduc". Supplements or additives include inhibitors that are added to the animal's diet with the aim of reducing CH₄ formation. They act with different mechanisms (Beauchemin et al., 2020; Palangi and Lackner et al., 2022: i) inhibition of CH₄ production by reacting with methyl-coenzyme M, which is involved in the last step of methane formation; ii) alternative H₂ sinks that move hydrogen ions away from methanogenesis; iii)

inhibition of methanogenic Archaea bacteria and reduction of the number of protozoa; iv) improved nitrogen metabolism; v) reduction in pH, increase in propionate and decrease in the acetate/propionate ratio. A variety of additives, including ionophores, essential oils, nitrates, algae, and yeasts, were examined. Although many strategies have been proposed for mitigating CH₄, many others are in the very early stages of development (*e.g.* phages, bacteriocins), or have low mitigation potential (*e.g.*, yeast, directly fed bacterial microbes, saponins, ionophores), or are difficult to apply on-farm (*e.g.*, protozoan defaunation) (Beauchemin et al., 2020).

According to Arndt et al. (2022) sustainable strategies for enteric CH₄ mitigation should preferably avoid socioeconomic and environmental trade-offs and, ideally, increase production yield per unit of input. Suggesting that other than the reduction of enteric CH₄ in absolute terms (g of CH₄/day), an additive must be evaluated for its effectiveness in modifying animal performance, which, therefore, influences the yield of CH₄. When choosing the feed additive, in addition to effectiveness and efficiency, the toxicity and potential environmental impacts/undesirable side effects must be considered (Palangi and Lackner et al., 2022). Arndt et al. (2022) in a meta-analysis carried out on 480 studies considering 98 mitigation strategies, reported that the most effective mitigation strategies in the context of "supplements" were: CH₄ inhibitors, tanniferous fodder, electron sinks, oils and fats and oilseeds. These strategies decreased CH₄ intensity by 17% on average (ranging from 12 to 32%) and daily CH₄ emissions by 21% on average (ranging from 12 to 35%) without negatively affecting animal performance. Among the CH₄ inhibitors, 3-nitrooxypropanol (3-NOP) is the one that has shown the greatest efficiency in CH₄ abatement and the minimum effect on animal performance (Arndt et al., 2022; van Gastelen et al., 2024). These compounds are specific inhibitors of methyl-coenzyme M reductase, an enzyme involved in CH₄ formation of methanogenic Archaea in the terminal phase (Duin et al., 2016).

The reduction of CH₄ from 3-NOP varies with dosage (Dijkstra et al., 2018; Melgar et al., 2020) and depends on the type of diet (Dijkstra et al., 2018; van Gastelen et al., 2022; 2024). Increasing level of 3-NOP in the diet decreased enteric CH₄ emissions per unit of body weight, CH₄/kg DMI, CH₄/L of milk and CH₄/kg of digested organic matter (Jayanegara et al., 2017; van Gastelen et al., 2022). van Gastelen et al. (2022) reported a greater reduction in CH₄ intensity and yield with diets containing higher starch content (based on corn silage) when supplemented with 3-NOP, compared to diets based on grass silage.

The long-term effects of 3-NOP administration in dairy cows have been evaluated. In their year-long study, covering all lactation phases, van Gastelen et al. (2024) reported that 3-NOP persistently decreased CH₄ emission, with a positive impact on milk fat and protein yield, energy-

corrected milk yield, and feed efficiency. The 3-NOP is currently a commercial product, available to farmers. Indeed, in 2022, DSM company received EU market approval for Bovaer® for dairy cows, following a positive EFSA opinion confirming that the product reduces enteric CH₄ emissions from dairy cows and is safe for the animal and the consumer. This is the first time the EU has marketed a product as a feed additive for its environmental benefits (DSM, 2024).

Topic 1 stood out from the others due to the presence of terms referring to animals like “intake”, “animal”, “performance”, “weight”, “group” so it was defined as a topic inherent to the interaction between EME and animal, for this reason it was called “methane emission-animal, ME-A”. Approximately 8% of the articles selected fall into this topic. Generally, CH₄ yield was reported to be affected by level of intake, diet quality, supplementation level (Jentsch et al., 2007) physiological state (lactating, non-lactating) (Holter and Young, 1992; Ricci et al., 2013) and cattle class (beef, dairy) (Grainger et al., 2011; Moraes et al., 2014). It has been suggested that CH₄ emissions vary during different physiological phase (Ricci et al., 2013; Lyons et al., 2018). For example, CH₄ levels have been reported to increase by up to 35% from early to late lactation (Bielak et al., 2016; Garnsworthy et al., 2018), and this increase was primarily due to an increase in DMI, the main driver of CH₄ production. Lyons et al. (2018) measured CH₄ emission in dairy cows at different times of lactation and reported an increase in CH₄ yield (L/kg DMI) from the 5th to the 42nd week of lactation (32.2 vs. 36.7 L of CH₄/kg of DMI, respectively). Furthermore, it has been observed that the lactation phase affects the level of reduction of the EME by the supplements. Adding 3-NOP to the ration of dairy cows, van Gastelen et al. (2024) observed a reduction, on average, of 16%, 20%, 16% and 26% in the CH₄ yield (g/kg DMI) for the dry, early, mid and late lactation diets, respectively. This variability has been partly attributed to the different composition of the diets for the different physiological phases, more specifically to the fiber content.

Oddy et al. (2019) carried out a study on dairy sheep and reported that the main determining factor of CH₄ production and yield was DMI. However, they also identified factors such as age, rumen volume, and pregnancy status as influential.

Dong et al. (2019) further elucidated that the increase in EME with increasing age is related to greater DMI. In contrast, Ramírez-Restrepo et al. (2016) reported similar average CH₄ yield between heifers and multiparous cows, both estimated with the SF₆ technique (25.3±0.52 and 24.1±0.55 CH₄/kg DMI, in heifers and multiparous cows, respectively) and measured in the RC (23.7±0.66 and 23.6±0.66 CH₄/kg DMI, in heifers and multiparous cows, respectively) suggesting that there are no differences based on age. A recent study on grazing dairy cows, Salas-Riega et al.

(2022) reported that dry cows produced less CH₄ than lactating cows (266 and 325 g CH₄/cow/day for dry and lactating, respectively). Body weight (BW) also affects CH₄ production. The effect of BW can be attributed to the relationship between BW and intestinal capacity, since intestinal volume is proportional to BW, thereby influencing DMI (Demment and Van Soest, 1985). Animals with higher BW typically have a greater intestinal volume, enabling them to consume more dry matter. This increased DMI is correlated with higher CH₄ production.

Topic 7 is the topic linked to dairy production. The terms that emerged for topic 7 are "milk", "cow", "yield", "dairy", "product" and therefore it was called "dairy production, DP". This topic includes studies conducted on dairy ruminants, particularly dairy cattle. Most studies on EME have been conducted on dairy cows as also confirmed by the meta-analysis by Della Rosa et al. (2022). Those authors reported that 62% of studies conducted on *Bos taurus* were on dairy cattle (4% growing, 50% lactating, 8% non-lactating) and 38% involved beef cattle (35% growing and 3% mature). The terms found in this topic have been discussed extensively previously.

CONCLUSIONS

The aim of this review was to analyse the literature referring to EME from the livestock sector from 1986 to May 2024 using machine learning methods. A growing trend has been observed in the number of publications relating to EME, especially in response to the global political interest in reducing global GHG emissions. At a geographical level, the publications reflect the major GHG emitting countries, *i.e.* those in the northern hemisphere. From the text mining results, terms strongly associated with EME emerged, covering various aspects such as milk, cow, diet, DMI, supplement, model, measure, and animal. The results of the topic analysis highlighted expected topics such as GHG from livestock, dairy production, diet composition, and ruminal fermentation, as well as unexpected topics like extensive livestock farming. Other subjects, such as beef production or genetic improvement, were evidently overshadowed by other themes.

Future prospects

The future of EME research in the livestock sector is promising, with several key areas for development. Research should continue across multiple fronts, emphasizing the need for stronger connections between them. Increased funding from governments, all aiming to identify effective strategies for reducing EME, presents significant opportunities for advancement in this field.

First, diet optimization remains critical, focusing on innovative feed additives to reduce EME while maintaining animal health and production yield and quality. However, the long-term use of

these additives, including 3-NOP, raises concerns due to the limited evidence available regarding their safety and efficacy over extended periods. Comprehensive studies are essential to assess the impact of these additives on livestock health and their environmental effects. In addition, there should be a greater focus on precision livestock farming. This approach can utilize phenotypic data to enhance EME estimation and improve the quality of diets through precise analysis. By leveraging precision systems, researchers can implement modifications that optimize animal nutrition, ultimately leading to more effective methane reduction strategies. Moreover, implementing more manageable and precise methane measurement systems will allow for a more accurate selection of individuals that emit less methane, enhancing breeding programs focused on low-emission livestock. Integrating these areas of research will be crucial for advancing our understanding of EME and developing sustainable solutions in the livestock sector. Collaboration among scientists, policymakers, and industry stakeholders will further enhance the effectiveness of these efforts.

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Chapter 3

Chapter 3

Application of Near Infrared (NIR) spectroscopy for feeding management practices in the livestock sector

Sub Chapter 3.1

Assessment of the predictive performance of a benchtop and portable NIR instrument for analysing the chemical composition and fatty acid profile of dairy buffalo's total mixed ration

Evangelista Chiara¹, Contò Michela², Renzi Gianluca², Basiricò Loredana³,
Bernabucci Umberto³ and Failla Sebastiana²

¹Department for Innovation in Biological, Agro-food and Forest systems (DIBAF), University of
Tuscia, Viterbo, Italy

²CREA, Consiglio per la Ricerca in Agricoltura e L'analisi dell'Economia Agraria, 00015
Monterotondo, Italy;

³Department of Agriculture and Forest Sciences (DAFNE), University of Tuscia, Viterbo, Italy

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ration

ABSTRACT

In the context of modern dairy buffalo production, achieving precision in feed management is crucial to meet the nutritional needs of high-yielding animals while promoting sustainability and efficiency. This study assessed the predictive performance of benchtop and portable near-infrared spectroscopy (NIRS) devices for analysing the chemical composition and fatty acid (FA) profiles of total mixed rations (TMR) for dairy buffaloes. Seventy-four TMR samples, analysed in duplicate, were evaluated using conventional wet chemistry and NIRS data acquisition. Calibration, and cross validation models, and prediction were developed using partial least squares regression (PLSR). The benchtop NIRS device exhibited good accuracy for most chemical parameters, achieving $R^2c \geq 0.90$ for components such as dry matter, crude protein, and fibre fractions. The portable device demonstrated moderate accuracy for chemical parameters, with R^2c values ranging from 0.75 to 0.91. FA predictions were less reliable for both instruments, particularly for the portable device, which showed low R^2 values. Models using selected variables improved predictions for some parameters by minimizing noise. These results highlight the potential of NIRS, particularly benchtop devices, for rapid TMR chemical composition assessment, while FA analysis requires further refinement. This study underscores NIRS's role in precision feed management, contributing to sustainable and efficient dairy buffalo production systems.

Keywords: near spectroscopy; fatty acids; total mixed ration; chemometric evaluation

INTRODUCTION

The feeding of dairy ruminants, including buffalo, has become increasingly complex, as it aims to incorporate a wide variety of dietary components with great precision. This approach is intended to meet the metabolic demands of high-yielding animals while promoting optimal rumen function (Hanigan and Daley, 2020). Buffaloes require a fibre-rich diet that varies with plant maturity stages and cultivation practices (Dwayne, 1996; Evangelista et al., 2024). Carefully formulated rations are essential to prevent nutrient oversupply, which helps to reduce feed costs minimizing environmental waste (Empel, et al., 2016). To achieve nutritionally balanced diets, total mixed rations (TMR) are commonly used. These rations combine on-farm forages with concentrates, as well as vitamin and mineral supplements. The use of TMR facilitates the integration of critical nutrients, thereby supporting productivity, animal health, and sustainable production (Schingoethe, 2017; Evangelista et al., 2024). The rising demand for buffalo-derived products, such as mozzarella, has led to increased mechanization on buffalo farms, boosting farmer incomes and making TMR the preferred feeding method. In this context, effective management of dairy

buffaloes now requires careful monitoring of rations and timely adjustments. Achieving optimal TMR composition not only requires a high-quality mixer-wagon but also continuous assessments of feed quality. This is particularly important in silage-based diets, where significant silage variability can impact the overall diet composition (Weiss et al., 2012; Piccioli-Cappelli et al., 2019; Buonaiuto et al., 2021). The evaluation of the chemical composition of ruminant diets, beyond merely providing balanced nutrition, is essential for enhancing the quality of animal-derived products (AdP). Among dietary components, starch and fibre fractions play a crucial role in modifying the nutritional and chemical quality of these products (Hanuš et al., 2018; Uzun et al., 2018). The fatty acid (FA) profile of TMR also can contribute, to a lesser extent, to product quality (Toral et al., 2018). Thus, although complex, a strong relationship exists between the quality of AdP and the diet provided for the animals. Nowadays, it is common practice to manipulate and enrich animal diets, aiming to deliver specific nutritional substances through the consumption of AdP (Halmemies-Beauchet-Filleau et al., 2018).

In this context, Near Infrared Spectroscopy (NIRS) emerges as a valuable tool for modern feed analysis, providing rapid, non-destructive, and cost-effective evaluations (Piccioli-Cappelli et al., 2019; Yakubu et al., 2020). The NIRS device enables swift assessment of chemical composition in samples using the near-infrared spectrum, specifically at wavelengths from 900 nm to 2500 nm. It is commonly used to analyse chemical components in raw materials such as corn, soybean meals, and forages, as well as to evaluate combined raw materials in TMR (Evangelista et al., 2021; Serva et al., 2021). NIRS is also a valuable tool for studying the digestion process in livestock, by analysing both diets and faecal samples (Jancewicz et al., 2016; Guerra et al., 2024).

Several NIR instruments with different features and performance levels have been employed to support the creation of robust predictive models and provide accessible, affordable devices for on-site analysis. Benchtop NIRS devices have long been a cornerstone in analytical contexts due to their high precision, stability, and ability to analyse a wide variety of samples with minimal interference (Hossain et al., 2024). Over the years, these instruments have undergone significant advancements in spectral resolution, data processing capabilities, and the development of sophisticated calibration models, establishing themselves as the benchmark for in-depth and highly accurate analyses (Evangelista et al., 2021).

A notable technological development in recent years has been the emergence and increasing adoption of portable NIRS devices. Designed to be compact and user-friendly, these instruments enable real-time analysis directly in the field (Hossain et al., 2024). Such capabilities are made possible by calibrations tailored for fresh or untreated samples, eliminating the need for prior

sample preparation (Yamada et al., 2024). Thanks to innovations such as longer battery life, advanced wireless connectivity, and integration with mobile platforms, portable NIRS devices have become practical and accessible tools even in remote settings (Guerra et al., 2024). However, the use of portable devices comes with certain challenges, such as the influence of moisture absorption bands and variations in sample composition (Yamada et al., 2024).

These instruments enable real-time data collection at the barn level (Marchesini et al., 2017). Both benchtop NIR devices with Fourier transform, typically covering a wide wavelength range (900-2500 nm), and portable spectrometers (900-1600 nm) have shown moderate to strong predictive performance, with R^2 values ranging from 0.70 to 0.98 (Mentink et al., 2006; Buonaiuto et al., 2021; Serva et al., 2021; Pereira-Crespo et al., 2022; de Oliveira et al., 2024).

Few studies validate the use of NIRS to determine the FA composition of forages. Foster et al. (2006) obtained validation results with R^2 values ranging from 0.86 to 0.97 for different types of forage. Other authors (Calderon et al., 2007) reported lower R^2 values ranging from 0.64 to 0.89. Recently, Arslan et al. (2025) evaluated the FA composition in different types of flaxseeds, obtaining R^2 values ranging from 0.71 to 0.85. However, they highlighted the excellent performance of NIR with Fourier Transform (FT-NIR) in evaluating proteins ($R^2 = 0.87$) and Neutral Detergent Fiber (aNDF) ($R^2 = 0.90$).

In NIR spectrometry, spectral bands correspond to specific molecular vibrations of chemical bonds (e.g., C-H, N-H, O-H), enabling the identification and quantification of several components (Ozaki et al., 2018). The NIR spectrum includes first, second, and third harmonic bands, as well as combination bands. Due to their overlap, NIR spectra can be complex, with broad peaks that may obscure finer details, particularly in biomolecular samples where multiple bond types contribute to the spectrum (Czarnecki et al., 2021). To interpret this complexity, chemometric methods like Principal Component Analysis (PCA), Partial Least Squares (PLS), Support Vector Machines (SVM), and Artificial Neural Networks (ANNs) are essential for capturing complex relationships in large datasets (González-Domínguez et al., 2022). Additionally, statistical and mathematical transformations such as Standard Normal Variate (SNV), first and second derivatives, and Multiplicative Scatter Correction (MSC) are used to enhance peak clarity and reduce spectral interference from overlapping bands (Zeaiter et al., 2005).

The high number of spectral variables in NIR data can introduce significant noise and interference, potentially compromising the reliability of predictive models. However, PLS helps to mitigate the noise by reducing dimensionality using latent variables (LVs), which minimize overlap and noise

(Yun et al., 2019). While LVs alone may not offer the precision required for robust models, selecting specific, real variables can improve predictive performance and model interpretability. By focusing on key spectral regions linked to distinct chemical bonds, more accurate and reliable models can be developed.

Few studies have focused on FA determination in individual feed components of animal diets (Foster et al., 2006; Calderon et al., 2007; Arslan et al., 2025), and the application of NIR for FA analysis in TMR is nearly non-existent. Fats constitute a minor component of the ration, approximately 2–3% of dry matter, compared to other chemical constituents. Moreover, these fats can originate from various sources within the ration, such as forages, concentrates, and vegetable oils, further contributing to variability. This study aims to address these gaps by developing predictive models to estimate the chemical composition and FA profile of TMR used in ten buffalo dairy farms across different seasons (April to November). Specifically, it evaluates the efficacy of NIR spectrometry, particularly using a benchtop model, in developing these predictive models. Additionally, the in-situ application of NIR technology directly on fresh TMR at the time of feeding is gaining popularity. However, the significant variability of fresh TMR before drying and grinding poses substantial challenges. The separation of fibrous, starchy, and oily components can often compromise measurement accuracy, making it difficult to obtain representative spectral determinations. Therefore, the two NIR spectrometry instruments, a benchtop model and a portable device each with distinct capabilities and performance characteristics, could provide useful information for different purposes if capable of generating predictive models.

MATERIAL AND METHODS

Study design

The TMR samples used in this study were obtained from the study by Evangelista et al. (2024), in which ten dairy buffalo farms in the Lazio region, supplying milk for buffalo mozzarella production, were visited monthly. During each visit, a TMR sample was collected, along with data on the composition of the TMR in terms of raw materials. The TMR composition included hay, silage, concentrates, and, during certain seasons, green forage. The samples were collected immediately after morning distribution. Three subsamples were taken from the feed bunk (corresponding to the beginning, middle, and end of the bunk), and then pooled together. A sample of approximately 1 kg was taken from this mixture and stored under refrigerated conditions (4°C) for transport to the laboratory for analysis.

NIR data collection was conducted during the final seven months (April to November). Spectrometric measurements with the portable NIR device were conducted simultaneously with the collection of fresh samples, whereas measurements with the benchtop NIR device were performed after sample drying, which was carried out in the laboratory and processed in parallel with the chemical analyses.

All chemical analyses and FA assessments were performed on two replicas of TMR, with NIR spectra obtained for each individual replicas, resulting in a total of 148 determinations from 74 TMR samples. The composition of the TMRs is reported in table S1.

The differences between the values of repeated determinations were tested using a basic statistic (Figure S1). The absolute differences for each constituent between the value and its repeated determination were compared to the standard deviation, which was used as a threshold. All the difference values exceeding this threshold were considered different. The percentage of data identified as different is reported in Tables S2a and S2b.

Additionally, the Compound Symmetry (CS) covariance structure of the sample and its replication is provided also in the supplementary materials (Table S2a and S2b). CS is a specific measure used in mixed models (calculated using the SAS statistical package, Systems v.9.4; SAS Institute Inc., Cary, NC, USA) to describe the relationships among repeated measures. The CS value ranges from 0 to 1, with values closer to 1 indicating minimal distance or high similarity between measurements.

Total mixed ration and fatty acids analysis

A total of 74 TMR samples were analysed by wet chemistry. Dry matter (DM) was measured after oven drying at 65°C to constant weight. The samples were ground through a mill (Retsch Müller, Germany) to pass a 1 mm screen. Samples were analysed for ash, crude protein (CP), ether extract (EE) (AOAC, 2006), and total starch using a K-TSTA assay kit (Megazyme International, Bray, Ireland). Neutral detergent fibre (aNDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) were analysed using an Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY) according to Van Soest et al. (1991). The crude fibre (CF) content was determined with the same instrument, according to the Weende method (AOAC, 2006). Chemical data are reported as percentages on a DM basis.

The extraction of FAs followed the method of Folch et al. (1957) using a chloroform:methanol mixture (2:1, v/v). Fat diluted in hexane was methylated with methanolic KOH, and a 1 µl sample

was injected into a gas chromatograph with a flame ionization detector (GC-FID) (Agilent Technologies, Santa Clara, CA, USA), equipped with a CP-Sil88 capillary column (100 m, 0.25 mm, 0.20 μ m, 100% cyanopropyl, Agilent Technologies). The fatty acid 19:0 (Sigma-Aldrich Merck, Darmstadt, Germany) was used as the internal standard. GC-FID conditions were as follows: splitless inlet at 250°C; FID at 250°C; helium carrier gas at 1.2 mL/min. The oven temperature program began at 60°C (held for 4 min), then ramped up by 13°C/min to 175°C (held for 27 min), followed by an increase of 4°C/min to 215°C (held for 25 min), and finally increased by 2°C/min to 220°C (held for 5 min) as previously reported by Contò et al. (2022). Fatty acid peaks were identified by comparing them to peaks in a Supelco mix 37 standard (Sigma-Aldrich Merck, Darmstadt, Germany), among which are considered the main fatty acids present in the TMR such as: myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1 cis-9), vaccenic acid (18:1 cis-11), linoleic acid (18:2 n-6), arachidic acid (20:0), γ -linolenic acid (18:3 n-6), α -linolenic acid (18:3 n-3), and arachidonic acid (20:4 n-6). Fatty acid methyl esters (FAMES) were expressed as percentage of the total FAME content. The total amounts of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids omega-6 (PUFA n-6), and omega-3 (PUFA n-3) were also calculated. All data were analysed in duplicate.

Collection of NIR spectra

Spectra were acquired using the portable PoliSPEC^{NIR} (ITPhotonics, Italy) instrument directly on the fresh samples, following the manufacturer's guidelines, the protocol included four acquisitions per sample and replicate, each lasting 7 seconds in reflectance. The control software is PoliDATA v.4. This device records 260 absorbance points in the spectral region ranging from 900 to 1700 nm.

Spectra for the benchtop NIRFlex 500 (Büchi, Switzerland) spectrometer equipped with a polarization interferometer were acquired on dried samples, which were ground to 1 mm prior to measurement.

The minced, dried sample was placed into a cylindrical quartz glass cup with a diameter of 25 mm and a height of 10 mm. To ensure better spectral averaging across the sample surface, a sample rotation accessory was employed. For each spectrum, 32 scans were taken, and each sample was scanned three times, using, as final data, their mean. Spectral acquisition was conducted at room temperature (approximately 20–21 °C).

The benchtop spectrometer collected 1501 data points within the standard spectral range of 10,000 to 4000 cm^{-1} (1000–2500 nm), with a resolution of 4 cm^{-1} . In addition, the spectrum was acquired using the Fourier-transform (FT-NIR). A detailed register of specifications of two spectrometers can be found in table 1.

Table 1. Type of NIRS used in the determination of reflectance measurements.

Type	NIRFlex BUCHI 500 ¹	PoliSPEC ^{NIR2}
Sensor	Extended range InGaAs (temperature controlled)	InGaAs, 256 pixels, cooled with single Peltier stage
Spectrum range	1000 – 2500nm	900-1700 nm
Numerical resolution	8 cm^{-1} (with boxcar apodization)	3.2 nm
Average optical resolution	HWHN 3.25 nm	HWHM3.25 nm
Supply	100 - 230 VAC $\pm$ 10 %, 50/60 Hz, 350 W	12 Vdc power supply with power supply provided, interchangeable rechargeable battery
Type of measurements	Reflectance / transmittance Measurement cells NIRFlex Solids	Reflectance / transmittance
Measurement Geometry	FT-NIR Internal and automatic	PIX Diffuse / 0°

¹ Benchtop NIR, ² Portable NIR

Spectral data from both instruments were initially recorded as reflectance (R) and subsequently converted to absorbance ($\log 1/R$). The spectra from both instruments were exported in Microsoft Excel spreadsheet format and acquired with the chemical data on statistical software. All chemometric processing and analyses were performed using Un-scrambler X, version 10.3 (CAMO Software, Oslo, Norway). Spectral data were used as the independent variable to predict the concentrations of chemical constituents as reported in table 2, and FAs reported in table 3, designated as the dependent variables.

Division of dataset

The dataset of 148 determinations was partitioned using Unscrambler X software, which randomly assigned samples to calibration and validation sets according to predefined experimental criteria. This random allocation process ensured that each set contained the specified number of samples. To maintain consistency and avoid any overlap, the original sample and its duplicate were always assigned to the same dataset (either calibration or validation). This approach ensured that replicate analyses did not inadvertently influence both sets, preserving the independence of the validation process. In table S1, we highlighted the validation dataset samples. Specifically, the dataset was divided into a calibration set consisting of 99 samples (67% of the total dataset) and a validation

set with the remaining 49 samples (33%). This stratified split enabled the development of a robust calibration model, whose predictive performance was subsequently assessed on the independent validation set. A basic statistical analysis was performed for each dependent variable. The mean, minimum, and maximum values were calculated, along with the standard deviation (SD) and coefficient of variation (C.V.). These statistical measures offered insights into the central tendency, range, and variability of the data, allowing for a comprehensive comparison of the measurement consistency and distribution between the two NIR devices.

Outlier determination

Sometimes, certain NIR spectra deviate significantly from the average due to incorrect acquisitions caused either by the chemical analysis of samples or the measuring instrument. To identify and exclude outliers' spectra, an unsupervised multivariate analysis using PCA was conducted. Hotelling's T^2 ellipse, a statistical tool that quantifies sample variability within the PCA model, was used to define a region that should encompass at least 95% of the samples. Any data points outside of this boundary were classified as outliers, indicating a significant deviation from the main dataset (Pasquini, 2018). As a result, six outliers were identified in the calibration set and three in the validation set, as shown in Supplementary Figure S2. Consequently, the calibration set was refined to include 93 samples, while the validation set consisted of 46 samples, after excluding the outliers.

Spectra pre-processing

Spectral data were subsequently pre-processed to optimize model performance for each chemical parameter analysed. Several mathematical transformations were applied, including MSC, which reduces scatter by generating a mean spectrum to isolate wavelength-dependent perturbations from residual spectral data, and SNV, that unlinking MSC, does not produce a mean spectrum but instead reduces scatter by subtracting the arithmetic meaning from each spectrum and dividing by the experimental standard deviation. Additionally, first and second derivatives were applied using the Savitzky-Golay (SG) algorithm with polynomial orders of 2 or 3 and with 5- or 7-points smoothing intervals to highlight overlapping bands. Other treatments included data normalization, baseline correction, and detrending (D) were applied to obtain the better PLSR model (Williams and Norris, 1987, Barnes et al., 1989; Yamada et al., 2024). The plots of pre-processed spectra for both NIR devices were reported in Figure 1.

Partial Least Squares (PLSR) regression models

PLSR was chosen for its ability to handle highly collinear spectral data by compressing multiple data points into fewer representative factors, known as latent variables (LVs). These LVs capture the main variance in the data while minimizing redundancy, allowing for effective model calibration. Calibration equations were specifically developed for the chemical constituent and FA fractions.

The calibration dataset was used to build the PLSR model by applying five-fold cross-validation, a method particularly well-suited for the relatively small dataset size and the presence of repeated measurements. This approach divides the calibration data into five subsets, with four subsets used for model training and the remaining subset for validation in each iteration. This method ensures that all samples are included in both the training and validation phases (Williams and Norris, 1987). The optimal calibration models for the two NIR instruments were selected based on key performance metrics reflecting the robustness of each model. Subsequently the PLSR model was used in the validation dataset to obtain the same coefficient to estimate the performances in prediction. This study considered several goodness-of-fit statistics, including the coefficients of determination for both calibration and prediction (R^2_c and R^2_p respectively). The standard errors of calibration (SEC), cross validation (SECV) and prediction (SEP) were also evaluated, with the ideal being as low as possible to indicate high predictive accuracy of the model. The residual predictive deviation ($RPD = SD/SECV$ or SEP) was also calculated, with its classifications outlined in table 2. In addition, the range error ratio ($RER = \text{range of reference values} / SECV$ or SEP) was calculated (Williams and Norris, 1987). High R^2 , RPD value greater than three and RER value greater than 10 suggested that the model retains very good predictive capabilities across different datasets (Yamada et al., 2024).

Table 2. Classification and success levels based on R^2 , RPD, and RER as per references (Williams, 2004; Malley et al., 2005; Yamada et al., 2024).

R^2	RPD	RER	Classification	Application
<0.80	<2.0	<7	Very poor	Not recommended
	2.0 to 2.5		Poor	Rough screening
0.80 to 0.90	2.5 to 3.0	<10	Fair	Screening
	3.0 to 3.5	>10	Good	Quality control
0.90 to 0.95	3.5 to 4.0		Very good	Process control
>0.95	>4.0	>20	Excellent	Any application

R^2 = coefficients of determination; RPD: Residual Predictive Deviation SD/SEC ; RER: Range of Reference Values.

Variable Selection in PLSR Calibration Models

To improve model interpretability and predictive performance, a discrete selection of spectral variables was applied after PLSR analysis. This process involved a systematic, one-by-one testing of spectral points, focusing especially on those within the Variable Importance in Projection bands, which reflect the contribution of each spectral point based on the variance explained by each PLSR component (Yun et al., 2019). Least Absolute Shrinkage and Selection Operator (LASSO) regression analysis using the procedure Glm-select of SAS statistical software was then performed, to identify spectral points that captured the most substantial variance associated with the target property (McNeish, 2015). Through iterative refinement, calibration models with a focused set of spectral variables were developed. A model using selected spectral points was considered effective if it demonstrated robustness equal to or exceeding that of models based on the entire spectral dataset (de Araújo, 2022). This target approach ultimately enhances the interpretability of the regression model.

RESULTS AND DISCUSSION

Sample composition

Nine chemical parameters and 11 individual FAs were analysed for each TMR sample. Additionally, four FA classes were calculated for each sample. The results of the descriptive analysis (mean, range, standard deviation and coefficient of variability) are presented in table 3 for the TMR chemical composition and in table 4 for the FA content, separately for the calibration and validation sets.

Buffalo rations in this study were inherently more fibrous and had a distinct nutrient profile compared to those formulated for cattle or other buffalo rations (Evangelista et al., 2022; Masucci et al., 2024). A wide variability was observed in the chemical composition of the TMRs in both the calibration and validation sets, particularly for starch, crude fibre, and, in the validation set only, ether extract (C.V. > 18%). Conversely, the lowest variability (< 14%) was noted for dry matter, aNDF, and ADF in both the calibration and validation sets.

Table 3. Descriptive statistics of the chemical composition (g/100 g of DM) of Total Mixed Rations (TMR) from both the calibration (n = 93) and validation (n = 46) sets.

	Calibration Set (93) *				Validation Set (46) *			
	Mean	Range	S.D.	C.V.	Mean	Range	S.D.	C.V.
DM	52.59	31.93	5.95	11.31	53.25	25.47	6.18	11.61
Ash	6.92	5.73	0.97	13.96	6.83	6.83	0.92	13.40
CP	12.10	9.62	1.87	15.45	12.11	7.18	1.89	15.58
EE	2.45	2.17	0.41	16.78	2.46	2.10	0.46	18.82
CF	29.52	19.23	5.53	18.72	29.32	21.49	5.88	20.04
aNDF	45.75	21.9	5.05	11.04	45.62	19.61	4.97	10.89
ADF	30.72	20.75	3.74	12.16	30.99	12.63	3.40	10.96
ADL	6.13	6.58	1.03	16.84	6.28	4.06	0.99	15.35
Starch	14.09	15.99	3.11	22.04	14.08	15.36	3.26	23.16

*= 6 outlier data for the calibration set and 3 outlier data for the validation set were removed before the PLSR analysis. S.D. = standard deviation; C.V.= coefficient of variation (S.D./Mean *100); DM= dry matter; C=: crude protein; EE= ether extract; CF= crude fibre; aNDF= Neutral Detergent fibre; ADF= Acid Detergent Fiber; ADL= Acid detergent Lignin.

The FAs present in the TMRs derive from both concentrate sources (sunflower, corn, soya, barley, field bean), fodder sources (green forage, hays, silage), and some supplements, such as linomix, providing significant sources of 18:3 n-3. As a result, there is considerable variability in the FA composition of the TMRs. For 18:3 n-3, 20:4 n-6, and PUFA n-3, the C.V. exceeds 40%, as well as for 18:3 n-6 in the validation set. In contrast, the lowest variability (< 10%) was observed for 18:1 cis-11, 18:2 n-6, MUFA, and PUFA n-6 in both datasets, and for 18:1 cis-9 in the calibration set and SFA in the validation set.

Although several studies have explored several characteristics of TMR for dairy buffalo, few have specifically examined the FA profiles. This makes it difficult to directly compare our findings with previous research. Existing studies have primarily focused on grazing systems, reporting low fat content (Foster et al., 2006; Pereira-Crespo et al., 2014), or on individual types of concentrates. In contrast, this study examined TMR, a mixture of forages and concentrates such as cotton, flax, soy, and green forage which collectively contribute to the higher fat content observed in the diet.

Table 4. Descriptive statistics of fatty acids (% of total FAME) in Total Mixed Rations (TMR) from both the calibration (n = 93) and validation (n = 46) sets.

	Calibration Set (93) *				Validation Set (46) *			
	Mean	Range	S.D.	C.V.	Mean	Range	S.D.	C.V.
14:0	0.47	0.54	0.10	20.64	0.47	0.44	0.09	18.51
16:0	17.10	10.19	1.75	10.20	17.09	9.41	1.78	10.39
16:1	0.45	0.55	0.11	24.22	0.44	0.53	0.11	24.55
18:0	3.01	5.24	0.54	17.97	3.05	3.22	0.52	17.64
18:1 cis-9	20.43	12.01	1.92	9.41	20.49	10.65	2.13	10.39
18:1 cis-11	1.01	0.67	0.09	9.31	1.02	0.33	0.08	7.35
18:2n-6	43.04	21.82	4.10	9.51	43.42	20.57	4.11	9.46
20:0	0.49	0.71	0.11	21.63	0.50	0.47	0.09	18.02
18:3 n-6	1.86	3.27	0.72	38.66	1.89	3.82	0.76	40.42
18:3 n-3	9.97	20.45	4.82	48.29	9.57	20.47	4.97	51.95
20:4 n-6	0.66	2.05	0.37	55.91	0.67	1.84	0.39	57.91
SFA	21.40	13.77	2.15	10.05	21.46	7.94	1.68	7.81
MUFA	22.39	7.12	1.62	7.22	22.42	7.28	1.80	7.98
PUFA n-6	45.93	19.9	3.76	8.19	46.33	17.21	3.54	7.63
PUFA n-3	9.97	20.45	4.92	49.30	9.57	20.47	4.57	47.77

*= 6 outlier data for the calibration set and 3 outlier data for the validation set were removed before the PLSR analysis. FAME= Fatty acid methyl ester; S.D.= standard deviation; C.V.= coefficient of variation; SFA= saturated fatty acid; MUFA= monounsaturated fatty acid; PUFA= polyunsaturated fatty acid.

In general, a robust calibration set should have exhibited sufficient variability to enhance the model's reliability, generalizability, and resistance to overfitting (Williams, 1987; Burns et al., 2007; Ripoll et al., 2021). In this study, the inclusion of data from 10 dairy buffalo farms checked in different seasons of the year, ensured high variability in terms of TMR composition.

Spectral characteristics

The NIR spectral characteristics of TMR samples differed significantly between fresh and dried samples, primarily due to variations in water content. These differences substantially influence absorption features and the calibration of predictive models (Acosta et al., 2020). Therefore, the two types of spectra obtained using the portable and benchtop NIR instruments not only cover

different wavelength ranges and use distinct measurement technologies but also capture the TMR samples in different physical states.

In fresh samples, water absorption bands around 1400 nm dominate the spectra, often overshadowing weaker signals from organic compounds. This dominance complicates the precise quantification of specific components. The water content contributes to broader, more diffuse peaks due to molecular interactions and light scattering. However, fresh samples retain the natural state of the feed, facilitating real-time analysis of TMR composition at the point of distribution to animals.

In contrast, dried samples produce sharper, more defined peaks, offering enhanced resolution for organic compounds such as proteins, fats, fibres, and FAs. Additionally, drying improves sample stability during storage and transport, allowing spectra to be collected alongside other analytical procedures. However, the drying process may alter water-soluble components, potentially distorting the nutritional profile of fresh forage. Identifying FAs in wet samples remains challenging. The C-H absorption bands associated with some FAs are detectable in the second and third harmonic overtones, where water typically obscures these signals. As reported by Giaretta et al. (2019) in their analysis of raw meat samples, this issue can be partially addressed, as certain FAs absorb within the C-H combination stretching region (2200–2500 nm), which is commonly analysed using NIR devices. Therefore, spectral limitations were particularly evident in portable NIR devices restricted to a range of 900–1700 nm (Figure 1), where only limited information is available before the water absorption peak. Chemical composition analysis in this range is heavily dependent on water, making it well-suited for proximate chemical composition determinations.



Figure 1. NIR spectra from NIRFlex BUCHI 500 and PoliSPEC^{NIR} with the typical band of chemical compound absorption.

In summary, NIR analysis of TMR samples involves a trade-off between the representativeness of fresh samples and the analytical precision of dried samples. Fresh samples are advantageous for real-time, field-relevant assessments, while dried samples provide higher accuracy for detailed chemical quantification. Careful calibration and interpretation are essential to address these differences and optimize the application of NIR in TMR analysis.

The harmonics of NIR refer to absorption phenomena within the NIR range (780–2500 nm), driven by molecular vibrations in functional groups containing bonds like C-H, O-H, and N-H (Workman, 2021). Proteins absorb primarily due to N-H and C-H bonds, with main absorption bands around 2050–2250 nm (amino groups) and 1600–1700 nm (secondary bands). Fats and FAs show absorption related to C-H groups, with key bands at 1700–1800 nm (stretching of CH₂ and CH₃), and 2300–2400 nm (specific to saturated/unsaturated fats). Fibers like cellulose and hemicellulose absorb in the 2100–2200 nm and 1700–1800 nm regions, associated with O-H and C-H bonds in complex carbohydrates (Shenk and Westerhaus, 1991). Water, due to O-H groups, absorbs strongly at 1400 nm and 1900 nm, critical for quantifying moisture. Pre-processing is required to mitigate water interference and allow the individuation of peaks associated to chemical compounds also if some treatment could introduce noise and compromise the robustness of predictive models.

Pre-treatment of NIR spectra

Pre-treatment of NIR spectra is essential because raw spectral data often contain noise, baseline shifts, and scattering effects, which may arise from the physical characteristics of TMR samples or from instrumental factors. These interferences can obscure chemical information in the spectra,

making it difficult to develop accurate predictive models. Some pre-treatment methods were tested both individually and in combination (Zeaiter et al., 2005). In particular, after baseline correction and normalization, three data sets were obtained and used for subsequent treatment.

NIR spectra typically produce low-resolution and poorly defined signals; therefore, mathematical treatments, such as the first derivative using the Savitzky-Golay (SG) algorithm, are used to transform subtle oscillations into discernible peaks. Before applying this mathematical treatment, it is crucial to remove random oscillations to prevent the amplification of noise. For this purpose, preprocessing techniques like Standard Normal Variate (SNV) or Multiplicative Scatter Correction (MSC) are employed to mitigate scattering effects (Azzouz et al., 2003; Zeaiter et al., 2005). As shown in Figure 2, however, part of the spectra, particularly before 1100 nm for the NIRFlex 500 and before 1000 nm for PoliSPEC^{NIR}, exhibited a region rich in noise, especially evident after mathematical transformations.

This stepwise approach should minimize information loss and could effectively reduce noise. The pretreated spectra were subsequently used in PLSR analysis to develop models that maximized R^2 and minimized the SEC for each analyte studied. The effectiveness of pretreatments varied depending on the analyte, as different compounds absorb in distinct spectral regions.

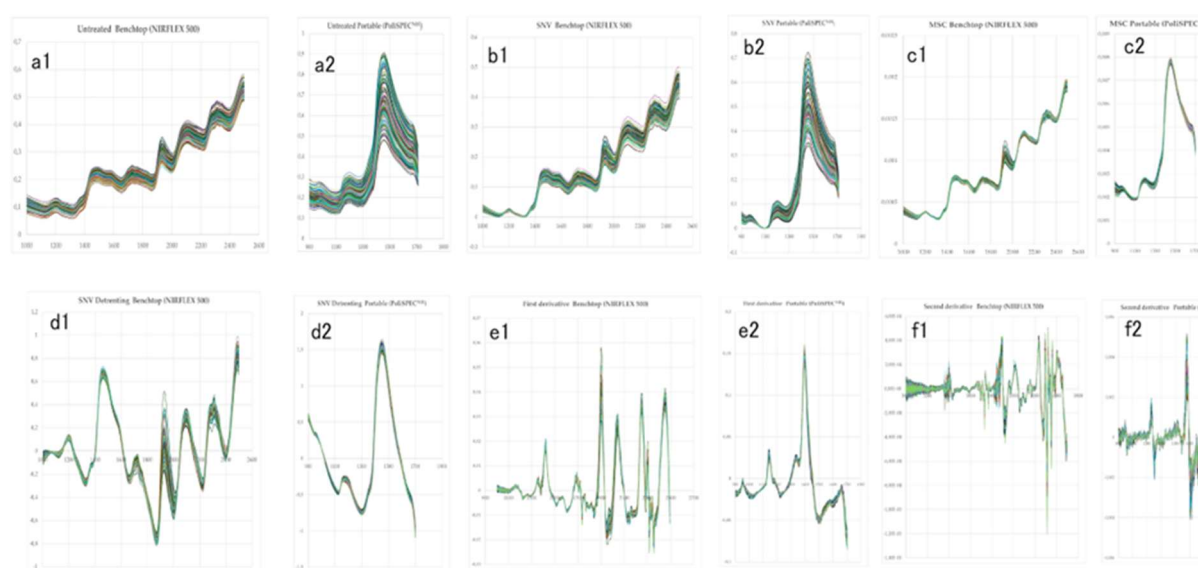


Figure 2. The trends of the spectra with the most frequently used pre-treatments for both the benchtop NIR (NIRFlex 500) “1” and the portable NIR (PoliSPEC^{NIR}) “2”. **Note:** **a**=untreated spectra; **b**=SNV; **c**=MSC; **d**=SNV+Detrending; **e**=first derivative; **f**=second derivative.

The spectral pre-treatments applied for the calibration of chemical composition and FAs in TMR samples are detailed in supplemental materials (Table S3a and S3b), while the most frequently used pre-treatments are shown in figure 2 for benchtop NIR and portable NIR.

Prediction models

The PLSR models were evaluated to determine their reliability and practical usefulness. For each chemical compound and FA, some models were tested to explore their predictive capabilities and identify the most effective approach. This chapter presents the results of these evaluations, focusing on the performance and applicability of the models.

Calibration, cross validation and validation statistics for the PLSR models using full spectra with selected spectral variables are presented in tables from 5 to 8 for the benchtop NIR, and from tables 9 to 12 for the portable NIR instrument. In the same tables the outlier and latent variable (LV) were reported for each compound (in Figure S4, the best PLSR model is reported).

The outliers in PLSR analysis were identified and removed using statistical tools such as Leverage and Q residuals (or Squared Prediction Errors). Leverage measures how far a sample is from the average in the predictor space. High leverage indicates that a sample has unique or extreme values. Q residuals (or Squared Prediction Errors) are statistical indicators used in multivariate analysis, particularly in PLSR. Mathematically, Q residuals are defined as the sum of the squared differences between observed values and those estimated by the model. High Q residuals suggest that the sample contains variability not captured by the model, either during calibration or validation. When both Leverage and Q residuals are significantly high, it is likely that the sample is an outlier. Removing them can improve the robustness of the model, reducing SEC.

After defining the calibration models, we aimed to reduce the number of variables and eliminate noise to improve model performance. For each model, the weight of each spectrometric measurement was determined, generating a regression curve (Figure S5). From these regression curves, some picks were isolated. Using the LASSO procedure, a multivariate regression approach that alternately added and removed variables models were estimated to achieve higher R^2 in cross validation and lower SECV values (McNeish, 2015). If these criteria were not met, the process was repeated, focusing on other previously overlooked bands. This iterative process led to models with higher R^2 values and lower or at least comparable SECV values to the calibration model obtained with the entire spectrum. The variables selected for each chemical compound were shown in supplementary figures S5 indicated with red asterisks. In addition, the selected picks are summarized in some bands corresponding to different overtones reported in figure S6.

In the Near-Infrared spectrum, overtones and combination bands are associated with specific spectral regions and enable the detection of various compounds. The first overtone, typically observed between 1450 nm and 1900 nm, is dominated by C–H, N–H, and O–H vibrations and is particularly useful for identifying water, proteins, and fats. The second overtone, found in the range of 900 nm to 1300 nm, involves the same types of bonds but exhibits lower intensity. This region is commonly employed for the analysis of fats, carbohydrates, and proteins. The third overtone, located between 750 nm and 900 nm, is characterized by very low intensity, making its detection reliant on highly sensitive instruments. Consequently, it is less frequently utilized in practical applications. Combination bands, generally observed in the spectral range of 1900 nm to 2500 nm, are more intense than higher-order overtones and are particularly important for analysing complex chemical compositions such as proteins, starches, and fatty acids (Czarnecki et al., 2021).

The statistical evaluation of these new calibration and validation models was reported in tables 6, and 8 for benchtop NIR and in 10 and 12 for portable NIR.

Prediction model for chemical analysis using the benchtop NIRFlex 500

For the models obtained with benchtop NIR, only the ash content identified six outlier values. The remaining parameters showed few or no outliers during calibration, with an average of 2.5 outlier samples. The outliers in validation did not exceed 3 units for all the models analysed, with an average of 0.8 for the chemical parameters.

In the same tables (from Table 5 to 9) the latent variables of the calibration model were reported, and they ranged from a minimum of 2 to a maximum of 7 for chemical constituents.

Considering the chemical composition, good calibration models ($R^2c > 0.90$) for almost all chemical parameters, except for DM, Ash, and ADL, achieved average values of 0.863.

Table 5. Calibration, cross validation and prediction statistics for chemical composition (g/100 g of DM) of Total Mixed Rations (TMR) from both the calibration and validation set by NIRFlex 500, using the full NIR spectrum.

mg/100g	Calibration set									Validation set			
	LV	OUT	R ² c	SEC	R ² cv	SECV	RPDcv	RERcv	OUT	R ² p	SEP	RPDp	RERp
DM	5	3	0.896	1.520	0.843	1.954	3.05	16.34	1	0.762	2.696	2.29	9.45
Ash	4	6	0.853	0.263	0.836	0.388	2.50	14.77	2	0.832	0.397	2.32	17.20
CP	6	5	0.914	0.515	0.842	0.721	2.59	13.34	2	0.741	0.873	2.16	8.22
EE	7	0	0.931	0.109	0.889	0.142	2.89	15.28	0	0.845	0.183	2.51	11.29
CF	7	0	0.937	1.194	0.812	2.086	2.65	9.22	1	0.743	2.606	2.26	8.25
aNDF	7	0	0.951	1.106	0.870	1.807	2.79	12.12	0	0.854	1.882	2.64	10.42
ADF	7	2	0.935	0.810	0.863	1.193	3.13	17.39	0	0.803	1.344	2.53	9.40
ADL	5	5	0.840	0.378	0.728	0.498	2.07	13.21	1	0.703	0.524	1.89	7.75
Starch	6	2	0.968	0.544	0.877	1.079	2.88	14.82	0	0.815	1.286	2.53	11.94

LV= latent variable; OUT= outlier; R²c= coefficient of determination on calibration set; SEC= standard error on calibration set; R²cv= Cross validation coefficient of determination on calibration set; SECV= Cross validation standard error on calibration set; R²p= prediction coefficient of determination on validation set; SEP= prediction standard error on validation set; RPDcv= SD/SECV; RPDp= SD/SEP; RERcv= Range (max-min value)/SECV; RERp= Range (max-min value)/SEP; DM= dry matter; CP= crude protein; EE= ether extract; CF= crude fibre; aNDF= Neutral Detergent fibre; ADF= Acid Detergent Fiber; ADL= Acid detergent lignin.

In the 5-fold cross-validation, the R² value decreased as normally for all the chemical components considered, falling below the threshold of 0.9. Values ranged from 0.812 for CF to 0.889 for EE, except for ADL, which exhibited a notably poor model with R²= 0.728. For all parameters evaluated, the SECV was higher than the SEC.

To assess model performance based on the RPDcv value, following the guidelines provided by Williams (2004), in Table 2, no parameters had an RPDcv lower than 2, thereby excluding any models classified as poor and not recommended for use. The RPDcv values exceeded the threshold of 2.5 for almost all parameters, except for ADL, which showed a value of 2.07. Meanwhile, DM and ADF surpassed the threshold of 3. Notably, only the RERcv for CF failed to reach the value of 10.

When these PLSR models were applied to an external dataset (validation set) to further validate their robustness, only five parameters (ash, EE, aNDF, ADF, and starch) were found to exhibit “fair” performance, with R²p values exceeding 0.8. Among these five chemical compounds, only four achieved an RPDp greater than 2.5. Furthermore, the RERp for this parameter did not exceed the threshold of 10.

If external validation is considered essential to evaluate the applicability of the model to independent datasets, we can conclude that only three models (EE, aNDF and starch) demonstrated "fair" performance, as defined by Malley et al. (2005).

NIRS studies related to TMR are relatively limited compared to those published on forages and concentrates, with a notable lack of research focused on TMR for dairy buffaloes. The study by Buonaiuto et al. (2021), carried out on rations for cows producing milk for Parmigiano Reggiano cheese, could serve as a useful point of comparison, as these rations are significantly richer in fibre than those typically used for dairy cows. However, it is important to note that the rations for Parmigiano Reggiano do not include silage, while silage is commonly used in buffalo diets, including the rations considered in this study. Buonaiuto et al. (2021) achieved good results for CP, starch, aNDF, and ADF ($R^2c > 0.80$), with lower values for ADL ($R^2c = 0.76$), and ash ($R^2c = 0.57$). The RPD values for these components were greater than 2.20, while they were lower for ADL, and ash (< 1.90). They reported RMSE for CP, ADL, and ash, while for the other parameters, the RMSE was found to be greater than 2. They utilized a benchtop NIR with a broad spectral range, analysing 205 TMR samples collected between 2016 and 2021.

Pérez-Marín et al. (2022) developed calibration models on 394 TMR for dairy cows obtaining coefficients of determination (R^2c) greater than 0.80, except for starch, which had an R^2c of 0.75. The SEC ranged from 0.71 to 1.25, and the RPD values ranged from 2.31 to 5.76. The best calibration results were achieved for DM. These models were developed using the Foss NIRSystems 6500 instruments (1100–2498 nm), and when transferring the calibrations to portable systems, comparable results to the benchtop instrument were obtained. Excellent calibration results were also published by Pereira-Crespo et al. (2022) in a study involving 125 samples of TMR for dairy cows. They achieved high coefficients of determination for calibration ($R^2c > 0.98$) with very low errors ($SEC < 0.67$) and cross-validation RPD values greater than 3, indicating excellent model capability according to the discussed metrics.

Another issue contributing to low R^2 and high SEC values could be excessive noise in the spectra, which compromises model accuracy.

The calibration model specifically obtained using spectral data collected with benchtop NIR, where 1500 values were recorded for each spectrum, showed potential estimation errors due to overfitting or excessive background noise, particularly when dealing with spectra pretreated with first- and second-order derivatives. This noise was especially pronounced in spectral regions below 1100 nm and above 2300 nm, this one coinciding with the peaks of fat, and protein (Barnes et al.,

1989). Several statistical treatments before application of derivative transformation and the use of a limited number of latent variables partially should overcome these issues. Furthermore, PLSR, despite using a limited number of LV, did not rely on actual values but rather on those calculated from the entire spectrum, which partially hindered the portability of equations to other NIR spectrometers with the same wavelength. Many researchers attempted to address these challenges, and one proposed solution was to reduce the variables used in the model. Eliminating variables constituting only noise and reducing the dimensionality of the model involved complex statistical systems developed in recent years (Xiaobo et al., 2010; Yun et al., 2019; de Araújo et al., 2022).

After defining the calibration models, we aimed to reduce the number of variables to improve model performance. The statistical evaluation of these new calibration and validation models was reported in table 6.

Table 6. Cross validation and prediction statistics for chemical composition (g/100 g of DM) of Total Mixed Rations (TMR) from both the calibration and validation set by NIRFlex 500 using only selected variables.

mg/100g	Calibration set with SV					Validation set with SV			
	SV	R ² cv	SECV	RPDcv	RERcv	R ² p	SEP	RPDp	RERp
DM	6	0.886	1.697	3.50	18.82	0.805	2.376	2.60	10.72
Ash	6	0.847	0.277	3.49	20.69	0.849	0.251	3.65	26.17
CP	8	0.905	0.524	3.57	18.36	0.887	0.701	2.69	10.24
EE	8	0.929	0.115	3.57	18.87	0.905	0.135	3.43	15.56
CF	7	0.942	1.190	4.67	16.16	0.875	1.855	3.17	11.58
aNDF	8	0.941	1.187	4.25	18.45	0.862	1.487	3.34	13.19
ADF	8	0.931	0.936	4.02	22.17	0.852	1.048	3.24	12.05
ADL	7	0.847	0.365	2.83	18.03	0.735	0.369	2.61	11.00
Starch	7	0.924	0.870	3.57	18.38	0.840	1.293	2.52	11.88

SV= selected variables; R²cv= Cross validation coefficient of determination on calibration set; SECV= Cross validation standard error on calibration set; R²p= prediction coefficient of determination on validation set; SEP= prediction standard error on validation set; RPDcv= SD/SEC; RPDp= SD/SEP; RERcv= Range (max-min value)/SEC; RERp= Range (max-min value)/SEP; DM= dry matter; CP= crude protein; EE= ethereal extract; CF= crude fibre; aNDF= Neutral Detergent fibre; ADF= Acid Detergent Fiber; ADL= Acid detergent lignin.

The PLSR model obtained with some selected variables (from 6 to 8), showed better performances compared to full spectrum in particular in C.V. models where CP, EE, CF, aNDF, ADF, and starch showed a R²cv > 0.9. Except for ADL, all parameters demonstrated RPDcv values greater than 3 (ranging from 3.49 to 4.67), and RERcv values exceeding 10 (ranging from 16.16 to 22.17).

The models developed with selected variables on the validation set demonstrated good predictive coefficients, with R²p values ranging from 0.805 to 0.905, except for ADL, which showed R²p=

0.735. All RPDp values considered were above 2.5, with ash, EE, CF, and aNDF exceeding 3.0. Additionally, the RERp values were all higher than 10, indicating a strong relationship between NIRS predictions and laboratory measurements.

Prediction model for FAs using the benchtop NIRFlex 500

For FAs, (Table 7) the outlier ranged from 3 to 7 for the calibration set, while in the validation set, they did not exceed 3 units for all the FAS analysed, with an average of 1.9.

Considering the PLSR models for FAs, the benchtop NIR system achieved good R^2 values during calibration for all FAs except for the 14:0, which showed $R^2 = 0.777$. Two of the polyunsaturated fatty acids (18:3 n3 and 20:4 n6), as well as the models for FA classes (except for PUFA n-6), showed $R^2 > 0.9$. Unfortunately, in cross-validation using the five-fold approach, the R^2_{cv} values decreased, and the SECV increased significantly compared to the SEC, revealing instability in the models.

Among the models in cross-validation, only 5 out of the 11 FAs achieved acceptable R^2_{cv} with values exceeding 0.8. Similarly, for FA classes, half of the models (SFA and PUFA n-6) did not yield satisfactory performance. Nevertheless, all RPDcv values, except for 16:0 and 20:0, exceeded 2.5, and the RERcv values were higher than 10 for all fatty acids, surpassing 20 for 18:1 cis 11 and 18:3 n-6.

The FAs classes exhibited lower performance, with particularly low RPDcv values, except for MUFA, which had RPD= 2.64. Additionally, the RERcv for PUFA n-3 was below 10, further highlighting the limitations in these models for about the 50% of the FA considered.

Table 7. Calibration, cross validation and prediction statistics for fatty acid composition (% of total fatty acids) of Total Mixed Rations (TMR) from both the calibration and validation set by NIRFlex 500, using the full NIR spectrum.

mg/100g	Calibration set								Validation set				
	LV	OUT	R ² c	SEC	R ² cv	SECV	RPDcv	RERcv	OUT	R ² p	SEP	RPDp	RERp
14:0	3	4	0.777	0.033	0.734	0.038	2.63	14.21	2	0.622	0.036	2.50	12.22
16:0	6	6	0.856	0.706	0.723	0.970	1.80	10.45	3	0.705	0.880	2.02	10.69
16:1	6	3	0.918	0.028	0.825	0.040	2.75	13.75	0	0.769	0.045	2.44	11.78
18:0	7	7	0.874	0.151	0.763	0.208	2.60	25.19	3	0.715	0.207	2.51	15.56
18:1 cis-9	7	6	0.853	0.553	0.769	0.714	2.69	16.82	2	0.747	0.845	2.52	12.60
18:1 cis-11	7	6	0.841	0.028	0.777	0.033	2.73	20.30	2	0.734	0.036	2.22	9.17
18:2 n-6	7	6	0.898	1.001	0.851	1.214	3.38	17.97	1	0.785	1.574	2.61	13.07
20:0	5	7	0.835	0.043	0.788	0.049	2.24	14.49	3	0.716	0.038	2.37	12.37
18:3 n-6	6	4	0.861	0.236	0.826	0.272	2.65	12.02	0	0.719	0.353	2.15	10.82
18:3 n-3	7	6	0.934	1.172	0.834	1.889	2.55	10.83	2	0.638	2.600	1.91	7.87
20:4 n-6	6	6	0.931	0.070	0.849	0.108	3.43	18.98	0	0.792	0.147	2.65	12.52
SFA	7	3	0.918	0.546	0.767	0.922	2.33	14.93	2	0.702	0.943	1.78	8.42
MUFA	6	3	0.900	0.471	0.835	0.613	2.64	11.62	2	0.732	0.818	2.20	8.90
PUFA n-6	7	4	0.895	1.032	0.778	1.597	2.35	12.46	3	0.639	1.812	1.95	9.50
PUFA n-3	7	4	0.928	1.318	0.813	2.191	2.25	9.33	3	0.689	2.524	1.81	8.11

LV= latent variable; OUT= outlier; R²c= coefficient of determination on calibration set; SEC= standard error on calibration set; R²cv= Cross validation coefficient of determination on calibration set; SECV= Cross validation standard error on calibration set; R²p= prediction coefficient of determination on validation set; SEP= prediction standard error on validation set; RPDcv= SD/SECV; RPDp= SD/SEP; RERcv= Range (max-min value)/SEC; RERp= Range (max-min value)/SEP; SFA= Saturated fatty acids; MUFA= Monounsaturated fatty acids; PUFA= Polyunsaturated fatty acids.

The FA models showed poor results when applied to an external dataset (validation set). No FAs reached even acceptable performance levels, with R²p ranging from 0.622 to 0.792. Similar results were highlighted for the FA classes where no PLSR models exceeded 2.5 value for RPDp, and 10 value for RERp.

When considering individual FAs, only 18:0, 18:1 cis-9, 18:2 n-6, and 20:4 n-6 surpassed the RPDp threshold of 2.5. These findings highlight the relatively low performance of NIR in measuring fatty acids.

To our knowledge, no studies have specifically developed NIR models for FA content in TMR. Some research on annual forages (Pereira-Crespo et al., 2014) has shown excellent calibration

results for only a few components, such as 18:3 n-3 ($R^2 = 0.93$, $SEC = 1.101$, $RER = 13.65$), total FA (TFA) ($R^2 = 0.89$, $SEC = 1.854$, $RER = 11.02$), and unsaturated FA (UFA) ($R^2 = 0.90$, $SEC = 1.431$, $RER = 11.46$). For the other FAs, their models were not satisfactory. Foster et al. (2006) reported impressive R^2c , ranging from 0.93 to 0.99, which suggests that the models developed have a strong fit to the data. Additionally, the reported values of the RPD greater than 3 indicate excellent predictive capability, further validating the effectiveness of their approach.

When PLSR models with selected variables (about 5 – 9 variables) were used for benchtop NIR spectra (Table 8), better R^2cv values were obtained, but also for these types of models no FAs exceeded 0.9. However, only the 14:0 recorded notably low values ($R^2cv = 0.791$). The $RPDcv$ for 14:0, 16:1, 18:0, 18:1 cis-9, 18:3 n-6, 20:4 n-6, and MUFA were higher compared to models obtained using the full spectrum, surpassing the threshold of 3. The $RERcv$ remained high, as observed in the full-spectrum models, ranging from 10.97 to 20.30. Furthermore, the $SECV$ of the models with selected variables was consistently lower than that of the full-spectrum models.

Table 8. Cross validation and prediction statistics for fatty acid composition (% of total fatty acids) of Total Mixed Rations (TMR) from both the calibration and validation set by NIRFlex 500 using only selected variables.

mg/100g	Calibration set with SV					Validation set with SV			
	SV	R^2cv	$SECV$	$RPDcv$	$RERcv$	R^2p	SEP	$RPDp$	$RERp$
14:0	6	0.791	0.029	3.34	18.62	0.693	0.034	2.56	12.94
16:0	8	0.855	0.656	2.66	15.53	0.831	0.638	2.78	14.75
16:1	9	0.893	0.035	3.11	15.71	0.817	0.039	2.77	13.59
18:0	8	0.830	0.172	3.15	30.47	0.712	0.212	2.54	15.19
18:1 cis-9	7	0.832	0.634	3.03	18.94	0.793	0.776	2.74	13.72
18:1 cis-11	6	0.775	0.033	2.85	20.30	0.748	0.035	2.14	9.43
18:2 n-6	7	0.844	1.417	2.89	15.40	0.802	1.601	2.57	12.85
20:0	7	0.820	0.044	2.41	16.14	0.781	0.041	2.20	11.46
18:3 n-6	8	0.873	0.235	3.06	13.91	0.832	0.291	2.63	13.13
18:3 n-3	8	0.834	1.864	2.58	10.97	0.788	1.895	2.62	10.80
20:4 n-6	7	0.880	0.104	3.55	19.71	0.823	0.125	3.10	14.72
SFA	8	0.834	0.771	2.79	17.86	0.709	0.752	2.23	10.56
MUFA	7	0.870	0.535	3.02	13.31	0.778	0.754	2.38	9.66
PUFA n-6	8	0.822	1.365	2.76	14.58	0.721	1.634	2.17	10.53
PUFA n-3	8	0.864	1.791	2.74	11.42	0.784	2.338	1.96	8.76

SV= selected variables; R^2cv = Cross validation coefficient of determination on calibration set; $SECV$ = Cross validation standard error on calibration set; R^2p = prediction coefficient of determination on validation set; SEP= prediction standard error on validation set; $RPDcv$ = $SD/SECV$; $RPDp$ = SD/SEP ; $RERcv$ = Range (max-min value)/ SEC ; $RERp$ = Range (max-min value)/ SEP ; SFA= Saturated fatty acids; MUFA= Monounsaturated fatty acids; PUFA= Polyunsaturated fatty acids.

The FA models that benefit from reducing the number of variables, thereby eliminating excessive noise compared to the full-spectrum model, were 16:0, 18:0, and 18:1 cis-9. However, the improved performances of these models were not confirmed when the PLSR models were applied to an external dataset (validation set). In this context, only 16:0, 16:1, 18:2 n-6, 18:3 n-6 and 20:4 n-6 achieve R^2 values greater than 0.8, and only the latter fatty acid has an RPDp value exceeding 3.

Prediction model for chemical analysis using the portable PoliSPEC^{NIR}

The spectral range of the portable NIR extends from 900 to 1700 nm, capturing only 260 spectral points within this range. Pre-treatment methods involving derivations significantly enhanced model performance during calibration.

The number of LV used ranged from 5 to 7 (Table 9). On average, 6 outliers were removed during calibration, while 3 outliers were excluded from the validation set to optimize the PLSR models.

The PLSR models derived from the portable NIR spectra achieved R^2 values above 0.9 for most parameters studied, except for ash, aNDF, and ADL. However, during cross-validation, model performance normally declined significantly, with R^2_{cv} values exceeding 0.8 for only five compounds. Additionally, only four RPD_{cv} values surpassed the threshold of 2.5, and the RER_{cv} consistently remained above 10, except for CF. The SECV increased compared to the calibration phase.

Table 9. Calibration, cross validation and prediction statistics for chemical composition (g/100 g of DM) of Total Mixed Rations (TMR) from both the calibration and validation set by portable PoliSPEC^{NIR}, using the full NIR spectrum.

mg/100g	Calibration set									Validation set			
	LV	OUT	R^2_c	SEC	R^2_{cv}	SECV	RPD _{cv}	RER _{cv}	OUT	R^2_p	SEP	RPD _p	RER _p
DM	6	8	0.923	1.519	0.886	1.889	3.15	16.90	2	0.848	2.303	2.68	11.06
Ash	6	10	0.812	0.288	0.701	0.370	2.62	15.49	4	0.704	0.368	2.50	18.56
CP	7	6	0.941	0.482	0.815	0.911	2.05	10.56	3	0.802	0.813	2.32	8.83
EE	7	10	0.954	0.0712	0.794	0.153	2.68	14.18	4	0.683	0.245	1.88	8.57
CF	7	0	0.956	1.022	0.850	1.939	2.85	9.92	3	0.793	2.136	2.75	10.06
aNDF	7	8	0.872	1.630	0.820	2.029	2.49	10.79	3	0.767	2.623	1.89	7.48
ADF	6	3	0.930	0.937	0.797	1.595	2.34	13.01	2	0.810	1.252	2.72	10.09
ADL	5	4	0.840	0.377	0.709	0.513	2.01	12.83	3	0.794	0.401	2.47	10.12
Starch	6	5	0.926	0.803	0.815	1.314	2.37	12.17	3	0.788	1.521	2.14	10.10

LV= latent variable; OUT= outlier; R^2_c = coefficient of determination on calibration set; SEC= standard error on calibration set; R^2_{cv} = Cross validation coefficient of determination on calibration set; SECV= Cross validation standard error on calibration set; R^2_p = prediction coefficient of determination on validation set; SEP= prediction standard error on validation set; RPD_{cv}= SD/SECV; RPD_p= SD/SEP; RER_{cv}= Range (max-min value)/SECV; RER_p= Range (max-min value)/SEP; DM= dry matter; CP= crude protein; EE= ethereal extract; CF= crude fibre; aNDF= Neutral Detergent fibre; ADF= Acid Detergent Fiber; ADL= Acid detergent lignin.

When these models were tested using an external validation set, only two compounds exhibited acceptable performance: DM ($R^2_p=0.848$, $RPD_p=2.68$, and $RER_p=11.06$), and ADF ($R^2_p=0.810$, $RPD_p=2.72$ and $RER_p=10.09$).

Using only 6–8 variables from the entire dataset (Table 10), the PLSR models in cross-validation demonstrated significantly improved performance, except for ash, which maintained an R^2_{cv} below the 0.8 threshold. In cross-validation, five compounds achieved R^2_{cv} values exceeding 0.9, classifying these models as "good," with four parameters with $RPD_{cv} > 3.5$. Notably, DM, CP, CF, ADF and starch showed strong predictive performance, consistent with results observed in models developed with selected variables from the benchtop NIR. Using the PLSR models with validation set promising results for DM, CP, and CF, were obtained achieving $R^2_p > 0.9$, $RPD_p > 2.5$, and RER_p values above 10. However, beyond these compounds, the PLSR models validated with an external dataset demonstrated unsatisfactory performance, particularly for EE.

Table 10. Cross validation and prediction statistics for chemical composition (g/100 g of DM) of Total Mixed Rations (TMR) from both the calibration and validation set by portable PolISPEC^{NIR} using only selected variables.

mg/100g	Calibration set with SV					Validation set with SV			
	SV	R^2_{cv}	SECV	RPD_{cv}	RER_{cv}	R^2_p	SEP	RPD_p	RER_p
DM	6	0.916	1.497	3.97	21.33	0.904	1.985	3.11	13.34
Ash	7	0.795	0.308	3.14	18.60	0.779	0.321	2.85	21.28
CP	8	0.919	0.555	3.37	17.33	0.905	0.655	2.88	10.96
EE	7	0.832	0.143	2.87	15.17	0.682	0.218	2.12	9.63
CF	8	0.926	1.322	4.18	14.55	0.927	1.809	2.54	11.87
aNDF	8	0.862	1.977	2.56	11.08	0.758	2.372	2.09	8.27
ADF	8	0.915	1.077	3.47	19.27	0.759	1.539	2.21	8.21
ADL	7	0.895	0.285	3.62	23.09	0.813	0.386	2.58	10.52
Starch	7	0.913	0.902	3.44	17.73	0.702	1.570	2.08	9.78

SV= selected variables; R^2_{cv} = Cross validation coefficient of determination on calibration set; SECV= Cross validation standard error on calibration set; R^2_p = prediction coefficient of determination on validation set; SEP= prediction standard error on validation set; RPD_{cv} = $SD/SECV$; RPD_p = SD/SEP ; RER_{cv} = Range (max-min value)/SECV; RER_p = Range (max-min value)/SEP; DM= dry matter; CP= crude protein; EE= ethereal extract; CF= crude fibre; aNDF= Neutral Detergent fibre; ADF= Acid Detergent Fiber; ADL= Acid detergent lignin.

Prediction model for FAs using the portable PolISPEC^{NIR}

The PLSR models for FAs were detailed in Tables 11 and 12. The number of LV ranged from 2 to 7, while the number of outliers per FA model varied from 3 to 14, with an average of 7.8 outliers per model. This excessive presence of outliers indicates that many data points are not adequately explained by the models. Despite this limitation, and during calibration, 50% of the fatty acids

considered achieved R^2 values exceeding 0.8. However, the remaining FAs showed R^2 values ranging from 0.481 to 0.559.

In cross-validation, the R^2_{cv} values further decreased, and the SECV increased. None of the tested models exceeded the R^2 threshold of 0.8. In these cases, the models failed to effectively extract useful information. Only models with an R^2_{cv} value greater than 0.65 were tested with an external validation set, but all tested cases yielded unsatisfactory predictions.

Table 11. Calibration, cross validation and prediction statistics for fatty acid composition (% of total fatty acids) of Total Mixed Rations (TMR) from both the calibration and validation set by portable PolISPEC^{NIR}, using the full NIR spectrum.

mg/100g	Calibration set								Validation set				
	LV	OUT	R^2_c	SEC	R^2_{cv}	SECV	RPD _{cv}	RER _{cv}	OUT	R^2_p	SEP	RPD _p	RER _p
14:0	2	6	0.567	0.052	0.538	0.054	1.85	10.00	-	-	-	-	-
16:0	2	8	0.707	0.827	0.620	0.959	1.82	10.63	-	-	-	-	-
16:1	7	6	0.878	0.025	0.677	0.041	2.68	13.41	3	0.680	0.043	2.56	12.33
18:0	5	7	0.059	0.166	0.636	0.207	2.61	25.31	-	-	-	-	-
18:1 cis-9	7	9	0.853	0.553	0.742	0.730	2.63	16.45	4	0.544	1.064	2.00	10.01
18:1 cis-11	7	10	0.816	0.033	0.722	0.041	2.20	16.34	5	0.548	0.042	1.90	7.86
18:2 n-6	7	6	0.896	1.124	0.745	1.858	2.21	11.74	4	0.639	1.816	2.26	11.33
20:0	5	14	0.481	0.043	0.358	0.048	2.29	14.79	-	-	-	-	-
18:3 n-6	7	8	0.844	0.281	0.732	0.376	1.91	8.70	4	0.516	0.439	1.73	8.70
18:3 n-3	5	10	0.851	1.481	0.765	1.892	2.55	10.81	2	0.737	1.913	2.60	10.70
20:4 n-6	2	10	0.664	0.070	0.618	0.144	2.57	14.24	-	-	-	-	-
SFA	2	10	0.632	1.035	0.621	1.067	2.01	12.91	-	-	-	-	-
MUFA	3	5	0.738	0.784	0.727	0.838	1.93	8.50	3	0.524	1.217	1.48	5.98
PUFA n-6	4	6	0.872	0.0992	0.663	1.638	2.30	12.15	4	0.507	2.095	1.69	8.21
PUFA n-3	7	3	0.883	1.505	0.739	2.331	2.11	8.77	4	0.517	2.197	2.08	9.32

LV= latent variable; OUT= outlier; R^2_c = coefficient of determination on calibration set; SEC= standard error on calibration set; R^2_{cv} = Cross validation coefficient of determination on calibration set; SECV= Cross validation standard error on calibration set; R^2_p = prediction coefficient of determination on validation set; SEP= prediction standard error on validation set; RPD_{cv}= SD/SECV; RPD_p= SD/SEP; RER_{cv}= Range (max-min value)/SECV; RER_p= Range (max-min value)/SEP; SFA= Saturated fatty acids; MUFA= Monounsaturated fatty acids; PUFA= Polyunsaturated fatty acids.

For the portable NIR, PLSR models developed with selected variables (Table 12) (6–10 variables) did not significantly improve R^2_{cv} , except for 18:1 cis-11, 18:2 n-6, 18:3 n-6, and 18:3 n-3, which exceeded the 0.8 threshold. Although the RPD_{cv} showed some improvement, it did not surpass the threshold of 3, and the RER_{cv} remained above 10 for all FAs except for 16:0 (9.82), 20:4 n-6 (8.76), MUFA (9.08), and PUFA n-3 (9.98). Lastly, the $SECV$ did not decrease compared to the full-spectrum model for most of the FAs considered. While the reduced-variable models demonstrated improved performance in certain aspects, they still fell short of expectations.

Table 12. Cross validation and prediction statistics for fatty acid composition (% of total fatty acids) of Total Mixed Rations (TMR) from both the calibration and validation set by portable PoliSPEC^{NIR}, using only selected variables.

mg/100g	Calibration set with SV					Validation set with SV			
	SV	R^2_{cv}	$SECV$	RPD_{cv}	RER_{cv}	R^2_p	SEP	RPD_p	RER_p
14:0	6	0.521	0.042	2.31	12.86	0.353	0.060	1.45	7.33
16:0	5	0.661	1.038	1.68	9.82	0.435	1.115	1.59	8.44
16:1	8	0.791	0.036	2.96	14.95	0.726	0.052	2.08	10.23
18:0	8	0.747	0.185	2.93	28.34	0.527	0.230	2.34	14.00
18:1 cis-9	7	0.672	0.896	2.15	13.40	0.659	0.916	2.32	11.63
18:1 cis-11	7	0.817	0.034	2.76	19.65	0.679	0.043	1.76	7.73
18:2 n-6	7	0.810	1.668	2.46	13.08	0.767	1.770	2.32	11.62
20:0	6	0.485	0.065	1.64	10.97	0.232	0.062	1.45	7.58
18:3 n-6	7	0.834	0.302	2.38	10.83	0.632	0.401	1.91	9.53
18:3 n-3	7	0.854	1.605	3.00	12.74	0.670	2.145	2.32	9.54
20:4 n-6	6	0.639	0.234	1.58	8.76	0.428	0.270	1.44	6.81
SFA	5	0.637	1.073	2.00	12.83	0.631	1.006	1.67	7.89
MUFA	5	0.739	0.784	2.06	9.08	0.602	0.907	1.98	8.03
PUFA n-6	5	0.629	1.832	2.05	10.86	0.678	1.753	2.02	9.82
PUFA n-3	8	0.771	2.050	2.40	9.98	0.774	1.358	2.60	11.64

SV= selected variables; R^2_{cv} = Cross validation coefficient of determination on calibration set; $SECV$ = Cross validation standard error on calibration set; R^2_p = prediction coefficient of determination on validation set; SEP= prediction standard error on validation set; RPD_{cv} = $SD/SECV$; RPD_p = SD/SEP ; RER_{cv} = Range (max-min value)/ SEC ; RER_p = Range (max-min value)/ SEP ; SFA= Saturated fatty acids; MUFA= Monounsaturated fatty acids; PUFA= Polyunsaturated fatty acids.

The analysis of nutrients in mixed feeds, such as TMR, using NIRS presents several challenges due to the variety of ingredients involved (White and Rouvinen-Watt, 2004). The spectral patterns of these feeds are highly variable, as each ingredient has a distinct spectral signature determined by its chemical and physical properties (Givens et al., 1997). Additionally, the physical characteristics of the samples, particularly the presence of unground particles (e.g., flakes, pellets, whole forages), can complicate analysis by affecting spectral baselines. The inherent variability of

unground particles poses significant challenges in obtaining representative samples (Brimmer et al., 2001).

When developing NIRS calibration models, it is essential to collect samples that are both relevant and representative of TMR. This requires careful consideration of all potential sources of variability, including formulation-related factors (e.g., concentration ranges of components), physical characteristics (e.g., particle size), and distribution issues (e.g., blending) (Buonaiuto et al., 2021). A large calibration set that maximizes variability in both parameters and ingredients is essential for achieving reliable calibration outcomes. In addition, the method of data acquisition plays a significant role in the accuracy of the results, as noted by Yamada et al. (2024) in their study on portable NIRS instruments. While portable NIRS devices hold promise for the rapid assessment of forage quality under field conditions, it is crucial to carefully consider the scanning methodology to ensure the development of accurate predictive models (Walelegne et al., 2023).

CONCLUSIONS

The study highlights the limitations of the portable NIRS device in accurately predicting the chemical composition of TMR directly on the farm for dairy buffaloes. The portable NIRS exhibited low accuracy, particularly for fiber fractions and ether extract, with lower R^2 values and higher prediction errors. Additionally, its limited spectral range (900–1700 nm) further restricted its predictive capacity, reducing its ability to detect specific chemical bonds essential for detailed feed nutrient analysis.

The benchtop NIRS, which analysed dried and ground samples, demonstrated relatively good predictive performance for some chemical parameters. However, no models for this device achieved an R^2 greater than 0.9 when tested with an external dataset. Fatty acid prediction was generally weak for both instruments, particularly for the portable NIRS, which was further affected by spectral interference due to the high moisture content of fresh samples. Although variable selection improved some models, especially for the benchtop NIRS, where the ether extract model reached $R^2 = 0.9$ when validated with an external dataset, FA prediction remained unreliable for both instruments. This limitation was particularly pronounced for the portable NIRS, which exhibited consistently poor predictive performance across all FA considered.

These findings suggest that while NIRS may be useful for basic TMR composition assessments, its application for detailed FA prediction requires significant refinement. This underscores, once again, that the efficiency of the instrument and the reproducibility of the analysis are improved by

the accuracy of the data collected, the size of the sample set, and the application of appropriate chemometric techniques.

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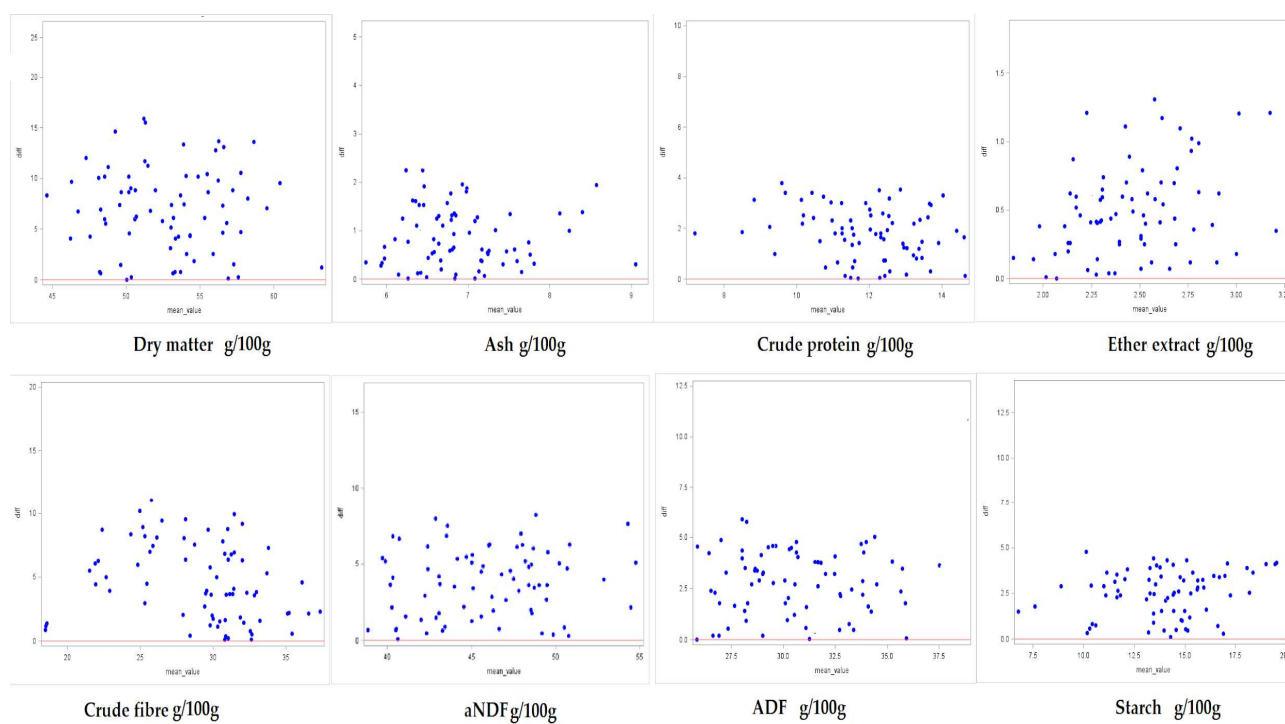
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SUPPLEMENTARY FILES



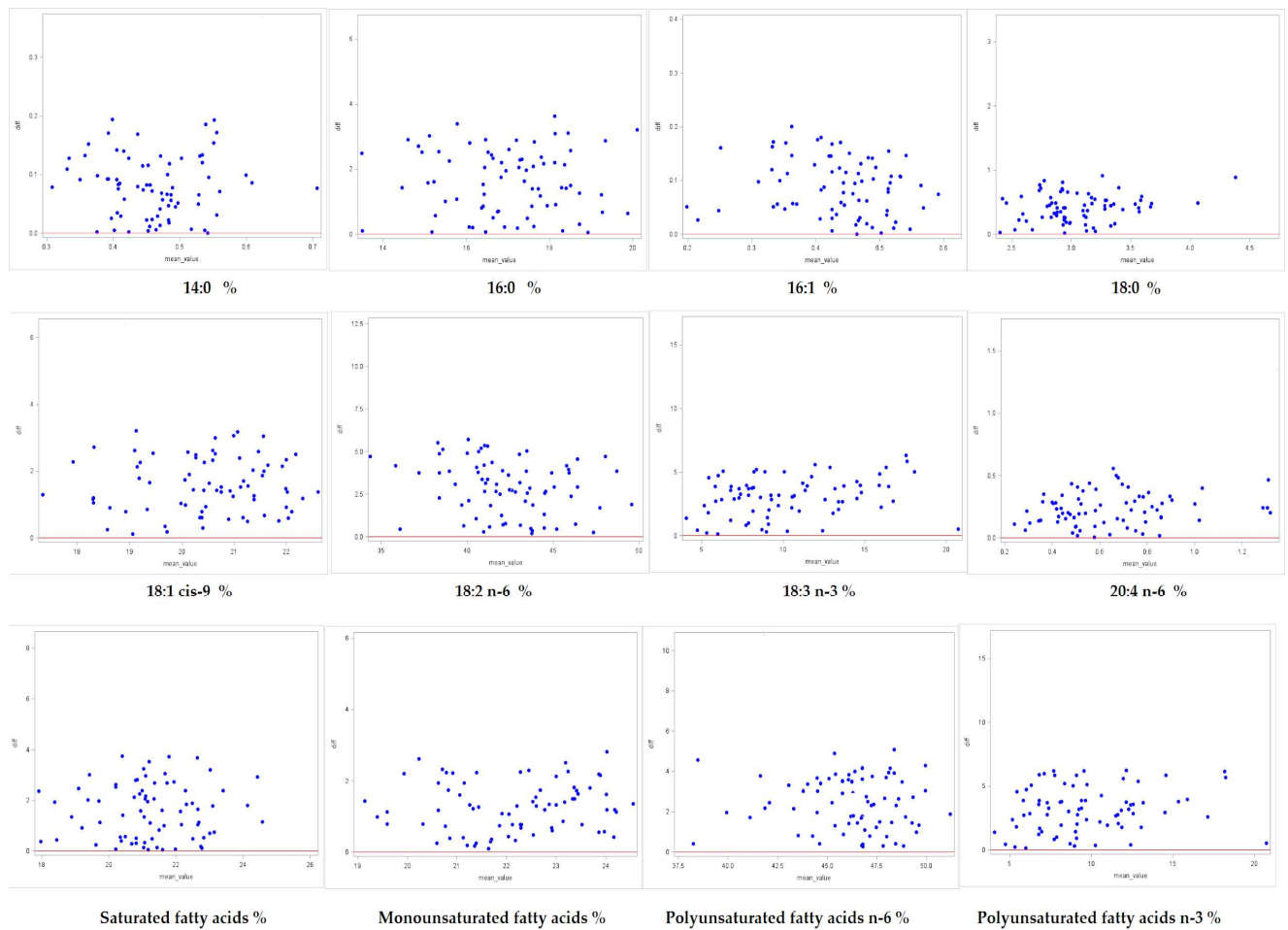


Figure S1: Distribution of differences in absolute value between proximate composition, fibres, fatty acids and classes of fatty acids of each sample and his replication, expressed as percentage.

Table S2a Percentage of values considered different because exceed the standard deviation in absolute value, and the Compound Symmetry (CS) describing the relationships among repeated measures (proximate composition and fiber).

Compound (g/100g)	Dry Matter	Ash	Crude Protein	Ether extract	Crude fibre	aNDF	ADF	ADL	Starch
% of Samples different	46	44	50	56	49	49	55	51	71
CS	0.110	0.133	0.103	0.059	0.089	0.084	0.054	0.063	0.057

aNDF: Neutral Detergent fibre; ADF: Acid Detergent Fiber; ADL: Acid detergent lignin.

Table S2b Percentage of values considered different because exceed the standard deviation in absolute value, and the Compound Symmetry (CS) describing the relationships among repeated measures (fatty acid and principal class of fatty acids).

%	14:00	16:00	16:1	18:00	18:1 cis-9	18:1 cis-11	18:2 n-6	20:00	18:3 n-6	18:3 n-3	20:4 n-6	SFA	MUFA	PUFA n-6	PUFA n-3
% of Samples different	56	46	50	47	57	52	55	51	54	63	49	53	46	47	56
CS	0.068	0.122	0.110	0.105	0.045	0.073	0.093	0.109	0.081	0.040	0.122	0.076	0.156	0.110	0.044

SFA= saturated fatty acids, MUFA= monounsaturated fatty acids; PUFA n-6= polyunsaturated fatty acids omega 6; PUFA n-3= Polyunsaturated fatty acids omega 3.

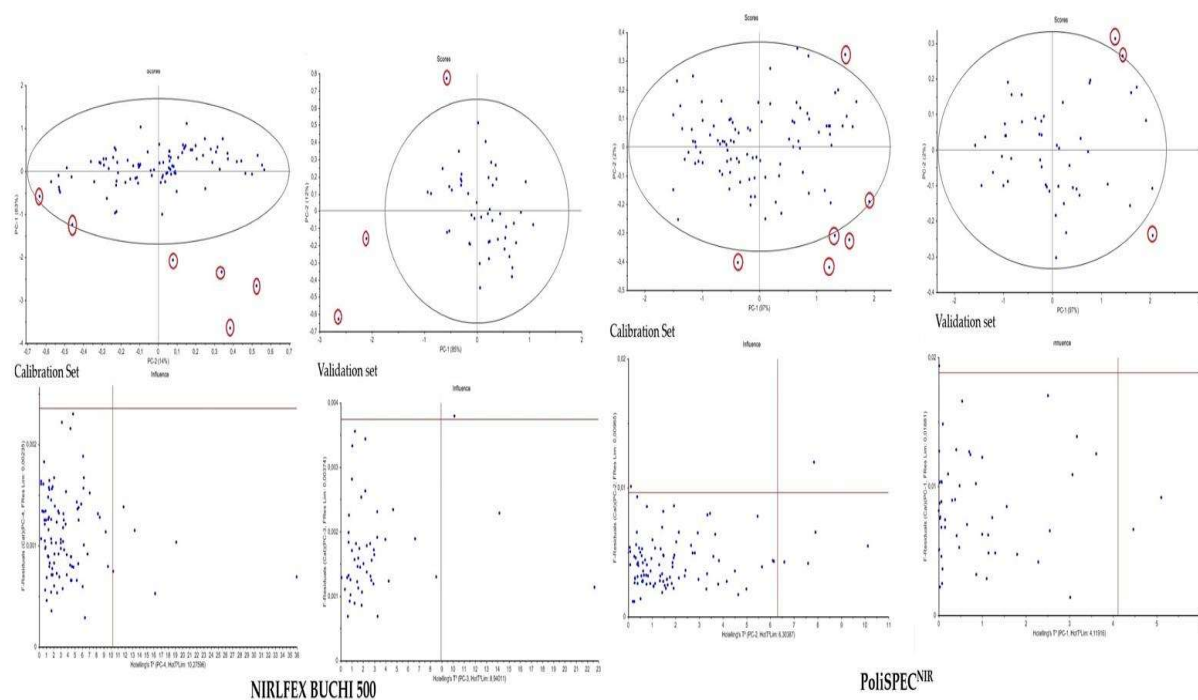


Figure S2: Outlier detection in calibration and validation sets using NIRFlex 500 and PoliSPEC^{NIR} instruments.

Table S3a. Spectral treatments, outlier handling, and latent variables (LV) used in the models for predicting the chemical composition of Total Mixed Rations (TMR).

	Baseline correction	Spectra normalization	Scatter correction	Mathematical treatment	Outli er	L V
DM ¹	-	-	-	SG-1-3-7	3	5
DM ²	-	Area	-	-	8	6
Ash ¹	-	Area	MSC	-	6	4
Ash ²	-	Area	MSC	-	10	6
CP ¹	-	-	SNV+D	SG-1-3-7	5	6
CP ²	-	-	-	SG-1-3-7	6	7
EE ¹	Offset	Area	-	-	0	7
EE ²	-	-	-	SG-1-3-7	10	7
CF ¹	-	-	D	SG-1-2-5	0	7
CF ²	-	-	SVN	SG-1-3-7	0	7
aNDF ¹	Offset	-	-	SG-1-2-5	0	7
aNDF ²	-	Area	MSC	-	8	7
ADF ¹	-	-	SNV+D	SG-1-3-7	2	7
ADF ²	-	-	SNV	SG-1-2-5	3	6
ADL ¹	Offset	-	-	SG-1-2-5	5	5

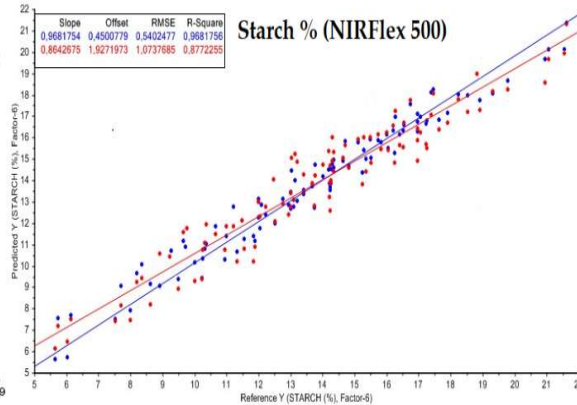
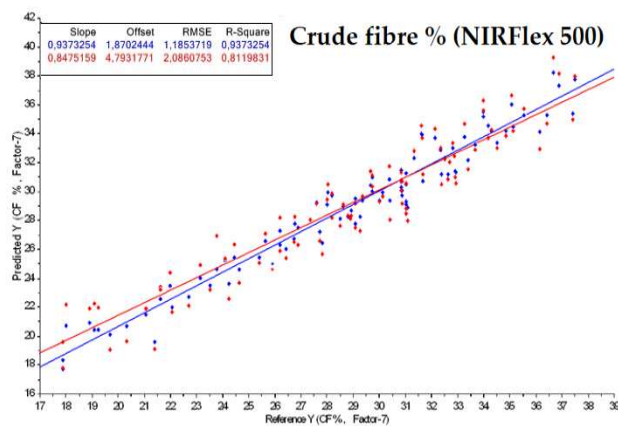
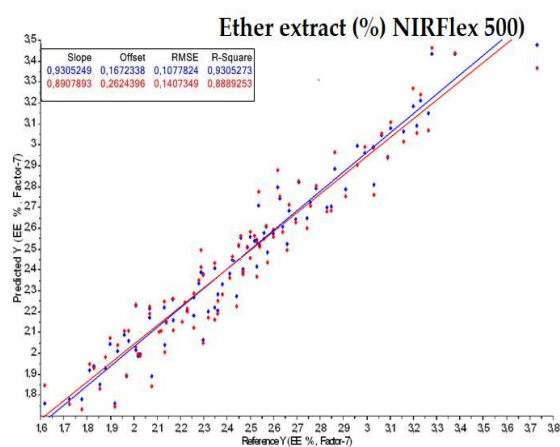
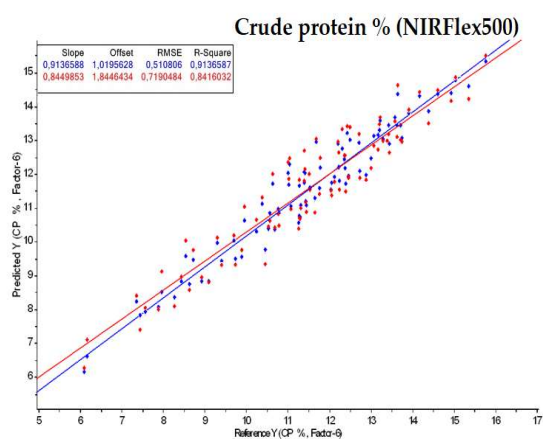
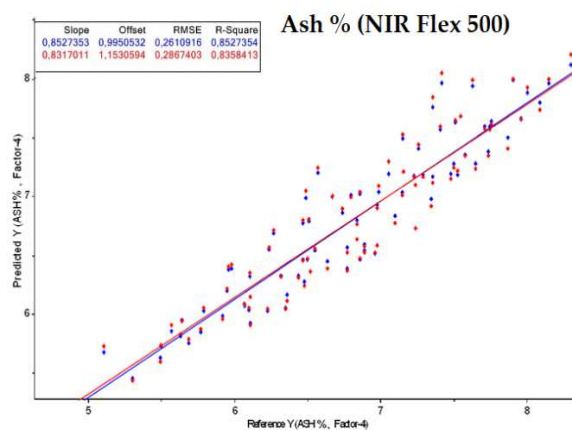
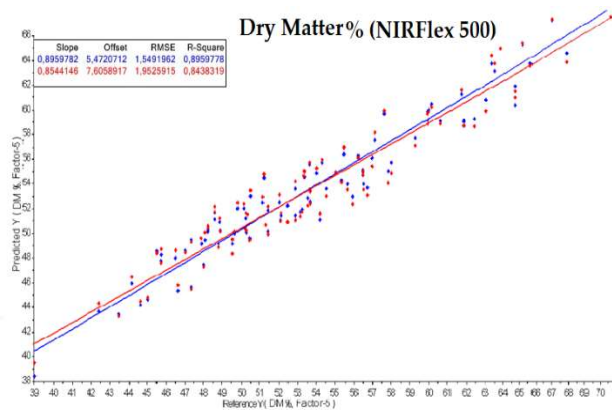
ADL ²	-	-	SNV+D	SG-1-3-7	4	5
Starch ¹	-	-	-	SG-2-2-5	2	6
Starch ²	-	-	SNV+D	SG-1-3-7	5	6

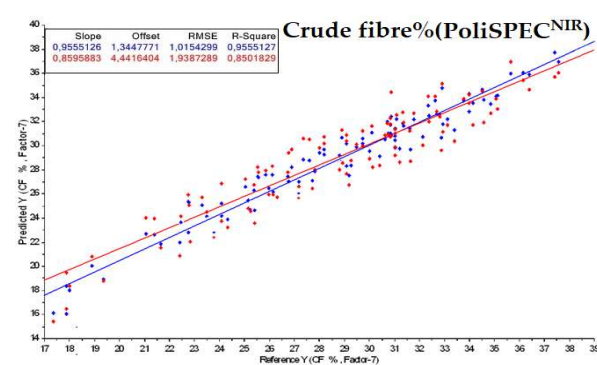
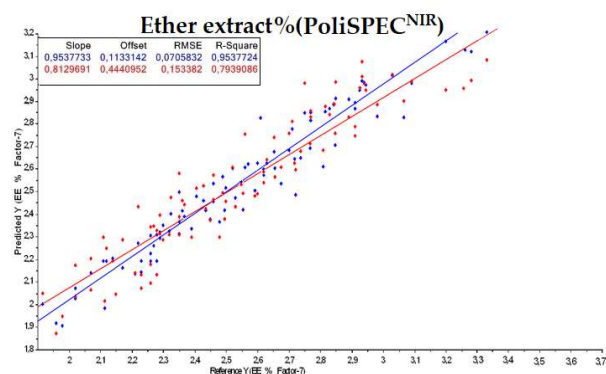
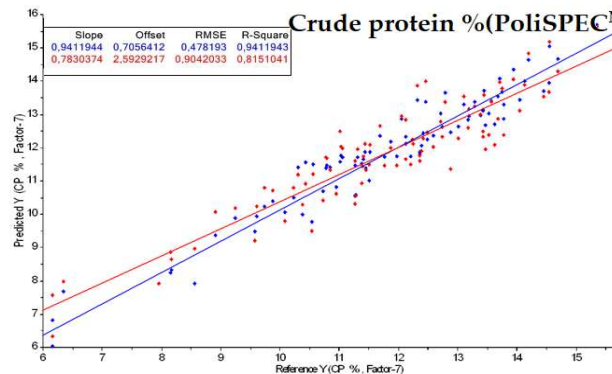
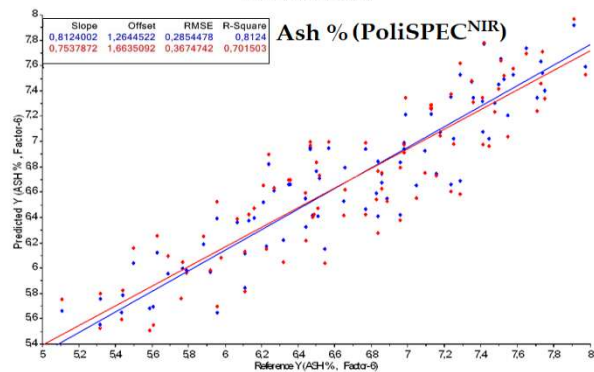
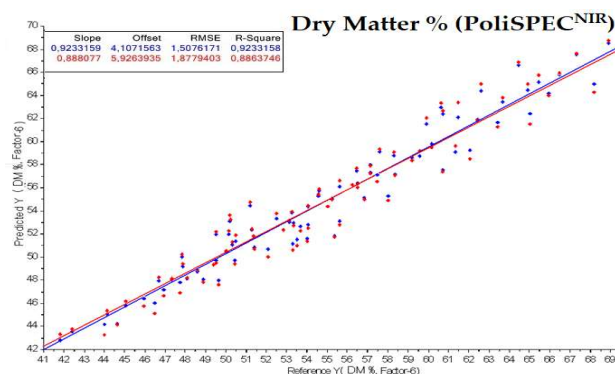
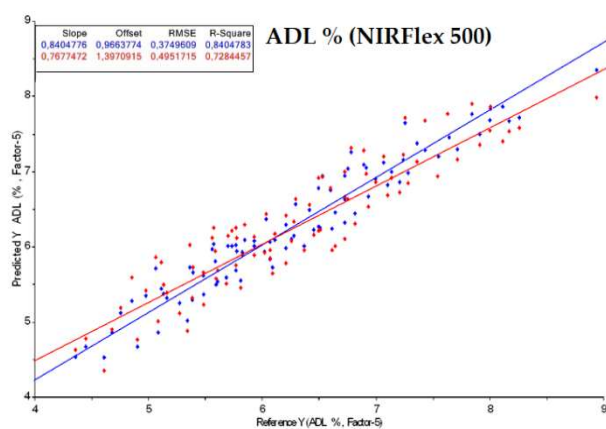
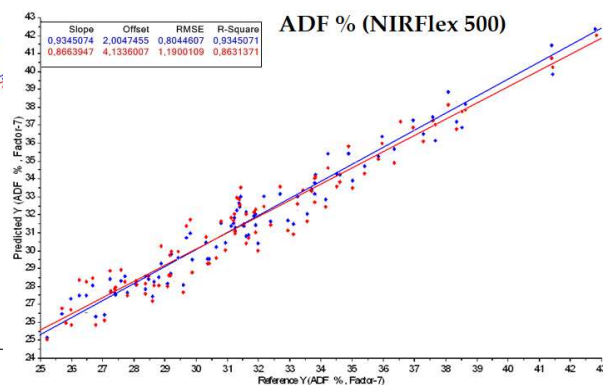
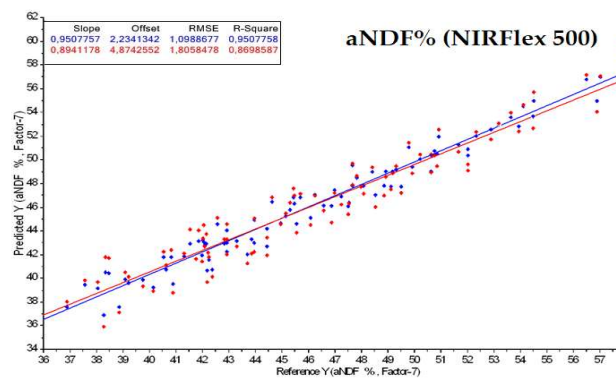
¹ Benchtop NIRFlex 500; ² Portable NIR PoliSPEC^{NIR}; LV: latent variables of model; MSC: multiplicative scatter correction; SNV: standard normal variate; D: detrending; SG: Savitzky-Golay derivative (the numbers indicated: – derivative order – polynomial order–smoothing points); DM: dry matter; CP: crude protein; EE: ethereal extract; CF: crude fibre; aNDF: Neutral Detergent fibre; ADF: Acid Detergent Fiber; ADL: Acid detergent Lignin.

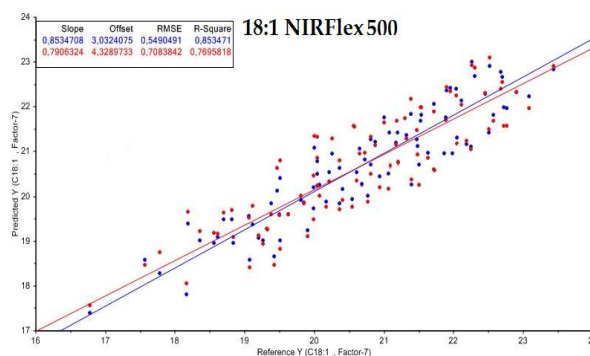
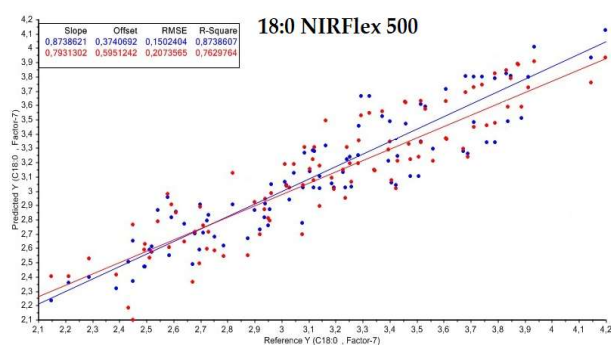
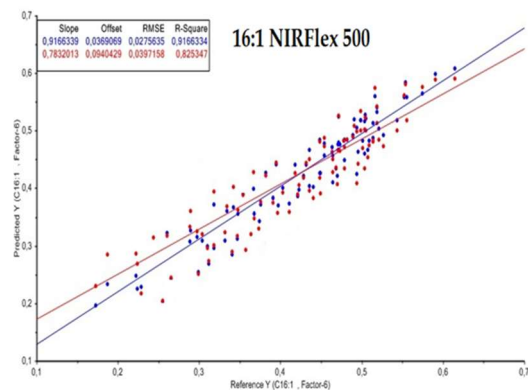
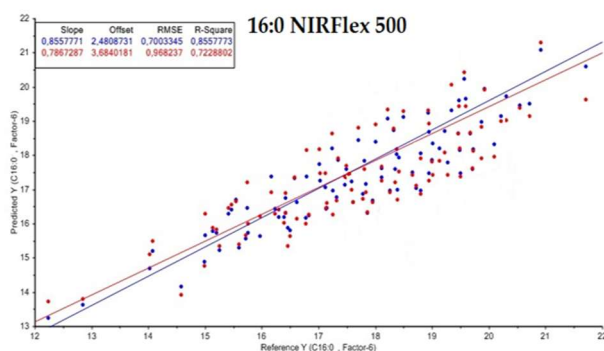
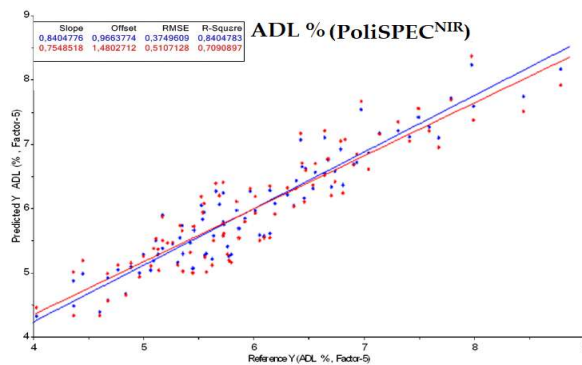
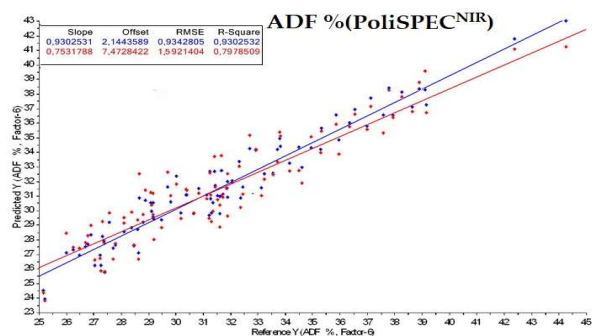
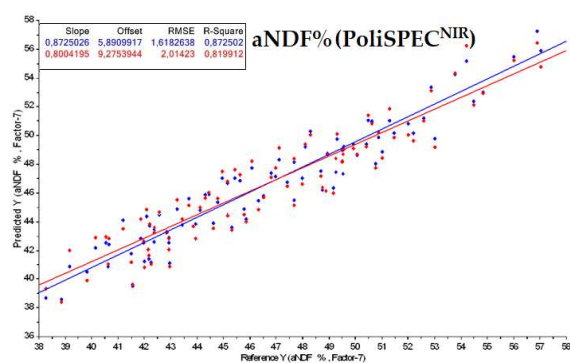
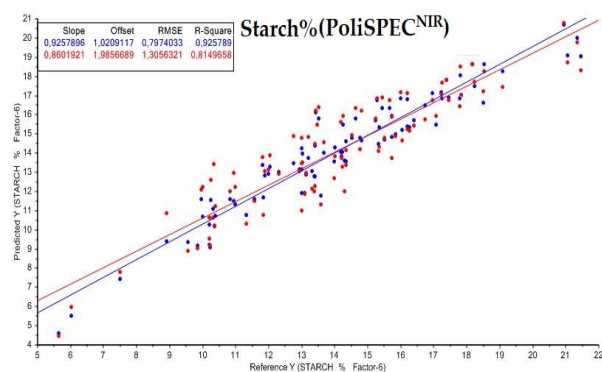
Table S3b. Spectral treatments, outlier handling, and latent variables (LV) used in the models for predicting the fatty acid composition of Total Mixed Rations (TMR).

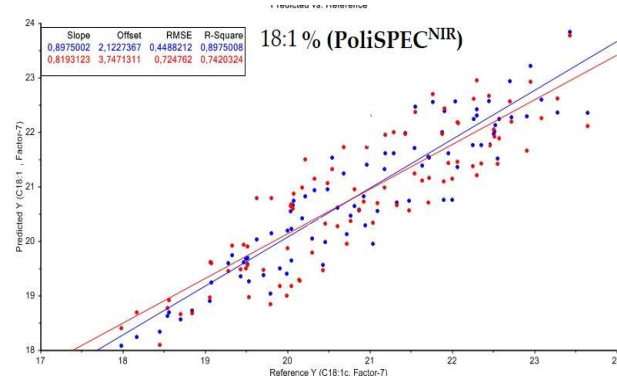
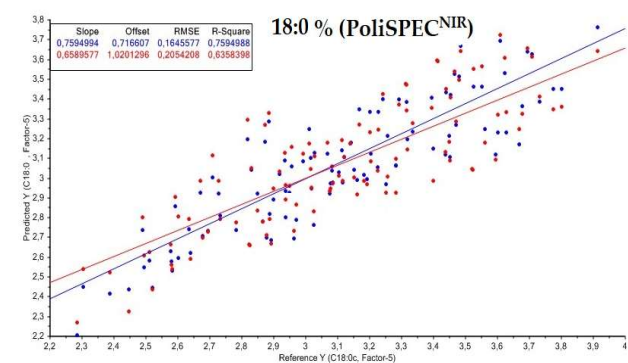
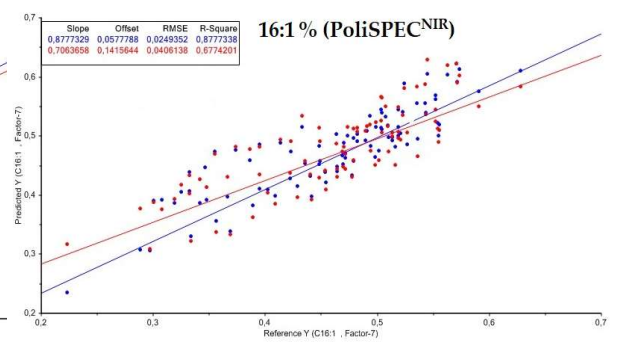
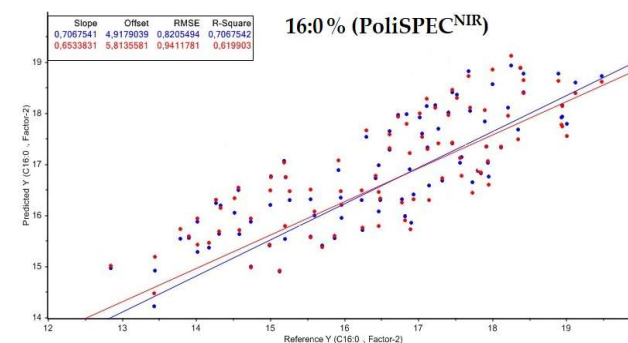
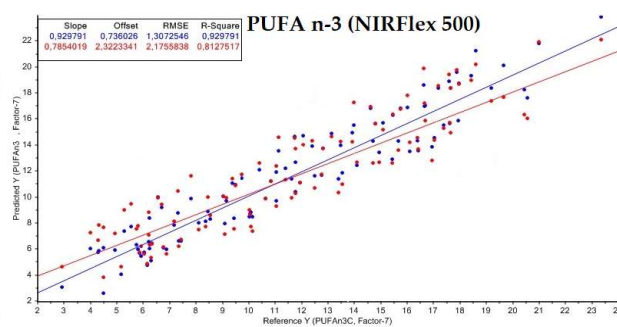
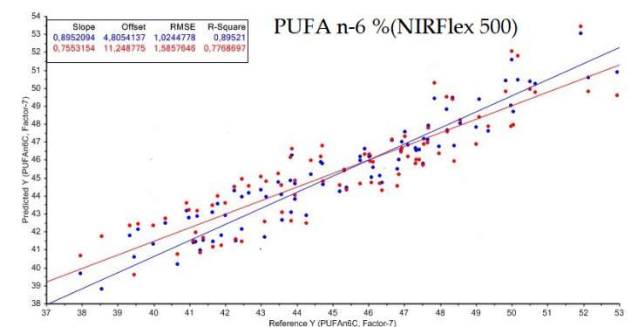
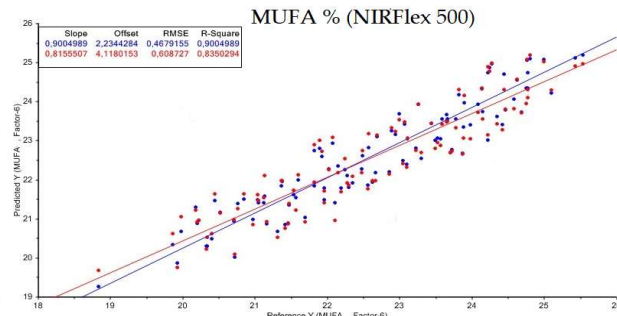
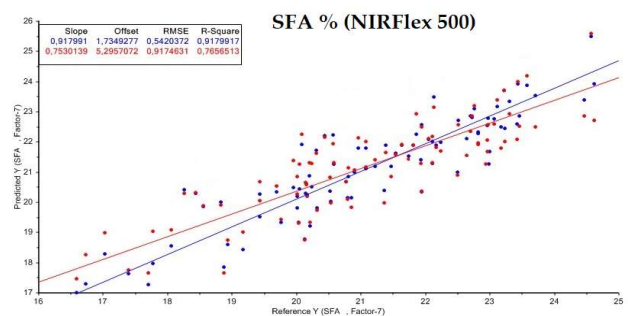
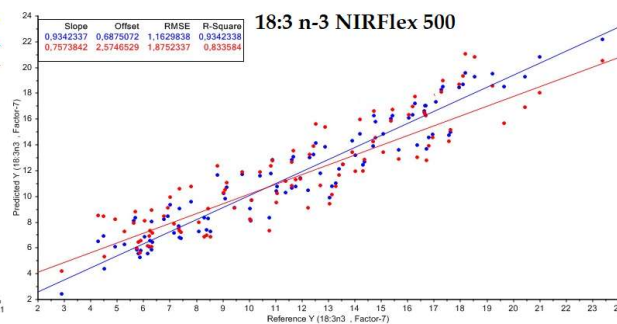
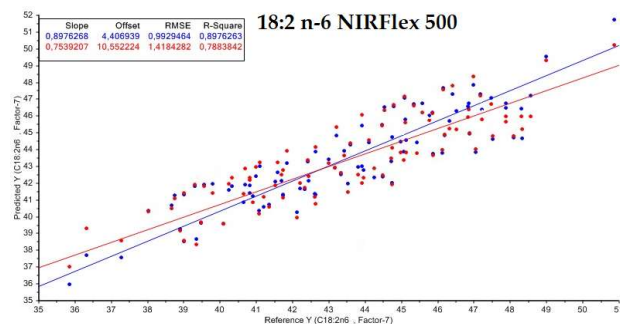
	Baseline correction	Spectra normalization	Scatter correction	Mathematical treatment	Outli er	<i>L</i> <i>V</i>
14:0 ¹	-	Area	-	-	4	3
14:0 ²	-	-	-	SG-1-3-7	6	2
16:0 ¹	-	-	D	SG-1-2-5	6	6
16:0 ²	-	-	-	SG-2-2-5	8	2
16:1 ¹	-	-	SNV+D	SG-1-3-7	3	6
16:1 ²	-	-	-	SG-1-3-7	6	7
18:0 ¹	Offset	Area	-	-	7	7
18:0 ²	-	-	-	SG-1-3-7	7	5
18:1 cis-9 ¹	Offset	Area	-	-	6	7
18:1 cis-9 ²	-	Area	SVN	SG-2-2-5	9	7
18:1 cis-11 ¹	-	Area	MSC	-	6	7
18:1 cis-11 ²	Offset	Area	-	-	10	7
18:2 n-6 ¹	-	-	-	SG-1-2-5	6	7
18:2 n-6 ²	-	-	-	SG-1-3-7	6	7
20:0 ¹	Offset	Area	MSC	-	7	5
20:0 ²	-	-	-	-	14	5
18:3 n-6 ¹	-	Area	SVN	-	4	6
18:3 n-6 ²	-	Area	MSC	-	8	7
18:3 n-3 ¹	Offset	-	-	SG-1-3-7	6	7
18:3 n-3 ²	-	-	SNV+D	SG-1-3-7	10	5
20:4 n-6 ¹	Offset	-	SNV	SG-1-3-7	6	6
20:4 n-6 ²	-	Area	-	-	10	2
SFA ¹	-	-	-	SG-1-3-7	3	7
SFA ²	-	Area	SNV+D	-	10	2
MUFA ¹	-	-	-	SG-1-2-5	3	6
MUFA ²	-	-	-	SG-1-2-5	5	3
PUFA n-6 ¹	-	-	-	SG-1-2-5	4	7
PUFA n-6 ²	-	-	-	SG-2-2-5	6	4
PUFA n-3 ¹	Offset	-	-	SG-1-2-5	4	7
PUFA n-3 ²	-	-	SNV+D	SG-1-3-7	3	7

¹Benchtop NIRFlex 500; ² Portable NIR PoliSPEC^{NIR}; LV: latent variables of model; MSC: multiplicative scatter correction; SNV: standard normal variate; D: detrending; SG: Savitzky-Golay derivative (the numbers indicated: – derivative order – polynomial order–smoothing points); SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids.









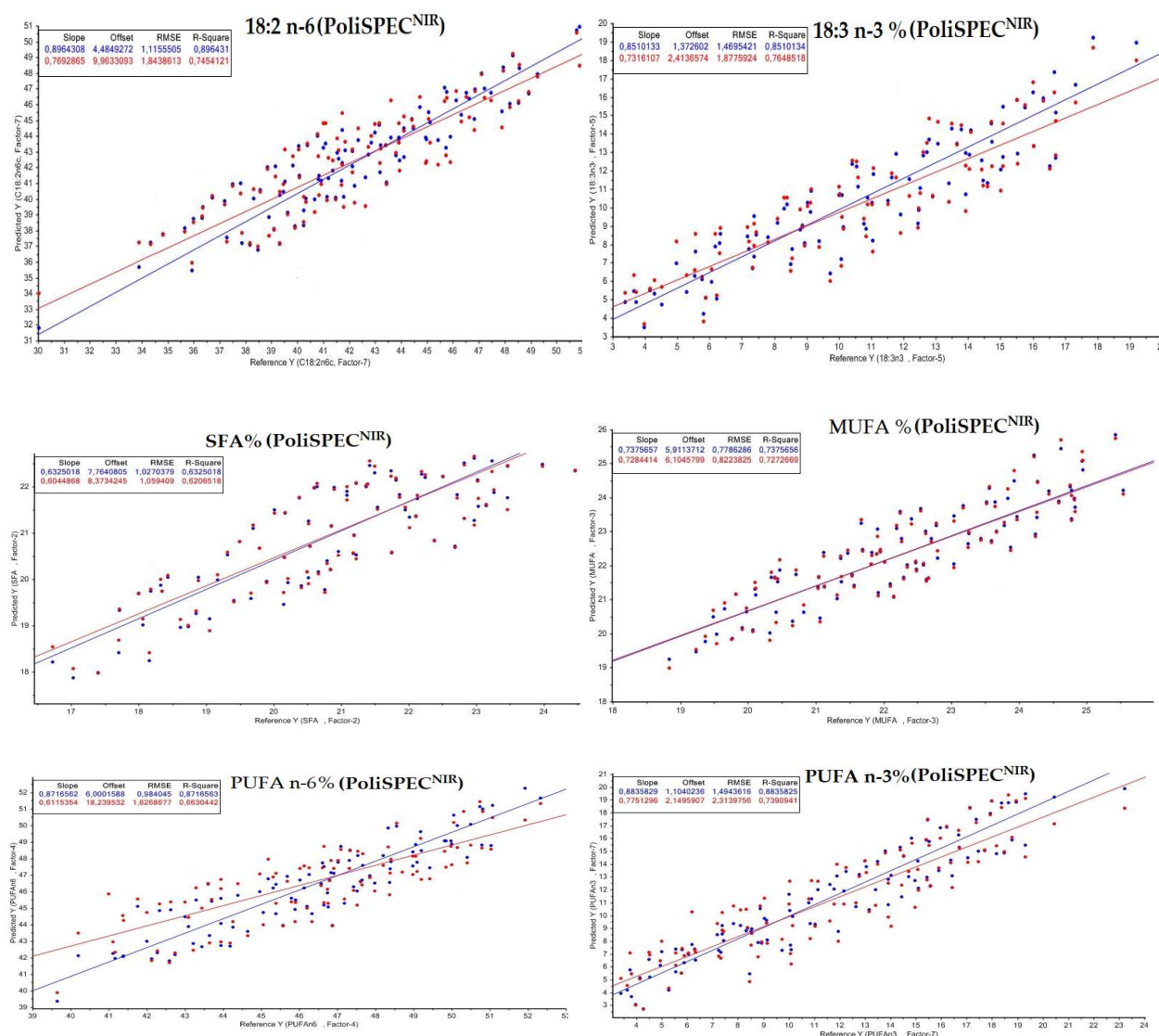
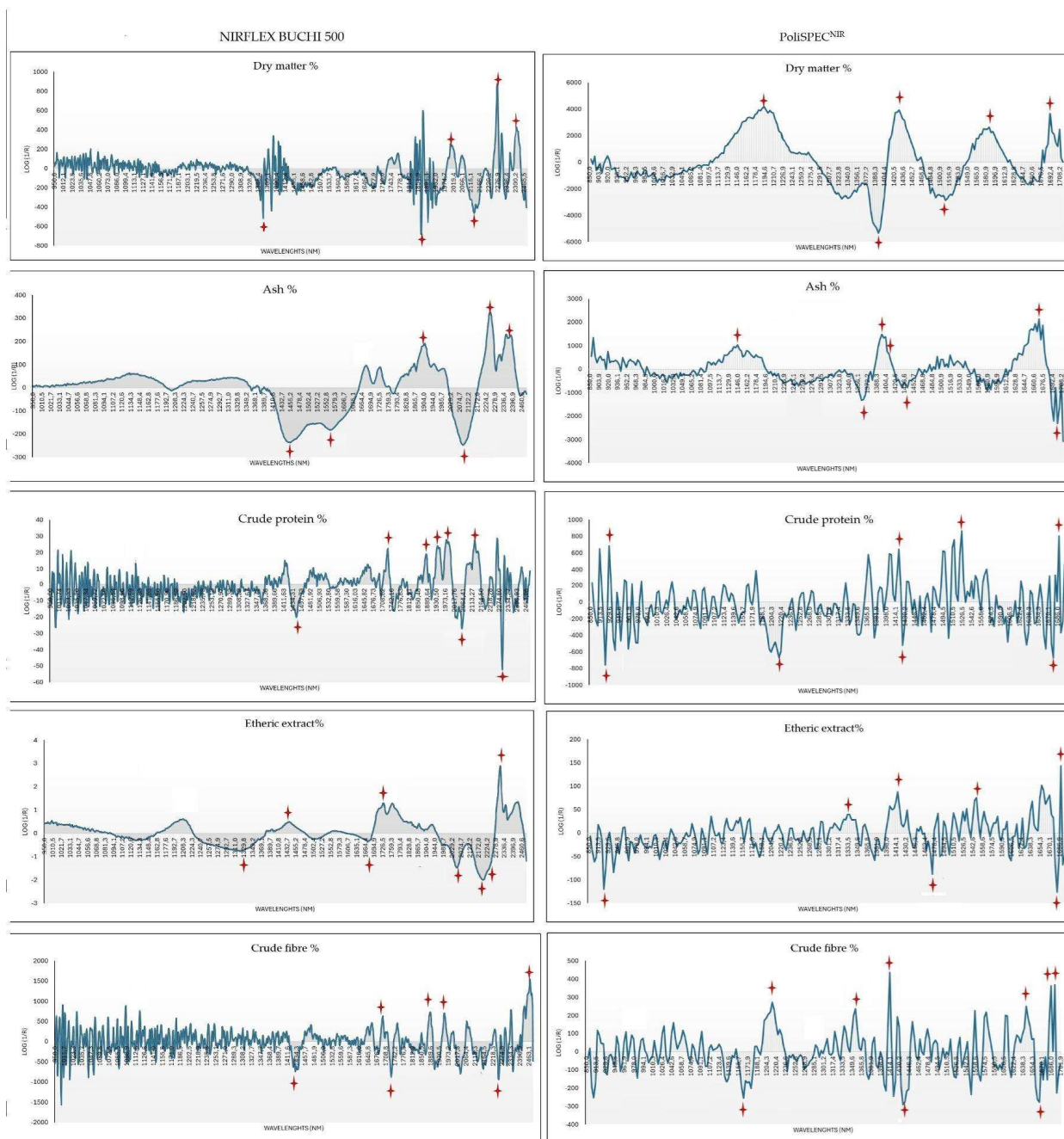
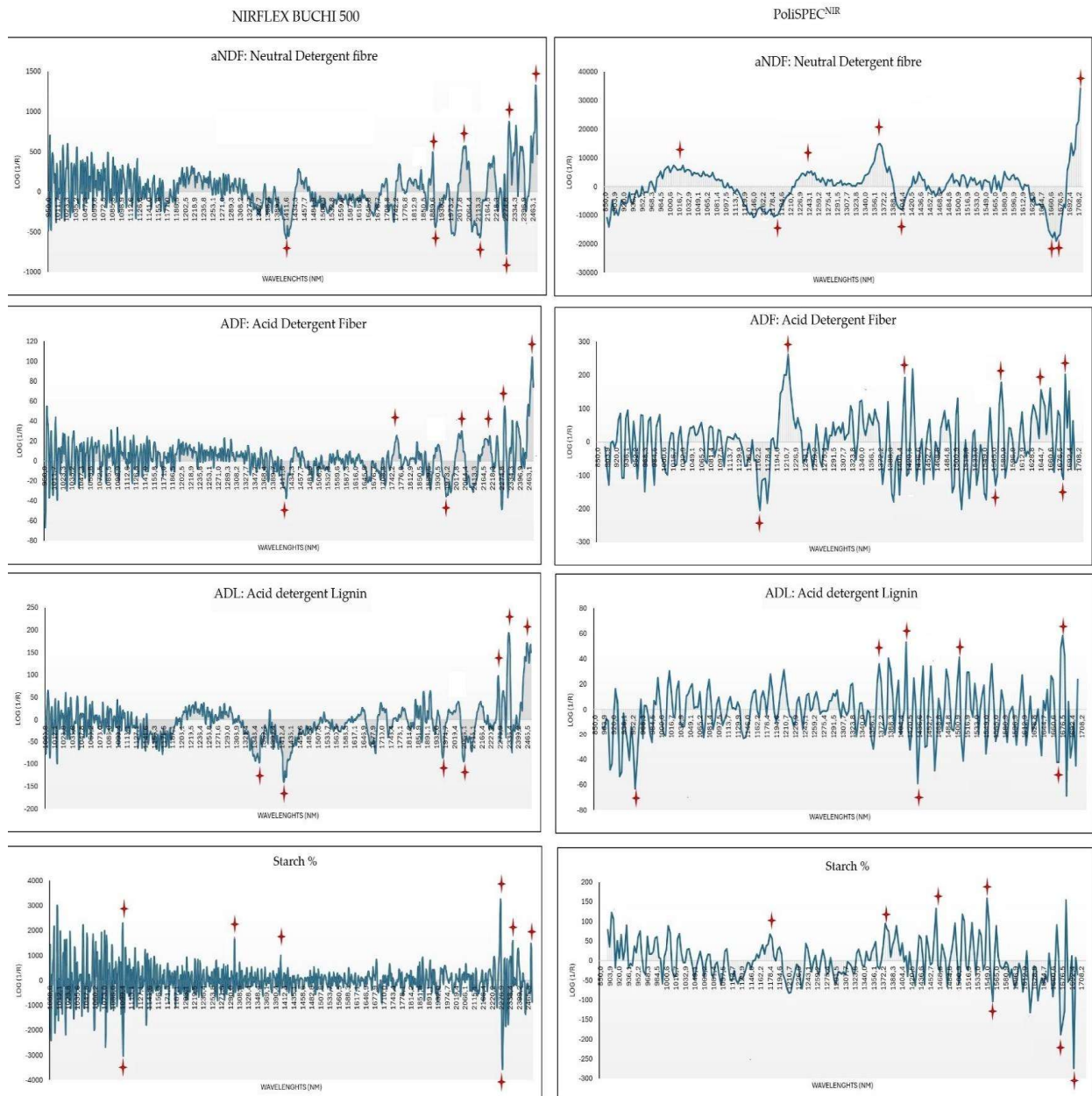
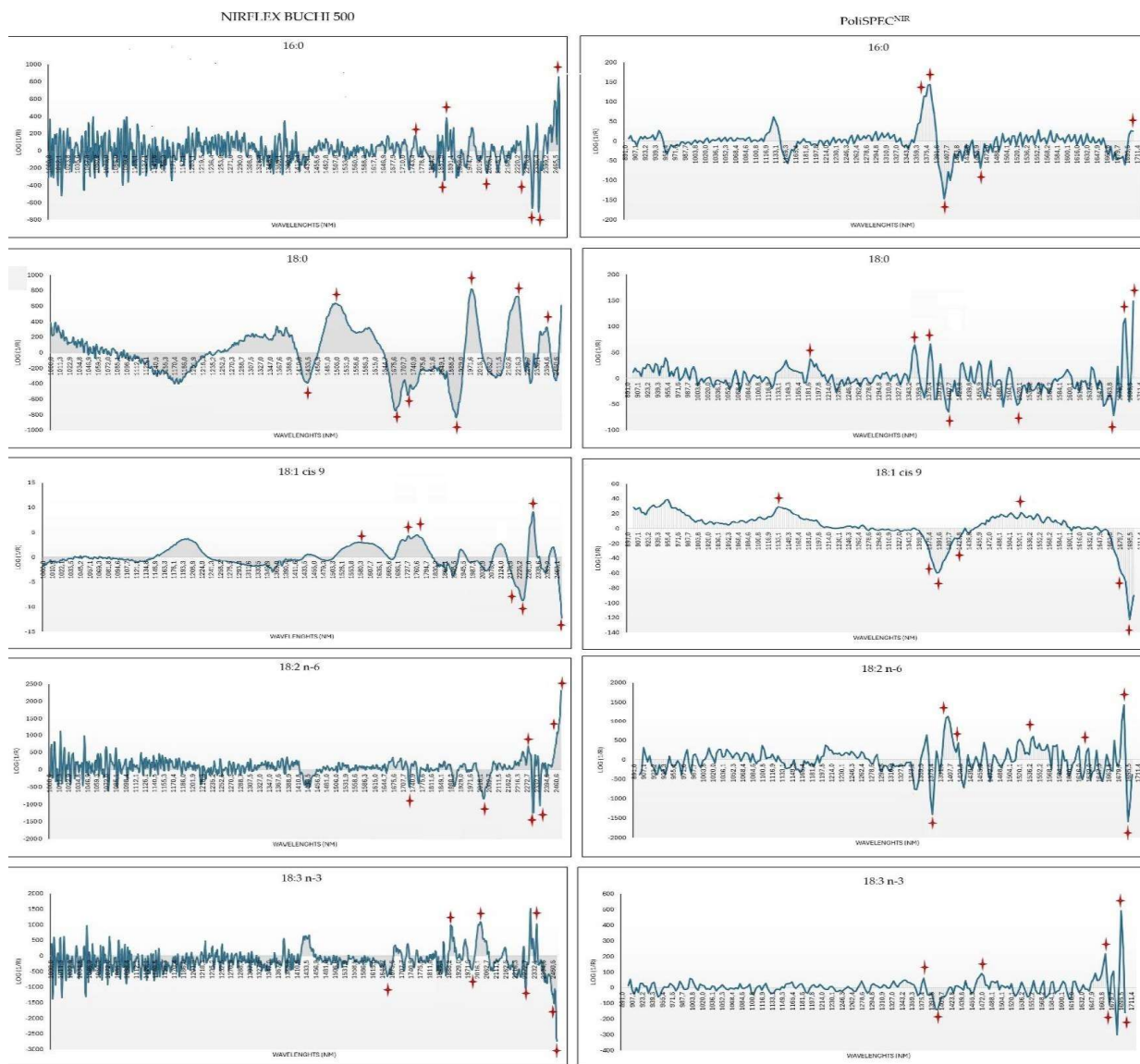


Figure S4. Plots of PLSR data analysis for prediction (blue) and cross-validation (red). Each plot corresponds to the best PLSR model obtained for each compound (proximate composition, fiber, fatty acids and principal classes of fatty acids). In the legend the slope, offset, RMSE (Root Mean Square Error), and R^2 values were reported.







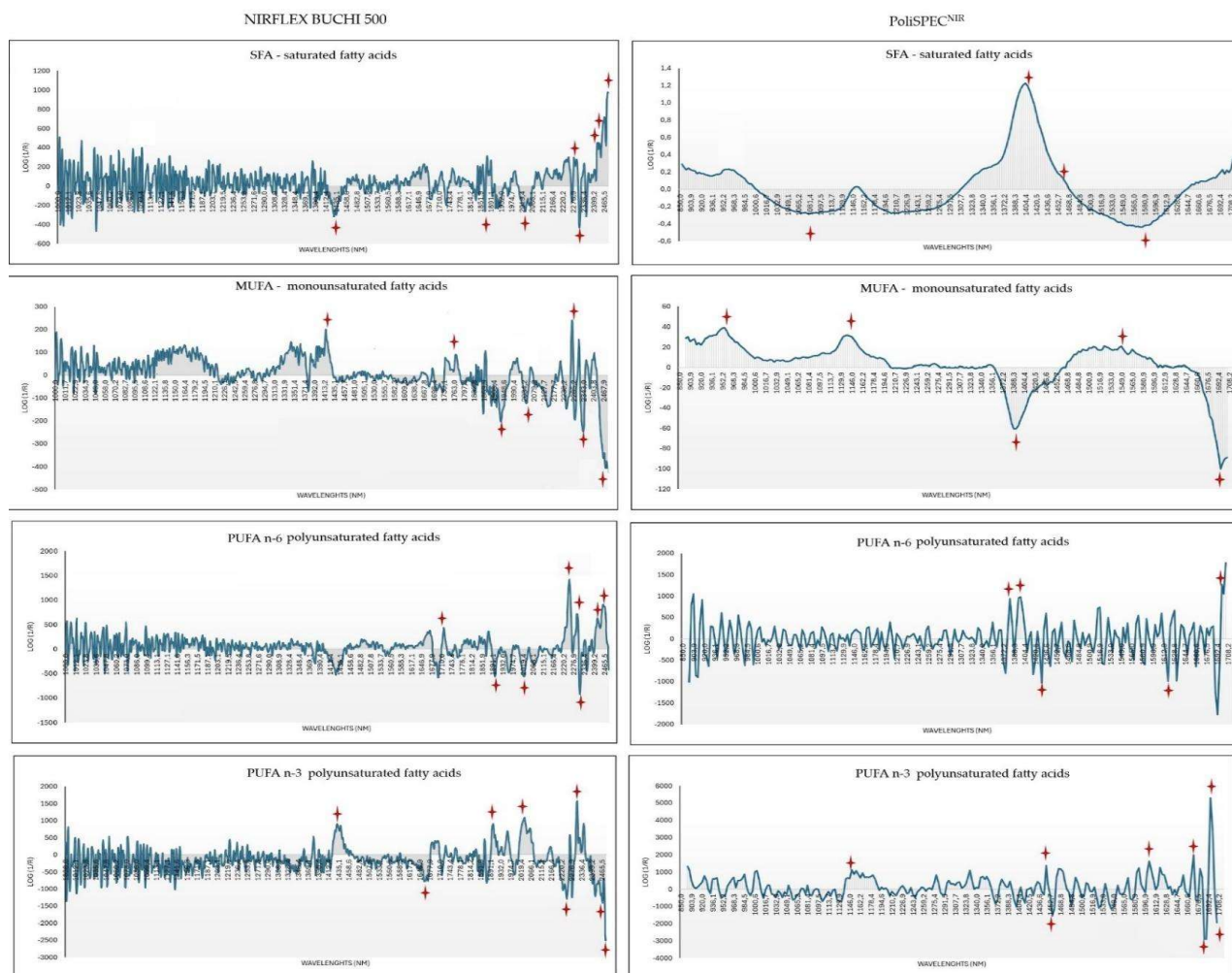


Figure S5. Regression coefficients and selected variables of PLSR models for chemical proximate constituents, fiber constituents, principal fatty acids and classes of fatty classes, obtained from the full spectrum of NIRFlex 500 and PoliSPEC^{NIR} instruments.

Selected variables for chemical compounds from spectra obtained with NIRFlex 500

	1050 1100	1320 1360	1410 1450	1650 1690	1710 1750	1840 1880	1900 1940	1950 1990	2010 2050	2150 2190	2260 2300	2330 2380	2410 2500	
DM%														6
Ash														6
CP														8
EE														8
CF														7
aNDF														8
ADF														7
ADL														7
Starch														8

Selected variables for FAs from spectra obtained with NIRFlex 500

	1410 1450	1530 1580	1650 1690	1710 1760	1840 1890	1900 1940	1950 1990	2000 2050	2200 2240	2250 2280	2290 2310	2330 2380	2400 2440	4440 2500	
14:0															6
16:0															8
16:1															9
18:0															8
18:1cis-9															7
18:1cis-11															6
18:2n-6															7
20:0															7
18:3n-6															8
18:3n-3															8
20:4n-6															7
SFA															8
MUFA															7
PUFA6															8
PUFA3															8

Selected variables for chemical compounds from spectra obtained with PoliSPEC^{NIR}

	920 950	1140 1190	1200 1230	1320 1360	1370 1400	1410 1450	1450 1490	1500 1540	1550 1590	1640 1670	1680 1700	
DM												6
Ash												7
CP												8
EE												7
CF												8
aNDF												8
ADF												8
ADL												7
Starch												7

Selected variables for FAs compounds from spectra obtained with PoliSPEC^{NIR}

	920 950	1140 1190	1370 1400	1410 1440	1440 1470	1500 1530	1550 1590	1640 1670	1680 1700	
14:0										5
16:0										5
16:1										6
18:0										8
18:1cis-9										7
18:1cis-11										7
18:2n-6										7
20:0										6
18:3n-6										7
18:3n-3										7
20:4n-6										6
SFA										5
MUFA										5
PUFA6										5
PUFA3										8

■ Third overtone
 ■ Second overtone
 ■ First overtone
 ■ Combination bands

Figure S6. Distribution of selected variables in the NIR spectrum bands for benchtop and portable NIR devices.

Sub Chapter 3.2

Machine Learning NIR wavelength selection: application for a low-cost portable instrument for livestock feed management

Milanesi Marco¹, Pietrucci Daniele¹, Serva Lorenzo², Renzi Francesco¹, Vignali Giovanni¹, Evangelista Chiara¹, Marchesini Giorgio², Andrighetto Igino², Bernabucci Umberto³, Valentini Riccardo¹, Chillemi Giovanni¹

¹Department for Innovation in Biological, Agro-food and Forest systems (DIBAF), University of Tuscia, Viterbo, Italy

²Department of Animal Medicine, Production and Health, Padova University, Padova, Italy

³Department of Agriculture and Forest Sciences (DAFNE), University of Tuscia, Viterbo, Italy

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The results presented in this chapter are completely based on the conference paper:

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ABSTRACT

In modern livestock farm the control of the production chain is very important, to ensure product quality and profitability. For example, in dairy farms, feed and diet control is essential to guarantee animal health and maximize the productive potential. Portable Near Infrared (NIR) instruments, applied at several points of the production chain, can be of help for the management of farm. However, the costs of commercial instruments make this type of application unsustainable, in particular for small-medium farms. To reduce the cost, a targeted instrument, that use a limited number of wavelengths, selected for their importance for the estimation, could be the solution. This study aimed to apply a machine learning algorithm to identify a set of most important wavelengths to estimate fiber fractions. In silico NIRs were here evaluated, in terms of accuracy and cost-effectiveness, to identify which portable NIR technologies could be applied throughout the feeding process.

Keywords: NIRS, portable instrument, variable selection, Random Forest, dairy cattle

INTRODUCTION

Near infra-red systems (NIRSs) are widely used in many fields, from medicine to biological science, including agriculture. In the livestock sector NIRS instruments are used to evaluate feed in non-destructive, fast, and reliable measurements (Beć et al., 2021; Kappacher et al., 2022). Usually, a spectrometer can be schematised as an emitter, an element that is able to select wavelengths (Wvs) and a receiver. The second is the element that mainly defines the instrument and costs (Yang et al., 2021). Different technologies could be used to analyse near-infrared spectra; however not all are low-cost and suitable to be portable (Table 1). Typical benchtop instruments use diffraction grating spectrometers, FT-IR (Fourier-transform infrared spectroscopy) spectrometers, and FPI (Fabry–Pérot interferometer) spectrometers. Diffraction grating works both in transmittance and reflectance, with a broadband emitter, with the Wv selection performed by the diffraction grating. Photodiode could be replaced by a camera with suitable algorithms (Loewen and Popov, 2017). The cost for a benchtop solution is ~25,000\$, and for the portable is around 10,000-15,000\$. The FT-IR uses a Michelson interferometer to obtain an interference pattern from a broadband light source. The pattern changes in accordance with the mirror movements. The spectrum is obtained using Fourier-transform on the obtained interference figures (Chai et al., 2020). The cost, for a benchtop, is around 30,000€. Finally, the FPI spectrometer is built using two closely spaced highly reflective, but partially transmitting surfaces, facing each other (Vaughan, 2017). The selected vs depends on the distance between the two elements. The

most recent technologies allow changing the distance via application of a voltage (MEMS-FPI), thus allowing the use of this technology in miniaturised NIRs (Otto et al., 2022). The cost for a benchtop FPI is ~5,000€ and for a portable MEMS-FPI is ~1,500€. The three technologies described above provide an entire spectrum, indeed, benchtop instruments are expensive and unsuitable to carry out on the field. To reduce the cost of a portable instruments, it is important to identify a set of few and important Wvs able to estimate the studied parameter(s). The advantages of this “application-driven spectrometers” are: i) a scalable cost (depending on the number of required Wvs); ii) a smaller form factor; iii) a minimal heating up during its use; iv) the absence of moving parts. However, the drawback is associated with the relatively wide full width at half maximum (FWHM) for low-cost options (generally $\pm 50\text{nm}$), and a loss of information compared to other technologies. This solution is affordable for a limited number of Wvs, until the cost matches the cheaper continuous spectrum spectrometer. The selection of the Wvs is possible using a set of emitters peaked on the required Wvs (i.e. light-emitting diode – led) or one broadband emitter and an array of photodiodes with suitable filters chosen accordingly (i.e. filter) (Malinen et al., 1998). Generally, the commercial portable solutions have a widely range scope and can read a large part of the NIR spectra, which increase the instrument cost. In dairy livestock production system, real-time evaluation of fibre fractions in feedstuff (i.e. forages or total mixed ration) is very important for farmers and nutritionists (Evangelista et al., 2021). Fibre plays an essential role in cow’s nutrition and feeding: its amount and quality may affect the feed intake and chewing activity (Dado and Allen, 1995), and reduce the risk of metabolic diseases, such as subacute ruminal acidosis (SARA) (Morgante et al., 2007), among others negative effects. The “application-driven spectrometer”, skilled in fibre characterisation for feeds and ration and applied to a tuned mixer wagon (for example, on the flank of the mixer container and on the loading device), able to analyse the chemical and physical parameters in real time, is a practical example of the Precision Livestock Farming concept (Lovarelli et al., 2020). The aim of this study is to identify, using Machine Learning (ML) approaches, the set of most relevant Wvs that are more important for the characterisation of fibre in several feeds. ML algorithms offer flexible, general-purpose approaches for learning complex relationships and patterns from existing (homogeneous and heterogeneous) data automatically and pinpoint hidden or difficult to identify relationships. ML approaches try to overcome the limits of traditional linear models (Jordan and Mitchell, 2015; Helm et al., 2020). Moreover, a second objective is to evaluate the potential of using the selected Wvs (partially or all) in in silico NIRs, comparing the expected performance with the potential cost.

Table 1. Prices of some commercial solutions.

NIR	Type	Wvs range	Price
Diffraction grating spectrometer	portable	900-1700 nm	15,500\$
Diffraction grating spectrometer	portable	1750-2150 nm	9,700\$
Diffraction grating spectrometer	benchtop	900-2500 nm	25,000\$
FT-IR spectrometer	benchtop	1000-2600 nm	28,800€
FPI spectrometer	benchtop - interferometer + other instruments	1275-2000 nm	5,000€
MEMS-FPI	portable	1350-1560 nm	1,550€

MATERIALS AND METHODS

A dataset of 335 samples was collected and analysed by the chemical-NIRS-XRF laboratory of the Department of Animal Medicine, Production and Health of the University of Padova (Padova, Italy). Four sample types composed the dataset: cow's total mixed ration (TMRC, $n = 168$), bulls TMR (TMRb, $n = 85$), grass silage (GS, $n = 39$) and corn silage (CS, $n = 43$). Samples were analysed using a benchtop instrument FOSS DS2500 (FOSS Analytical, Hillerød, Denmark) operating in a spectral range between 850-2500 nm, step 0.5 nm. Absorbance data, expressed as $\log(1/R)$, where R represented reflectance values, were recorded. Samples were also analysed, using reference methods (Segato et al., 2022), to evaluate the dry matter (DM) and fibre composition fibre composition: neutral detergent (aNDFom) and acid detergent (ADF) fibre fractions, and lignin in sulfuric acid (sa-lignin). Statistic calculations were performed using R. To reduce the batch effects, an empirical Bayes frameworks-based ComBat function was used (Johnson et al., 2007). After this, the Boruta analyses were run for each component (Kursa and Rudnicki, 2010). A feature importance analysis, using a Random Forest algorithm (Liaw and Wiener, 2002) was performed to order the features, from the most important to the less one (Pietrucci et al., 2022). Next, the feature importance was evaluated. The features were sorted according to the Gini Index. Following the order, the model performance was evaluated by adding one feature at time. The root mean squared error (RMSE) was used to evaluate the model performance and identify the most important features for each fibre fraction. These were then merged in a common list. Then, Random Forest models were run to identify a common order for the features. The Shapley Additive exPlanations (SHAP) algorithm was used to explain how each Wv contributes to the prediction (Lundberg and Lee, 2017). The final ordered list was used to simulate in silico NIRs that use from one to the entire list of Wvs. For each NIR, the Pearson

correlation coefficient between observed (*i.e.* values obtained applying the ML model) and expected value (*i.e.* obtained in the laboratory) of DM, aNDFom, ADF, and sa-lignin was obtained. Finally, the estimated cost for three different NIR solutions (led, filter or FPI) were obtained using commercial quotations. Based on its FWHM value, one emitter could read a range of Wvs. However, the efficiency is lower as we move away from the central point. In this analysis, for the sake of simplicity, this behaviour was not considered. For FPI, Wvs not covered by this technology were covered by led.

RESULTS AND DISCUSSION

After the independent feature selection, 5, 4, 15 and 10 Wvs for DM, aNDFom, ADF and sa-lignin, respectively, were identified (Table 2).

Table 2. Selected Wvs for the four characteristics and final order.

WAVE	ADF	aNDFom	DM	sa-lignin	Final order
1360			1		1
1381			3		2
1953	4				3
1925				7	4
1309				5	5
1919				1	6
1921.5		4			7
1936.5	3				8
1364.5			2		9
1921				6	10
1218				10	11
1974.5			4		12
1904			5		13
1284.5				9	14
1940				4	15
1286.5	9				16
1142.5	5				17
912	1				18
862	6			3	19
868.5	10				20
912.5	7				21
883.5		3			22
894		2			23
874	14				24
861	13				25
861.5	2				26
906	11				27
859	15				28
858				2	29
904.5	12				30
1131.5	8				31

1105.5		8	32
897	1		33

A total of 33 Wvs (one as in common between ADF and sa-lignin) were identified and analysed to determine a common order among the fibre characteristics. The correlation values between the expected and for aNDFom and ADF greater than 0.80 are reached using 3 and 4 Wvs, respectively (Figure 1).

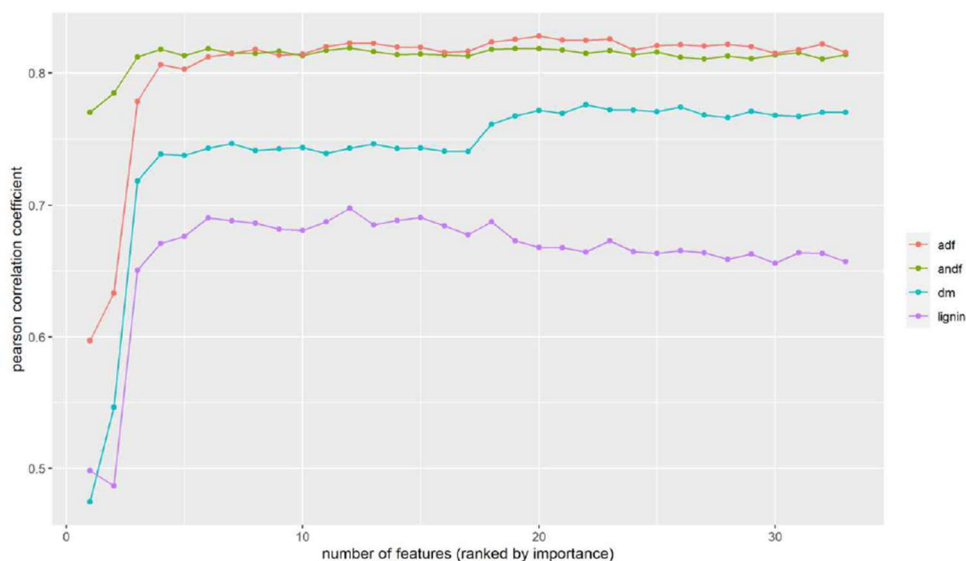
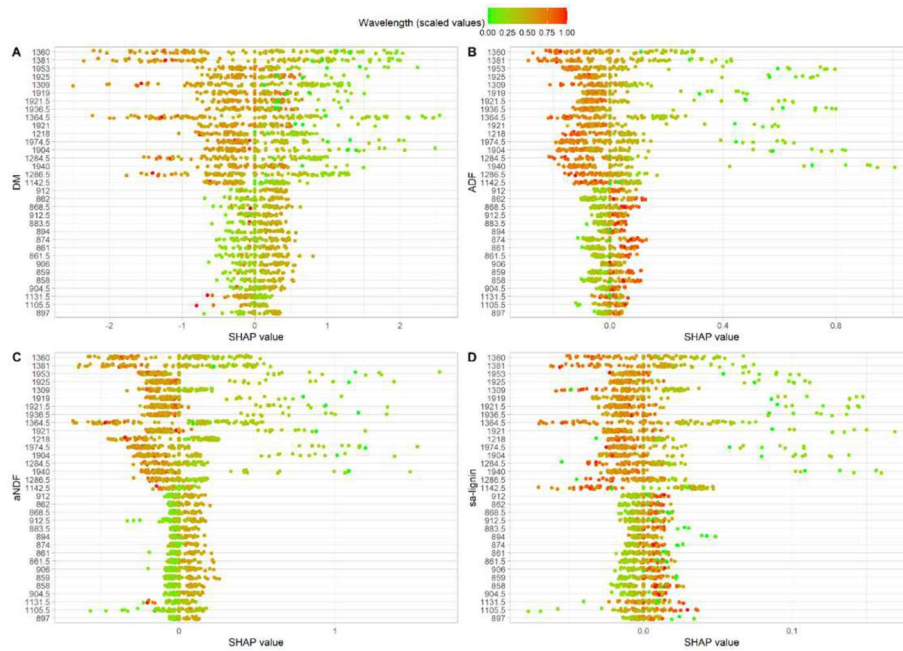


Figure 1. Correlation between observed and expected values using an increasing number of features (*i.e.* Wvs).

For DM the highest correlation is 0.77 using, at least, 20 Wvs. For sa-lignin the correlation coefficient is never higher than 0.70. Using the SHAP algorithm, the explainability for each Wv in the Random Forest model has been evaluated (Figure 2).



and possible other extra costs (*i.e.* photodiodes). For each of these theoretical NIRs, the minimum, maximum and median correlation in all the four fibre characteristics and all the Wvs in the group were calculated (Figure 3).

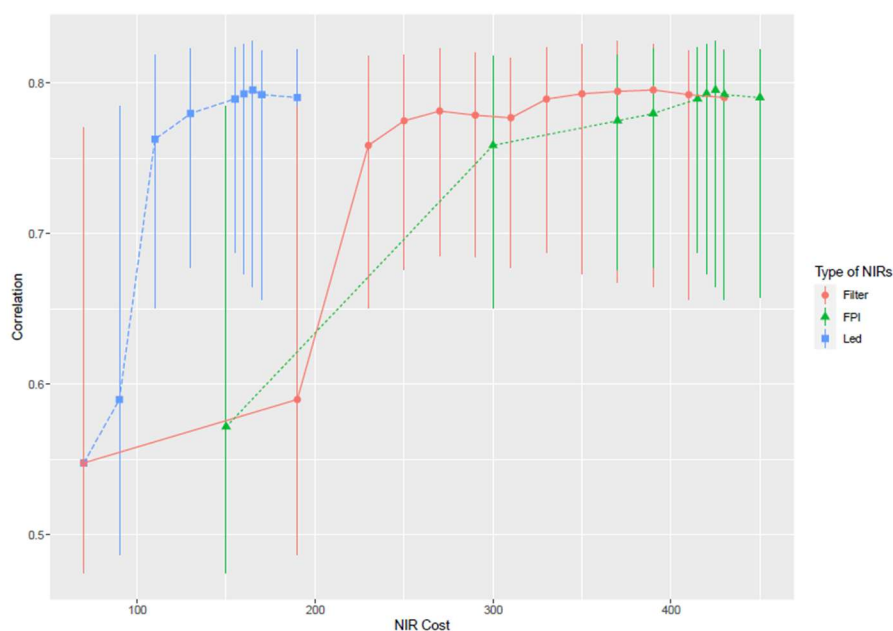


Figure 3. Comparison between the correlation (observed vs. expected), and the cost of the in silico NIRs. For the correlation, the bar represents the minimum and the maximum of the correlations in all the four fibre characteristics in the emitter block, the point is the median value.

Evaluating the results obtained with the in silico NIRS, the led solution is the most efficient in cost benefit terms. By using only three emitters, the led solution was able to achieve a median correlation coefficient of 0.76 for the four characteristics under investigation, including DM and all three fibre fractions. The estimated cost of this led solution was around €110, which is considerably lower than available commercial solutions. The filter and the FPI solutions are more expensive. To reach a similar median correlation coefficient of the led solution, ~230€ and ~300€ would be the cost for the filter and the FPI, respectively. The estimated total cost (using all the 33 identified Wvs) for led, filter and FPI solution is ~190€, ~430€ and ~450€, respectively. In all the cases, the estimated cost is considerably lower than the available commercial solutions. It is important to underline that the signals captured by the receiver is not the simple sum of the selected Wvs, but a more complex formula need to be used (*i.e.* the area covered by the FWHM). Moreover, not selected Wvs also contributes because present in the FWHM. For this reason, the correlation obtained in the present study could be underestimated.

CONCLUSION

This in silico trial showed that low-cost and (theoretical) reliable instruments are feasible to be used in specific on-field and on-farm applications using available technologies, such as led.

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Sub Chapter 3.3

Investigation the factors affecting homogeneity index of total mixed ration in dairy buffalo herds

Evangelista Chiara^{1*}, Basiricò Loredana², Bernabucci Umberto²

¹ Department for Innovation in Biological Agro-Food and Forest Systems, University of Tuscia,
Italy

² Department of Agriculture and Forests Sciences, University of Tuscia, Italy

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ABSTRACT

An important aspect in the field of precision feeding (PF) is to verify the homogeneity of the final total mixed ration (TMR). There is currently a portable tool available (PoliSPEC^{NIR}, ITPhotonics) able to measure homogeneity of ration and provide an output (homogeneity index, HI). However, HI is influenced by several factors such as type of mixer wagon (MW), mixing time (MT), feed loading sequence (SEQ), feed type and composition, and others. The objective of the present work was to investigate the possible factors influencing HI in dairy buffalo farms. The investigations were carried out in 10 dairy buffalo farms throughout an entire year. Each month, information on MW preparation (mixing time, feed loading sequence, composition of TMR) were collected and HI was analysed. Furthermore, a 120 TMR samples were collected for laboratory analysis. A Multiple regression analysis (MRA) was performed (a stepwise procedure) to determine the factor that best explained the HI results. Statistical analyses were performed using R software version 4.2.2 (R Core Team, 2022), significant differences were declared at $p < 0.05$, and the trend at $p < 0.10$. Results of MRA showed that among the factors considered, SEQ had a greater weight on the determination of HI ($p = 0.00$) followed by MW type ($p = 0.002$), TMR dry matter content ($p = 0.006$) and month of sampling ($p = 0.048$), while MT showed a minor effect on HI ($p = 0.076$). The model explains 30% of the variability, this means that there are other important factors influencing HI which were not investigated in this study.

Keywords: TMR, Buffalo farms, precision feeding, NIRS

INTRODUCTION

Precision Feeding (PF) is a strategic approach aimed at supplying animals with nutrients tailored to their specific requirements. By doing so, PF enhances physiology and overall health, thereby optimizing production efficiency and yielding superior quality animal products such as milk, meat, and eggs. Moreover, it contributes to environmental sustainability by minimizing waste, thereby ensuring profitability in animal husbandry practices. Precision Feeding relies on employing tools to assist farmers in managing and preparing animal rations. Among these tools, one of the most significant advancements of the last century is undoubtedly the mixer wagon (MW). Research has extensively shown that improper management of MW can have detrimental effects on the quality of the final ration. These effects manifest in terms of suboptimal fiber length, chemical composition, and homogeneity of the total mixed ration (TMR) (Piccioli-Cappelli et al., 2019; Costa et al., 2019; Marchesini et al., 2020; Serva et al., 2021; Carneiro et al., 2021; Tangorra et al., 2022). Significant disparities between the formulated diet and the actual feed provided to dairy

cows were demonstrated by Carneiro et al. (2021). Using the ration sieving technique, researchers observed notable differences between the offered diet and the formulated TMR. The offered diet exhibited a higher Neutral Detergent Fiber (NDF) content (+7.3%), while levels of Crude Protein (CP) (-6.5%), Acid Detergent Fiber (ADF) (-10.1%), and Crude Ash (-18.6%) were lower compared to the formulated TMR. These variations were attributed to the daily variability in commodity composition, with silage being particularly significant. The daily fluctuations in feed composition can have adverse effects on milk quality and yield, contributing to several issues such as abomasum dislocation, subclinical acidosis, fluctuations in daily milk yield, and influencing daily intake capacity (Sova et al., 2014). Piccioli-Cappelli et al. (2019) emphasizes the efficacy of precision control systems, such as portable Near-Infrared Spectroscopy (NIRS), when directly applied to MW in dairy cattle farming. The NIR system was tasked with analysing online the dry matter (DM) content directly from the forage harvester. Their study demonstrates that integrating such systems leads to a more consistent ration closely resembling the formulated diet, resulting in a reduction of DM usage by 1.35% while simultaneously increasing total daily milk production by 2.4%. Their study highlights the significant advantages of implementing ration monitoring and management systems in dairy cow operations, including cost reduction and enhanced performance (Piccioli-Cappelli et al., 2019). Accurate determination of DM content is particularly crucial due to the impact of humidity variations, especially in forage like silage, which can rapidly alter ration composition (Weiss, 2012; Piccioli-Cappelli et al., 2019). NIR systems offer versatility, being deployable both on the MW and manually by operators on the farm, facilitating real-time assessment of TMR composition and raw material composition (Evangelista et al., 2021). A recent use of NIR spectroscopy to evaluate the homogeneity of TMR through indices has been reported by Evangelista et al. (2021) and Serva et al. (2021). Homogeneity, understood as the degree of mixing and the relative granulometric composition, is of fundamental importance. If the ration is not mixed correctly, animals may choose the most palatable particles and ingest an unbalanced ration, which can lead to alterations in rumen efficiency, production efficiency, and increased variability between individuals (Serva et al., 2016). For this reason, the ability to verify the homogeneous distribution of TMR nutritional characteristics along feed bunk becomes economically significant for farm's management. Numerous factors can affect TMR homogeneity, including the type of MW, mixing time, filling level of the MW, feed loading sequence, chemical composition of raw materials, wear of the knives, and more (Buckmaster, 2009; Costa et al., 2019; Marchesini et al., 2020; Moallem & Lifshitz, 2020; Serva et al., 2021; Tangorra et al., 2022). To date, the causes of TMR inhomogeneity remain unclear. For this reason, the objective of the

present study was to investigate the possible factors influencing TMR homogeneity in dairy buffalo farms.

MATERIAL AND METHODS

Experimental design and Farms characteristics

The study lasted 12 months (from December 2021 to November 2022) and was carried out in 10 dairy buffalo herds representative of the Amaseno valley, in Lazio region (Central Italy). The size of the farms was on average 140 (± 87) lactating buffaloes. Feeding management for all farms was based on the use of TMR prepared by MW. The farm samplings took place once a month. For each visit, samples of TMR were collected and, homogeneity index (HI) was measured immediately after morning distribution by using a portable NIRS instrument named PoliSPEC^{NIR} (diode array spectrometers with a spectral range from 902 to 1680 nm, ITPhotonics, Italy) according to the protocol provided by the manufacturer. The protocol consists in dividing the feeding area into 4 sectors, each with 4 points, for a total of 16 points; each point was scanned with PoliSPEC^{NIR} (Figure 1).

A total of 120 TMR samples were collected and analyzed. One aliquot of TMR was sieved according to Heinrichs (2013) protocol. To determine the ration particles length (RPL) of TMR, 10 random particles were measured with a ruler for each sieve (for a total of 60 values for each sample). Dry matter (DM) was measured after oven drying at 65°C to constant weight. Dry matter is reported as percentages, RPL is reported in cm, HI is reported as pure number according to procedure indicated by Serva et al. (2021).

During each visit, the information involving the diet preparation process were monitored and recorded. These were the kind of MW, the order in which the ingredients were loaded (SEQ), the mixing time (MT) recorded from the time the last ingredient was loaded until the blend was finished, the filling level (FL) of MW, expressed as a percentage, was calculated by comparing the total load of the feed to the maximum load level. Finally, information on the composition of the ration was collected.

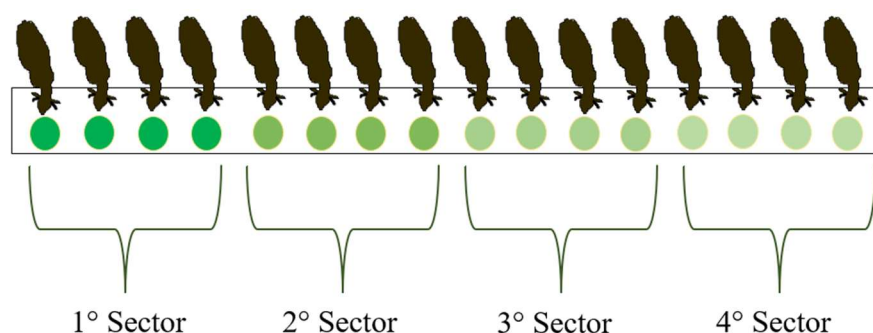


Figure 1. Protocol for homogeneity index analysis using the PoliSPEC^{NIR}.

Statistical analysis

The multiple linear regression model (a stepwise procedure) was performed using the 'dplyr', 'broom', and 'ggplot' packages in R software version 4.2.2 (R Core Team, 2022). The model included the HI as the response variable. The independent variables tested were Farms (F), Months (M), SEQ, MT, DM, RPL, FL and the presence of green forage (GF). Assumptions of the model were tested, including normality of residuals using the Shapiro-Wilk normality test, homoscedasticity of residuals using the Breusch-Pagan test, as well as correlations and collinearity among variables. To assess the difference in the HI for the variables that were found significant in the regression analysis, an analysis of variance (ANOVA) with interaction between the factors was conducted. The statistically significant differences were declared at $p < 0.05$ and the tendency at $p < 0.10$.

RESULTS AND DISCUSSION

In Table 1, the characteristics of individual farms and the TMR ration preparation are reported. Seventy percent (70%) of farms were equipped with a barrel MW, while 30% had a vertical MW. Regarding the loading sequence of the feed, 30% used sequence no. 1 (hay-concentrates-silages), while 70% used sequence no. 2 (hay-silages-concentrates). The mixing time varied among farms, as did the filling level of the MW.

Table 1. Descriptive statistics of farms characteristics (means $\pm$ sd).

Farms	Type of MW	SEQ	Mixing time (min.)	Filling level (%)
F. 1	Vertical	1	7 $\pm$ 2	53 $\pm$ 10
F. 2	Barrel	2	18 $\pm$ 23	37 $\pm$ 8
F. 3	Vertical	1	13 $\pm$ 5	64 $\pm$ 10
F. 4	Vertical	2	13 $\pm$ 6	50 $\pm$ 9
F. 5	Barrel	2	13 $\pm$ 8	52 $\pm$ 13
F. 6	Barrel	1	11 $\pm$ 3	42 $\pm$ 10
F. 7	Barrel	2	10 $\pm$ --	59 $\pm$ 7
F. 8	Barrel	2	10 $\pm$ 4	49 $\pm$ 12
F. 9	Barrel	2	12 $\pm$ 3	50 $\pm$ 12
F. 10	Barrel	2	10 $\pm$ 1	57 $\pm$ 13

SEQ: feeding loading sequence: 1= hay-concentrates-silages; 2= hay-silages-concentrates.

The results from MRA (Table 2) revealed that the most significant factor that affected TMR homogeneity was SEQ, followed by the type of MW, TMR dry matter content, and the month of sampling. Meanwhile, MT showed a minor influence on HI. All the other factors considered (farms, RPL, presence of GF and FL) did not potentially affected HI under condition of the present study. The model explains 30% of the variability (R^2), indicating that there are other important factors influencing homogeneity that were not investigated in this study.

Table 2. Results from multiple regression analysis.

	estimate	SE	t value	p value
Intercept	13.820	14.935	0.925	0.356
F	0.898	0.558	1.608	0.110
M	0.664	0.333	1.993	0.048
SEQ	12.599	2.607	4.832	0.000
MT	0.454	0.253	1.787	0.076
DM	0.548	0.199	2.757	0.006
RPL	-1.163	1.289	-0.902	0.369
GF	3.469	3.188	1.088	0.279
MW	-11.862	3.655	-3.245	0.002
FL	-0.133	0.101	-1.324	0.188
R^2	0.30			
RSE	12.22			

F: Farm; M: Month; SEQ: feed loading sequence; MT: mixing time; DM: dry matter; RPL: ration particle length; GF: green feed; MW: mixer wagon; FL: filling level of MW; RSE: residual standard error.

According to several authors (Oelberg, 2011; Zebeli et al., 2012; Schingoethe, 2017), the recommended order of adding ingredients to the MW to achieve a homogeneous diet is hay first, followed by minerals and concentrates, silage, and finally liquid feeds (such as whey or water). On the contrary, in our study the highest HI was achieved for the sequence that involved hay, silage, and concentrates (Figure 2, on the left). This is obvious because the humidity of the silage allows the flour particles to adhere perfectly to those of the silage, mixing optimally inside the hopper of the MW. Regarding the type of MW, the highest HI was observed for type 1, which is the vertical mixer wagon (Figure 2, on the right). Based on our knowledge, there are no studies that have assessed the difference in TMR HI between vertical and barrel mixer wagons using a portable NIR spectrophotometer. However, Moallem & Lifshitz (2020) examined the variations between vertical towed and self-propelled wagons in terms of ration homogeneity, measured with Penn State sieve and chemical analyses at different points of the feed bunk. They observed that while both types of MWs produced chemical differences between the distributed diet and the theoretical diet of approximately 5.7%, the self-propelled MW exhibited superior homogeneity in the chemical composition of the ration. In the present study, the barrel wagons were all self-propelled, whereas the vertical wagons consisted of both types. Hence, the improved homogeneity observed for the vertical type is not specifically linked to the self-propelled type as reported by Moallem & Lifshitz (2020).

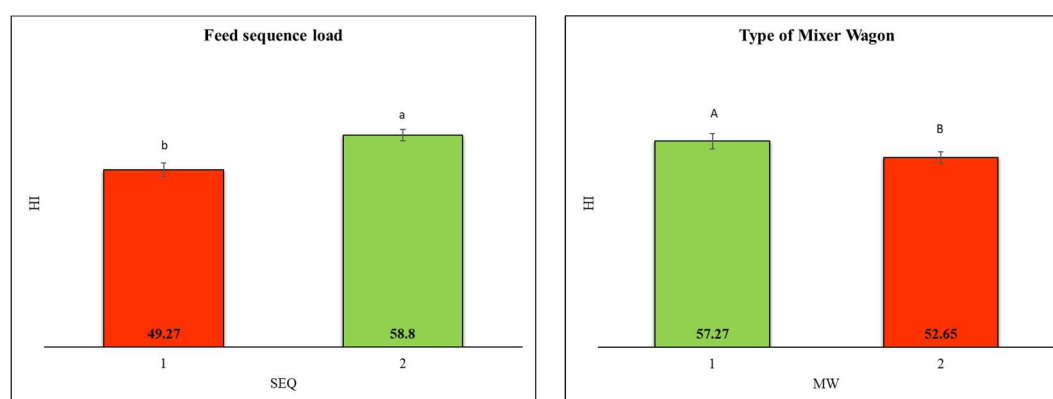


Figure 2. Comparison of the homogeneity index level for feed loading sequences (on the left) and for types of mixer wagons (on the right).

SEQ 1: hay-concentrates-silages; SEQ 2: hay-silages-concentrates; MW 1: vertical mw; MW 2: barrel mw.

a,b = $p < 0.05$; A,B = $p < 0.01$

The interaction between SEQ and MW confirms a higher level of HI for SEQ n. 2 and the vertical MW. For both types of MWs, SEQ n. 2 tends to increase HI (Figure 3). The worst HI was obtained for the barrel mixer wagon with SEQ n. 1, while the other combinations (SEQ*MW) are statistically comparable. Therefore, under condition of the present study, the best SEQ is the n. 2 (hay-silage-concentrate) and the best MW is the n. 1 (vertical type).

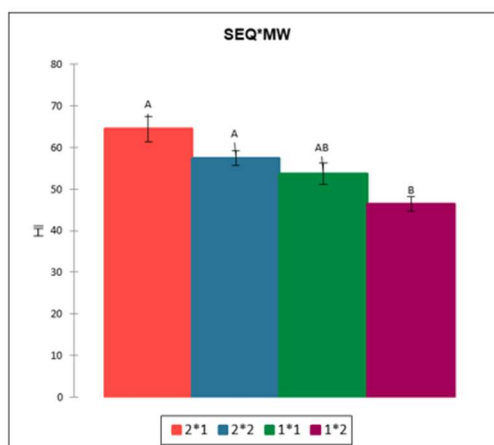


Figure 3. ANOVA interaction between feed loading sequence (SEQ) and type of mixer wagon (MW). SEQ 1: hay-concentrates-silages; SEQ 2: hay-silages-concentrates; MW 1: vertical MW; MW 2: barrel MW. A,B = $p < 0.01$

The DM content, according to Serva et al. (2016), is one of the three factors potentially influencing HI, with the other two being fiber amount and MW overload. In the present study, an improvement in the HI was highlighted with increasing DM (Figure 4, on the left), in agreement with findings by Serva et al. (2021). The better homogeneity with increasing DM could be due to the higher presence of raw materials with a high DM content (concentrates), also to the higher presence of finely chopped dry forages. This is supported by the negative correlation ($r = -0.265$, $p = 0.003$) between RPL and HI. This finding is consistent with previous studies, which have shown that more homogeneous TMR tends to have a lower presence of long particles compared to non-homogeneous ones (Perricone et al., 2018; Moallem & Lifshitz, 2020; Marchesini et al., 2020).

As shown in Figure 4 (on the right), HI tends ($p = 0.076$) to increase with the increasing MT. This is consistent with several studies (Perricone et al., 2018; Marchesini et al., 2020). An undermixing may cause the TMR to be very inhomogeneous along the feed bunk, while overmixing may cause an excessive particle size reduction (Serva et al., 2021). Perricone et al. (2018) suggested to adopt a mixing time greater than 7 min with horizontal cutter-mixer wagons. Marchesini et al. (2020), with a self-propelled vertical single-auger MW, found that mixing the ration for at least 30 minutes is more consistent over time. On the contrary, Tangorra et al. (2022) using NIRS instrument mounted directly on the MW, found that complete homogeneity of the TMR was achieved in 3.31 ± 0.87 min after the last ingredient was loaded with a horizontal self-propelled MW. In general, the MT depends on the type of MW and the raw materials used, as well as the filling level of the MW.

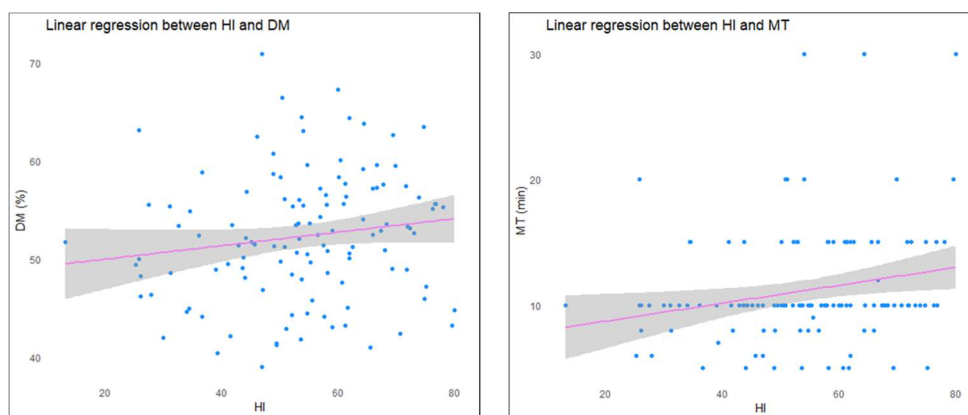


Figure 4. Linear regression between dry matter content (DM) and HI (on the left) and between mixing time (MT) and HI (on the right).

The filling level of the MW has a notable impact on the final homogeneity of the TMR diet (Costa et al., 2019; Carneiro et al., 2021; Serva et al., 2021). Overloading or underloading wagon may also compromise the mixing efficiency. In our study, FL was approximately 51%, much lower compared to what is suggested in the literature (80-100). Filling level did not affect HI in the present study. In other studies, it has been indicated as a limiting factor in achieving a homogeneous ration, suggesting a FL around 70% or higher (Costa et al., 2019, Serva et al., 2021) as optimal value.

CONCLUSION

Proper preparation and distribution of the diet are crucial aspects of precision feeding. Today, the availability of portable NIRS instruments capable of assessing the HI of ration distribution empowers farmers to enhance ration preparation, thereby improving animal welfare and health. Our investigation within a limited territory highlighted that the loading sequence of feeds, which affects particle adhesion properties, significantly influences HI in dairy buffalo farms. The type of mixer wagon and dry matter content were also identified as influential factors. Understanding these factors allows farmers to manipulate them to increase the homogeneity of the ration. Our study represents an initial exploration into the factors influencing HI, but it is limited in scope and does not comprehensively elucidate all variables and their modes of influence. Future research should encompass a broader range of farms and factors to provide deeper insights

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Chapter 4

Chapter 4

Approaches to feed strategies: enhancing nutrient efficiency and livestock production

Sub Chapter 4.1

Effect of Antioxidant Supplementation on Milk Yield and Quality in Italian Mediterranean Lactating Buffaloes

Evangelista Chiara¹, Bernabucci Umberto² and Basiricò Loredana²

¹ Department for Innovation in Biological Agro-Food and Forest Systems, University of Tuscia, Italy

² Department of Agriculture and Forests Sciences, University of Tuscia, Italy

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SIMPLE SUMMARY

Buffalo milk has good dairy properties that allow its transformation into excellent cheeses, in particular mozzarella. What greatly influences the quality of buffalo milk, and its properties is the diet administered; for this reason, we wanted to test whether a supplement of antioxidants (SOD, Zn, and Se) in the diet of lactating buffaloes could affect the quantity and quality of their milk. The experiment was carried out on sixty-six lactating buffaloes on a farm in central Italy. Individual milk samples were collected every four weeks at morning and evening milking. The results obtained showed that treatment had no effect on feed intake, feeding behaviour, and feed efficiency and improved milk yield and milk clotting properties (MCP).

ABSTRACT

Buffaloes are raised mainly to obtain milk, which although not much, is a milk that is nutritionally very rich. The technological characteristics of buffalo milk are optimal for processing into cheese, which is why the milk is mainly used to produce mozzarella cheese. Under stressful conditions, buffaloes, like other animals, produce milk qualitatively poorly. The stressors that can affect the quality of production are, in addition to other factors, deficiencies in nutrients such as vitamins, antioxidants, and minerals. In this study, we evaluated the effect of antioxidant supplementation on the quality of buffalo milk. Sixty-six buffalo were enrolled, subdivided into two balanced groups. The ZnSe group received 0.2 kg/head/day of Bufalo Plus® containing antioxidants and CaCO₃ and MgCO₃ mix; the control group was supplemented with 0.2 kg/head/day of CaCO₃ and MgCO₃ mix. The two groups were fed ad libitum with a total mixed ration (TMR). The amount of diet distributed was recorded daily and residue in the trough manger was recorded three times per week. TMR samples were taken every two weeks for each group. Daily Milk yield was recorded twice a week. Milk samples were collected every four weeks and analysed for chemical and technological properties. Furthermore, milk total antioxidant capacity was determined. The results obtained showed that the antioxidant supplement had no effect on feed intake, feeding behaviour, and feed efficiency. The treatment positively influenced milk production while it did not affect the chemical characteristics of milk. In addition, the supplement of antioxidants has improved the milk clotting properties (MCP). The supplement did not affect the antioxidant activity of the milk.

Keywords: Dairy Buffalo; milk; antioxidant; clotting properties

INTRODUCTION

Buffalo milk is very nutrient-rich milk containing a high percentage of both fat and protein and has double the energy content and lower cholesterol levels (6.5 mg/100 g milk) than cow's milk (Zicarelli, 2004). It is a good source of vitamins A, D, C and B₆, and minerals. It is very rich in calcium, magnesium, phosphorus, potassium, zinc (Salzano et al., 2021), and iron (Guo and Hendricks, 2010) and has more conjugated linoleic acid (CLA) compared to cow's milk (Pegolo et al., 2017). Buffalo milk, being very rich in nutrients, has a strong tendency to be transformed into cheese and has a high cheese yield relative to cow's milk (Bonfatti et al., 2012). The high concentration of nutrients, if on the one hand, it is particularly interesting for the cheese yield, on the other hand it is an aspect that potentially represents a drawback for the development of microorganisms, which use the high nutritional content to develop, worsening the nutritional characteristics, and hygiene of milk.

Milk and milk products are nutritious food items containing numerous essential nutrients such as oleic acid, conjugated linoleic acid, omega-3 fatty acids, vitamins, minerals, and bioactive compounds such as antioxidants (Khan et al., 2019). Antioxidants are chemical substances that scavenge/neutralise the free radicals and foods should contain enough concentration of antioxidants to prevent oxidative stresses (OS) (Khan et al., 2017). OS is defined as an imbalance between the production of free radicals and antioxidant defences in favour of the former and is closely related to the immune system and inflammatory status (Lykkesfeldt and Svendsen, 2007). The antioxidant capacity of milk and dairy products is due to the amino acids containing sulphur acids like cysteine, vitamins A, E, carotenoids, enzyme systems, superoxide dismutase, catalase, and glutathione peroxidase. Fresh buffalo milk has a greater antioxidant capacity compared with fresh cow's milk (Khan et al., 2017) because buffalo milk has a higher concentration of many antioxidant compounds such as vitamin E (5.5 mg/100 ml in buffalo milk and 2.1 mg/100 ml in cow milk), or vitamin C (3.66 mg/100 ml in buffalo milk and 0.94 mg/100 ml in cow milk) (Ahmad et al., 2013) and other antioxidant compounds such as selenium (Se), zinc (Zn), tyrosine, cysteine and catalase; furthermore, catalase activity is 2–4 times higher in buffalo milk as compared with cow milk.

There is a range of dietary antioxidants that can be provided in feed, which include vitamin E, carotenoids, polyphenolics, antioxidant enzymes, Zn, and Se (as a precursor to selenoproteins). Zn, Se, and Cu supplementations have been associated with the increased antioxidant capacity of superoxide dismutase (CuZnSOD), glutathione peroxidase (GSH-Px), and serum ceruloplasmin (CP), respectively, resulting in reduced milk somatic cell counts (SCC) (Cortinhas et al., 2010).

Zinc and Se are essential for the health and performance of all animal species because they are involved in several aspects of cellular metabolism (Rychen et al., 2018). There are several publications showing that trace mineral supplementation can affect the health, reproductive status, immune function, and lactation performance of dairy cows (Ashmead et al., 2004; Meglia et al., 2004; Griffiths et al., 2007). Weiss et al. (2005) reported that Zn, Cu, and Se are needed to maintain the antioxidant activity of dairy cows' immune system, in particular the defensive reactions of the mammary gland against mastitis.

The use of trace minerals from organic sources in animal nutrition can be an important tool for increasing milk production and maintaining the health status of animals (Cortinhas et al., 2012). However, the literature on the role of micronutrient and antioxidant supplementation in the diet of buffaloes and its effect on the production and quality of milk is very limited.

Therefore, this study aimed to evaluate the effect of antioxidant supplementation on milk yield and quality of lactating dairy buffaloes.

MATERIALS AND METHODS

Animals and feeding

Sixty-six healthy lactating buffaloes were subdivided into two groups of thirty-three animals balanced by parity, days in milk (DIM), and daily milk yield (Table 1). The two groups were separated by gates, but both in the same building with the same housing conditions (Figure S1). The research and the animal care protocols were in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes (European Union, 2010).

Table 1. Characteristics of the two groups at the beginning of the experiment.

	ZnSe	Control
	Mean±SD	Mean±SD
Parity (n)	5.2±3.2 ^a	5±3.2 ^a
Days in milk	91.5±26.7 ^a	91.2±25.6 ^a
Daily milk yield (L/d)	10.67±2.37 ^a	10.55±2.16 ^a

The two groups were: ZnSe and Control groups. The ZnSe group was fed with a standard diet supplemented with antioxidants (treated group); the control group was fed with a standard diet (control group). The composition of the diet distributed is shown in table 2. The ZnSe group received 0.2 kg/head/day of Bufalo Plus® (SIVAM, Italy) added to TMR. The supplement (Bufalo Plus®) composition was the following: CaCO₃, MgCO₃, barley meal, Melofeed® (rich in the antioxidant enzyme superoxide dismutase, SOD), hydrated amino acid zinc chelate-3b606 (4,000 mg/kg), selenium zinc-L-selenomethionine-3b818 (16 mg/kg), Ca (20.5%) and Mn (11%). The control group was supplemented with 0.2 kg/head/day of CaCO₃ and MgCO₃ mix.

The experimentation started in early May and ended in mid-August and lasted 103 days.

The buffaloes were milked two times a day in a herringbone milking parlour with 11 stalls, equipped with measuring jars with the possibility to measure milk yield and take individual milk samples. The two groups were fed *ad libitum* with a total mixed ration (TMR), distributed once a day at 7:30 a.m. with a two-augers vertical mixer wagon. The amount of diet distributed was recorded daily. The residue in the trough manger was recorded three times a week.

TMR samples were taken every two weeks for each group. A total of 8 TMR samples per group were collected.

Table 2. Composition of the standard diet.

Feed	Kg/head (w.b. ¹)
Corn silage	10.0
Grass silage	10.0
Commercial mixed feed ²	5.0
Alfalfa Hay	4.0
Mixed Hay	3.0
Sodium bicarbonate	0.02

¹w.b.: wet basis; ²Composition: shelled soybean meal, wheat middling, shelled sunflower meal, corn meal, soy husks, wheat bran, corn gluten meal, barley meal, dried beet pulp, soybean oil, hydrogenated fat, calcium carbonate, sodium bicarbonate, byproducts from L-glutamic acid production, sodium chloride, magnesium oxide. Supplements per kg: Vit. A 25,000.00 UI; Vit. D3 1,800.00 UI; Vit. E 30 mg; Niacin 50.00 mg; Vit. B1 0.40 mg; Vit. B2 0.80 mg; Vit. B12 0.05 mg; Choline chloride 20 mg; Trace elements (mg): Fe (20.70); Mn (50.00); Zn (75); Co (0.50); I (0.66); Se (0.25); Cupric sulphate (1.00).

The TMR was analysed for chemical and physical characteristics. Samples were sieved using the 3-screen (19, 8, and 4 mm) Penn State Particle Separator (PSPS; Kononoff et al., 2003) for particle size determination. For chemical analysis, DM was measured after oven drying at 65 °C to constant weight. Then, TMR samples were ground through a mill (Retsch Müller, Germany) to pass a 1 mm screen. To maintain optimal preservation conditions, sealed polyethylene containers were used to store prepared samples. Samples were analysed for crude protein (AOAC, 2005), ash (AOAC, 1990), ether extract (AOAC, 2000), starch (AOAC, 2005), using a K-TSTA assay kit (Megazyme International, Bray, Ireland), neutral detergent fibre (aNDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) using an Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY) according to Van Soest et al. (1991). TMR was, also, analysed on-site for determining homogeneity index (H.I.) and selection index (S.I.) at 1.5 h from distribution, with portable NIRS (PoliSPEC^{NIR} diode array spectrometers with a spectral range from 902 to 1680 nm, ITPhotonics, Breganze, Italy).

All data are reported as percentages on a dry matter basis, except for H.I. and S.I. which were expressed according to procedure indicated by the manufacturer.

Milk yield and analysis

Individual daily milk yield was recorded twice a week. Milk samples were collected every four weeks at morning and evening milking, directly from the measuring jars attached to the milking machine and packaged in 50 mL plastic tubes containing Bronopol® (2-Bromo-2-Nitropropane-1,3-Diol) as a preservative. Milk samples were refrigerated (4 °C) and analysed within 24 to 36 h after collection. Content (%) of fat, protein, lactose and solid not fat (SNF), and pH were determined by F.T.I.R. spectrophotometry, using a MilkoScanTM 7 RM (Foss Analytics, Hillerød, Denmark) calibrated with appropriate buffalo milk standards. Somatic cell counts (SCC, thousand cells/mL) were determined by fluoro-optoelectronics method using a FossomaticTM FC (Foss Analytics, Hillerød, Denmark). To achieve a normal distribution of the SCC, the somatic cell score (SCS, units) was calculated as follows (Wiggans and Shook, 1987):

$$SCS = \log_2 (SCC/100,000) + 3$$

The corrected milk of the total daily milk was converted into buffalo energy-corrected milk (ECM) using the following formula (Campanile et al., 1998):

$$Y = \{[(F \times MY - 40) + (P \times MY - 31)] \times 0.01155 + 1\} \times MY$$

where Y is the quantity (kg) of ECM equivalent to 1 kg of milk produced; MY is the milk yield; F is the percentage of fats; P is the percentage of proteins.

Feed efficiency ratio (FEr) was calculated relating the ingestion of DM, in the days before and after milk sampling, to the ECM.

Milk clotting properties (MCP) (RCT: rennet coagulation time, min; k20: curd firming time, min; a30: curd firmness, mm) were determined by using a Formagraph instrument (Mape System, Firenze, Italy) according to Zannoni and Annibaldi (1981). For MCP determination, milk samples (10 mL) were heated to 35 °C, and 200 µL of commercial calf rennet (Clerici s.p.a.-Sacco s.r.l., Cadorago, Italy; 75% chymosin and 25% bovine pepsin; 175 international milk clotting units/mL; strength 1:15.000 in Italian commercial units) diluted to 1% (wt/wt) in distilled water was added to the milk samples. Milk clotting properties were determined over the next 30 min after the addition of calf rennet.

Total antioxidant activity (TAA) of buffalo milk was determined using the Oxy Adsorbent Test (Diacron International, Grosseto, Italy) and a spectrophotometric plate reader (Sunrise Plate Reader, Tecan Trading AG, Männedorf, Switzerland) at 540 nm wavelength. The test is used to evaluate serum or plasma antioxidant barriers and for the evaluation of milk antioxidant capacity (Bianchi et al., 2011; Beghelli et al., 2016). Antioxidant capacity was assessed according to the producer's instructions, with slight modifications. The antioxidant activity of the milk was evaluated by measuring the ability of milk samples to resist the great oxidant action of hypochlorous acid (HClO), a potent and physiological oxidant. All antioxidant molecules in milk samples, scavengers, and shock absorbers related to neutralisation of free radicals were measured with this assay. Briefly, the sample of buffalo milk was incubated with an excess of HClO solution, and then the residual HClO reacted with a chromogenic mixture and developed a coloured complex. The measured optical densities were proportional to the concentration of HClO and indirectly associated with the antioxidant capacity of samples. TAA values were expressed as µmol of HClO/mL, according to the formula reported in the producer's instructions. For the assay, whole buffalo milk samples were diluted 1:75 (v/v) with distilled water.

Statistical analysis

A nested ANOVA with a mixed-effects model (an extension of one-way ANOVA in which each group is subdivided into subgroups) was used to test for a significant effect of “controls”, “groups”, and “samples” (*i.e.*, factors) on the measured parameters. The analyses were then performed by contrasting each parameter against each factor and their interactions by using “animal” as a random variable in the models. A multiple pairwise comparison test was performed by using a Bayes factor analysis between group levels with corrections for multiple testing. All analyses were performed by using the function “lme” and “pairwise_comparisons” in the packages “nlme” (Pinheiro et al., 2020) and “pairwiseComparisons” (Patil, 2019) of R (R core Team, 2021). Results are expressed as Lsmeans $\pm$ SE, and significance was declared at $p < 0.05$.

RESULTS

Feed intake and diet characteristics

Changes of feed intake during the experimental period is reported in figure 1. The average amount of feed intake (mean $\pm$ DS), as it is, was 27.97 ± 1.55 vs. 27.58 ± 1.76 Kg/head/day for ZnSe and control group, respectively (Figure 1a). Dry matter intake (Figure 1b), on average (mean $\pm$ DS), was 16.65 ± 0.73 vs. 16.52 ± 0.88 Kg/head/day in the ZnSe and control group, respectively. Both feed intake and dry matter intake did not differ between the two groups.

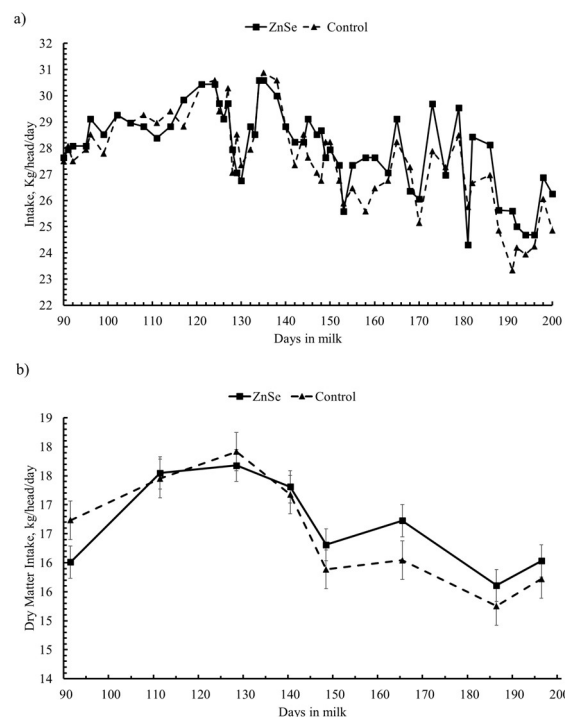


Figure 1. Trend of daily feed intake between the two groups. (a) feed intake; (b) dry matter intake.

The average composition of the diets distributed to the two groups is shown in Table 3. Both chemical composition and physical characteristics were not different between the two diets.

Table 3. Characteristics of total mixed ratio (TMR) distributed to the two experimental groups.

Parameters	ZnSe	Control
	Mean±SD	Mean±SD
Dry Matter, %	59.77±1.7	60.2±1.6
Ash, %	7.5±0.36	7.58±0.48
Crude Protein, %	11.24±1.05	11.23±0.71
Ethereal Extract, %	2.19±0.3	2.33±0.52
aNDF, %	41.98±2.94	42.37±2.26
ADF, %	29.48±2.88	29.94±2.24
ADL, %	3.81±0.7	4.1±0.43
Starch, %	20.23±1.34	20.13±0.83
S1, %	10.2±4.23	10.37±3.72
S2, %	29.95±4.49	29.55±4.31
S3, %	35.61±1.38	34.74±1.68
Bottom, %	24.24±3.56	25.33±3.48

DM: Dry matter; Ash; Crude Protein; Ethereal Extract; aNDF (Neutral Detergent Fiber); ADF (Acid Detergent Fiber); ADL (Acid Detergent Lignin) and Starch are on DM basis; S1 = % of ration retained by a sieve with holes of 19 mm; S2 = % of the ration retained by a sieve of 8 mm; S3 = % of the ration retained by a sieve of 4 mm; Bottom = % of ration with dimensions <4 mm.

Table 4 shows the results on the H.I. and S.I. of TMR distributed to the two groups. According to the classification scale of homogeneity the H.I. of TMRs were classified as "sufficiently homogeneous" ($50 < HI < 65$). The homogeneity of TMR distribution was not different between two groups. As regards the selection index at 1.5 h after TMR distribution buffalo did a "modest selection" ($20 < SI < 40$). Also, the selection index was not different between the two groups. This means that preparation and distribution of ration was identical for the two groups.

Table 4. Homogeneity (H.I.) and selection (S.I.) indices of total mixed ratio (TMR) distributed to the two experimental groups.

	ZnSe	Control
	Mean±SD	Mean±SD
H.I.	55.42±12.17 ^a	50.12±19.87 ^a
S.I.	38.17±8.35 ^a	39.78±12.02 ^a

*Milk yield and milk quality traits**Milk yield*

The trend of milk yield (Lsmeans ± SE) during the experimental period (L/head/day) is shown in Figure 2a. Figure 2b shows the overall average (Lsmeans ± SE) daily milk yield. Figure 2c reports the total milk yield (Lsmeans ± SE) in the two groups estimated by calculating the area under the lactation curve. The average daily milk yield was greater ($p < 0.001$) in the ZnSe group compared with the control group (9.12 ± 0.02 vs. 8.81 ± 0.02 L/head/day for the ZnSe group and control group, respectively) with an improved milk production of 0.31 L/head/day. Moreover, the total milk yield was greater (994.93 ± 32 vs. 964.99 ± 44 L/head; $p < 0.001$) in the ZnSe group compared to the control group. During the experimental period, the ZnSe group produced 30 L/head more than the control group.

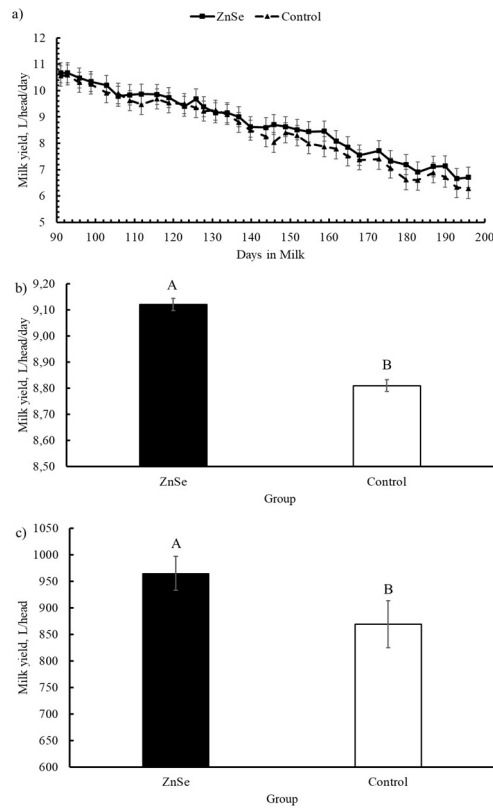


Figure 2. Milk yield trend (L /head/day), for the two groups; (a) changes during the trial; (b) average milk yield (L/head/day); (c) total milk yield (L/head) calculated as area under the curve. A, B = $P < 0.001$.

Milk quality traits

The results ($Lsmeans \pm SE$) on milk quality traits and SCS, ECM, Feed efficiency ratio are shown in table 5. There was not any difference for main constituents of milk between two groups (Table 5).

Table 5. Results of milk quality traits, energy corrected milk (ECM) and feed efficiency ratio (FEr) between the two groups under examination.

Parameters	ZnSe	Control
	Mean $\pm$ SE	Mean $\pm$ SE
Fat, %	8.20 $\pm$ 1.48 ^a	8.33 $\pm$ 1.61 ^a
Protein, %	4.64 $\pm$ 0.35 ^a	4.64 $\pm$ 0.47 ^a
Lactose, %	4.66 $\pm$ 0.20 ^a	4.66 $\pm$ 0.19 ^a
Solid Not-fat, %	10.00 $\pm$ 0.35 ^a	10.03 $\pm$ 0.50 ^a
pH	6.63 $\pm$ 0.14 ^a	6.61 $\pm$ 0.17 ^a

SCC, thousand/cells/ml	118.78±201.75 ^a	131.62±188.41 ^a
SCS, log ₂	2.76±0.78 ^a	2.73±0.97 ^a
ECM, Kg/h/d	14.34±1.10 ^a	13.64±1.06 ^a
FEr	0.57±0.09 ^a	0.56±0.08 ^a

ECM: Energy Corrected Milk; SCC: Somatic Cell Count; SCS: Somatic Cell Score; FEr: Feed Efficiency ratio.

The clotting parameters of milk were positively influenced by treatment (a_{30} and RCT $P < 0.01^{**}$; k_{20} $P < 0.05^{*}$). The average ($\pm$ SE) for RCT was 15.14 $\pm$ 0.85 vs. 16.37 $\pm$ 0.85 minutes; 2.49 $\pm$ 0.26 vs. 2.74 $\pm$ 0.26 minutes for k_{20} and 51.31 $\pm$ 2.46 vs. 47.94 $\pm$ 2.47 mm for a_{30} , for ZnSe and control group, respectively (Figure 3).

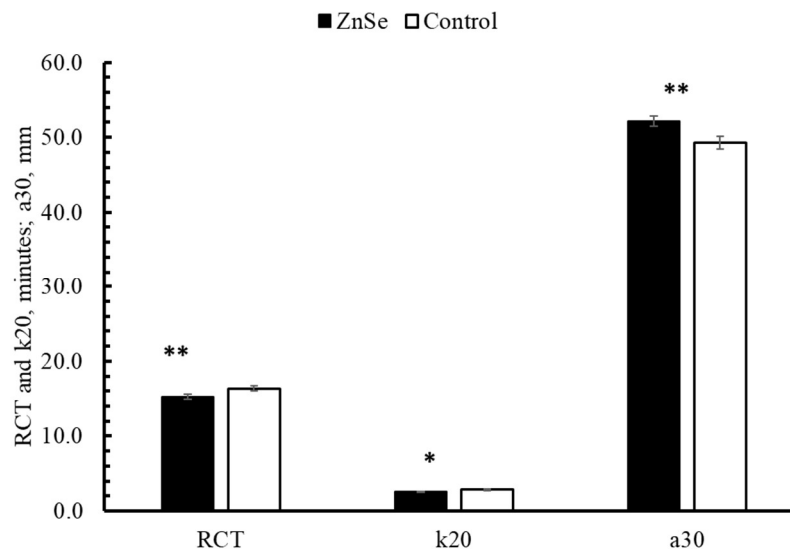


Figure 3. Results of technological characteristics of milk between two groups being tested. RCT: Rennet coagulation time, min. k_{20} : Curd firming time, min; a_{30} : Curd firmness, mm; * $P < 0.05$, ** $P < 0.01$.

Milk total antioxidant activity (TAA) was not affected by treatment. TAA was 408.15 $\pm$ 24.18 vs. 432.97 $\pm$ 25.64 μ mol neutralised HClO/ml for ZnSe and control group, respectively (Figure 4).

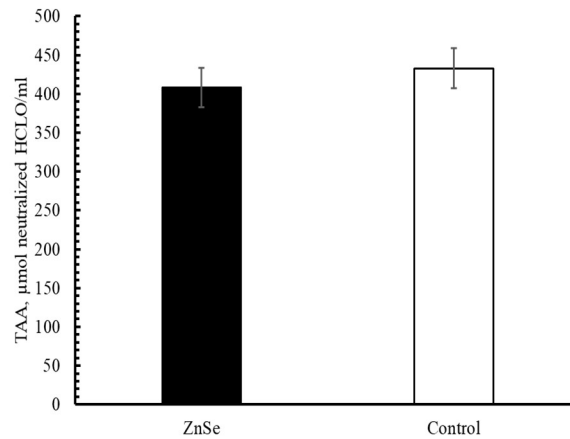


Figure 4. Total antioxidant activity (TAA) of milk in the experimental groups.

DISCUSSION

The results of the present study showed that antioxidant supplementation, SOD, Zn, and Se, in the diet of lactating buffaloes positively influenced milk yield and improved the milk clotting properties (MCP). The addition of an organic source of mineral antioxidant supplement did not affect dry matter intake, as well as in other studies carried out in buffaloes (Singh et al., 2021) and in dairy cows (Cortinhas et al., 2012), and it had no effect on the chemical characteristics of the milk.

Dry matter intake observed in this study is in line with what was recently reported by Salzano et al. (2021) for lactating buffaloes fed by TMR, and it is lower compared with that observed by Tufarelli et al. (2008) in a study carried out during spring. This study was carried out during summer and most likely could have reduced feed consumption in both groups. Both chemical composition and physical characteristics were not different between the two diets, and both distribution H.I. and S.I. were not different between the groups. These data indicate that preparation and distribution of diets and feeding behaviour of buffaloes were identical for the two groups.

Daily milk yield agrees with the survey conducted by Costa et al. (2020) on a large population of Italian water buffalo over a period of five years (8.81–9.24 kg/head/day), and it is lower than that reported by the A.I.A. statistics (AIA, 2022) (9.65 kg/head/day) referring to buffaloes raised in the Lazio region. Our results are lower than what was reported by Salzano et al. (2021). These differences could be due to different factors such as parity and the stage of lactation of the subjects used in the experiments (Singh and Ludri, 2001; Salari et al., 2013).

In the current study, the daily milk yield was improved by antioxidant supplementation (SOD, Zn, and Se). These results agree with findings by Singh et al. (2021), who found that the antioxidant supplementation (Cu, Zn, and vitamins A and E) improved milk production in buffaloes. Moreover, Kantwa et al. (2021) and Tanwar et al. (2019) reported a significant increase in the milk yield of buffaloes supplemented with chelated mineral mixture. Other studies have reported an increase in the milk yield in dairy cows supplied with organic mineral mixtures (Kellogg et al., 2004; Riad et al., 2018) and area-specific mineral mixtures (Kumar et al., 2020).

In agreement with other studies (Gupta et al., 2017; Kantwa et al., 2021), the greater milk yield of the treated group compared with the control group might be due to the effect of adding the minerals responsible for the stimulation of basket cells or myoepithelial cells of the udder and, in particular, Zn may improve udder health (Kellogg et al., 2004). Furthermore, Miranda et al. (2009) reported that low levels of antioxidants impaired oxidative status in the mammary gland, which was linked to a reduction in the number of mammary gland epithelial cells. Other authors (Wang et al., 2021) reported a reduction of apoptotic mammary gland epithelial cells after antioxidants supplementation.

Based on the literature and the results of the present investigation, in which an increased production performance was observed, with a comparable dry matter intake between the ZnSe group and control group, it is possible to hypothesise that the antioxidant treatment was able to improve the oxidative status of animals and milk-producing cells in the udder, leading to a more efficient utilisation of energy for milk production.

The content (%) of fat, protein, and lactose between the treatment and control group was not improved by the antioxidant supplementation in the buffaloes' diet. According to Kumar et al. (2020), the effect of area-specific mineral mixture supplementation in dairy cattle has positively influenced milk yield, but no significant differences were observed in the milk's fat, protein, and lactose contents. Moreover, Kellogg et al. (2004) showed that the supplementation of Zn-methionine in dairy cows improved the milk yield, and milk composition did not change. In contrast, De Andrade et al. (2016) reported that Se supplementation reduced SCC, fat, and protein percentages in buffalo milk. In a study by Singh et al. (2021), supplementation of antioxidant micronutrients in buffaloes improved milk fat and protein content and had no effect on the lactose content.

Milk fat content observed in the present study agrees with data reported by Costa et al. (2020a) and was greater than that reported by A.I.A. (2022). The protein percentage was similar to data

reported by A.I.A. (2022) and by Costa et al. (2020a). Lactose content was lower than in other studies on buffalo milk (Tripaldi et al., 2010; Costa et al., 2020a) and was higher compared with data reported by Niero et al. (2018). Differences for fat, protein, and lactose between our study and data from the literature are probably due to the different experimental conditions including days of lactation, number of lactations, seasons (Salati et al., 2013), age, health status, feed intake, concentration of the ration, etc.

Milk pH can be considered within the range of normality for buffalo milk (Tripaldi et al., 2010; Niero et al., 2018) and was not affected by treatment. To the best of our knowledge, there is a lack of information about the effects of antioxidant supplementation on the pH of buffalo milk. A recent study (Vizzotto et al., 2021) where extracts of oregano or green tea, endowed with antioxidant properties, were used as feed additives in Jersey cows during the transition period, reported that only the extract of oregano showed a tendency ($0.05 < p < 0.10$) in the reduction of milk pH compared to the control and green tea extract. Those authors explained the tendency of a lower pH with the lower content of SCC observed in the oregano-treated group compared to the other two groups.

Milk somatic cell count, and milk somatic cell score were not affected by the antioxidant treatment, as already reported in other studies (Kellogg et al., 2004; Tufarelli et al., 2008; Singh et al., 2021). Somatic cell count was lower than the values reported by Tripaldi et al. (2010) (314×10^3 cells/mL) and by Pasquini et al. (2018) for buffaloes bred in the Marche region (from 152.84 ± 25.22 to $199.73 \pm 23.43 \times 10^3$ cells/mL) and was higher compared with mid-lactating Murrah buffaloes in India (Singh and Ludri, 2001). The milk somatic cell score falls within the range reported by Costa et al. (2020b), which reported a range from 2.71 to 3.11. Differences between the results of the present study and findings from others might be also due to the different analytical methods (opto-fluoro-electronic vs. California Mastitis Test) applied.

The content of somatic cells is affected by the health of the udder, by buffalo productivity, by season, by parity, and by the lactation phase (Singh and Ludri, 2004; Bombade et al., 2018). Tripaldi et al. (2010) recommended 200×10^3 cells/mL as the limit for early identification of an animal affected by subclinical mastitis. Moreover, any change in environmental conditions, poor management practices, and stressful conditions significantly increases the number of somatic cells in milk. The level of somatic cells observed in the present study indicates good hygiene and proper nutrition and a low risk of pathogen infection in both groups, as well as optimal udder health conditions (Patil et al., 2015).

The result obtained for ECM agrees with what was reported in a survey by Costa et al. (2020a) (ECM: 14.36–15.75 Kg/d). Salzano et al. (2021) reported ECM values of 17.4 ± 0.4 kg/d due to the higher milk yield and fat percentage compared to data of the present study.

The FEr was not different between the two groups. Our result agrees with that reported by Khattab et al. (Khattab et al., 2010) in Egyptian buffaloes. Those authors found an FEr of 0.57 (with an average production of 8.9 L/head/d and DMI of 15.69 kg/head/d).

The data reported here showed that integration to the treated group with the SOD, Zn, and Se positively affected MCP. Milk clotting properties' values observed in the present study are similar to those reported by Costa et al. (2020b) in a study on 99 farms in the Lazio region ($RCT = 13.25 \pm 4.53$ min.; $k_{20} = 3.96 \pm 2.63$ min.; $a_{30} = 49.28 \pm 10.09$ mm). It is well known that a different coagulation time and rennet coagulation properties come from differences in milk composition linked to other factors than merely fat and protein (Ariota et al., 2007). In fact, the clotting properties of milk are affected by SCC, pH, titratable acidity (Politis and Ng-Kwai-Hang, 1988; Potena et al., 2007; Cecchinato et al., 2012; Bobbo et al., 2016), and the content of some minerals including Ca, P, and Ca/P ratio (Ariota et al., 2007), as well as days in milk (Cecchinato et al., 2012). In addition, the total casein content (CN) and the percentages of casein fractions affect them as well. It has been reported that a low concentration of κ -CN and a low proportion of κ -CN relative to the total CN were associated with poor and non-coagulating samples (Wedholm et al., 2006; Bonfatti et al., 2013). Post-translational modifications of caseins also are important; a higher proportion of glycosylated κ -casein seems to be associated with shorter rennet coagulation times (Jensen et al., 2012; Bonfatti et al., 2011). Furthermore, it is demonstrated that MCP are strongly influenced by the season (Malacarne et al., 2005; Bernabucci et al., 2015); in particular, the hot season makes these characteristics worse, making the milk less suitable to produce cheese. Regarding the rheological properties of milk, in the present study, the treatment significantly influenced the ability of milk to clot. It is not easy to explain the improvement of MCP while also considering the lack of differences for the other milk characteristics.

To date, there is no literature concerning the effects of dietary supplementation with SOD, Zn, and Se on the rennet characteristics of buffalo milk; therefore, this topic needs further investigation. In dairy cows, a supplement of mineral complex (Fe, Mn, Zn, Ca, Mg, P, Na, Cu, Cl, Calodat, Se, K) applied through the animals' drinking water improved milk yield, protein content, and a_{30} but had no effect on RCT and k_{20} (Angelova et al., 2021).

Some studies in dairy cows indicate that the use of trace minerals from organic sources in animal nutrition is important to maintain the antioxidant activity of the immune system and the health status of animals (Weiss and Hogan, 2005; Cortinhas et al., 2012). Faulkner and Weiss (2017) also suggested that trace minerals improved rumen fermentation and positively affected the nutrient digestibility. In addition, Pino and Heinrichs (2016) reported that, in dairy heifers, the supplementation of organic trace minerals (Cu, Zn, Mn, Se, and Co) had no effect on nutrient digestibility but increased total volatile fatty acids production. Therefore, in the present study, the dietary supplementation with SOD, Zn, and Se likely might have provided a greater available amount of energy to the buffalo's udder, to be used for daily milk production and probably also to carry out the post-translational modifications of the caseins, which play a role in milk clotting properties.

Integration with SOD, Zn, and Se had no effect on buffalo milk TAA. The lack of antioxidant integration on milk TAA in the treated group compared to the control group might be due to the buffalo's use of antioxidants supplementation to maintain their health status and to increase milk production. It is particularly difficult to compare the different milk TAA values reported in the literature because they were obtained by means of diverse analytical methodologies, but the antioxidant capacity of buffalo milk is greater than that of milk from other dairy species (Cloetens et al., 2013), probably because buffalo milk has a high concentration of many antioxidant compounds (Ahmad et al., 2013). TAA values obtained in this study can be compared to those reported by Beghelli et al. (2016), who adopted the same assay to measure milk TAA in different dairy species (goats, ewes, cows, and donkeys) and confirmed that buffalo milk has greater antioxidant capacity than milk from other species.

CONCLUSIONS

The effect of Zn, Se, and SOD supplementation on the productivity and quality of buffalo milk has been investigated. Feed intake, feeding behaviour, and feed efficiency were not affected by the treatment. The treatment positively improved milk yield (+3.1%) and the coagulation properties of the milk, and no differences were found for other milk parameters. It is not easy to explain the improvement of MCP, but the benefits of an appropriate antioxidant supplementation for improving the cheesemaking properties of milk are therefore of interest to the profits of farmers, since 100% of buffalo milk is used for cheese production. Further research is needed to investigate more in depth the effects of supplementation with Zn, Se, and SOD on milk coagulation properties considering other parameters that are strictly linked with milk coagulation attitude.

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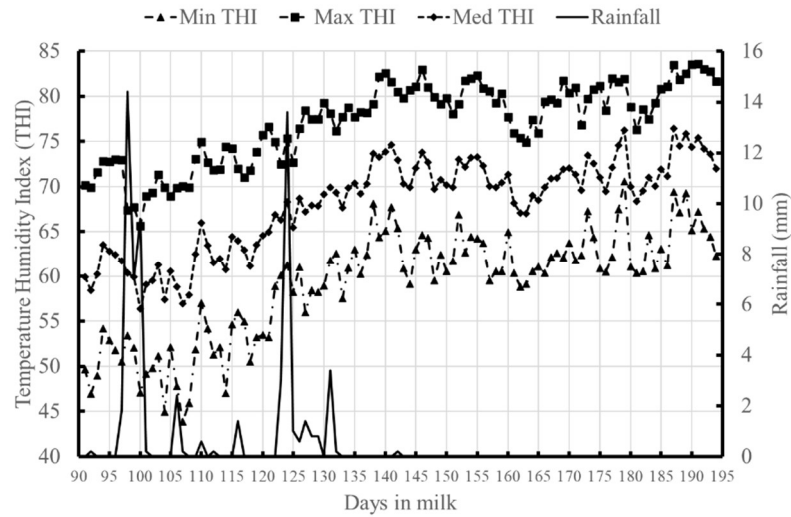


Figure S1. The temperature-humidity index (minimum, maximum, and average) and rainfall from the nearest weather station (Cotarda-Pontinia, located 4 km from the farm) during the experimental period.



Figure S2. The two groups of buffaloes tested were housed under the same environmental conditions.

Sub Chapter 4.2

Particle size distribution of total mixed rations fed to Italian Mediterranean buffaloes measured by the PSPS: impact on milk quality and digestibility

Evangelista Chiara¹; Petrocchi Jasinski Francesca²; Basiricò Loredana², Turriziani Giovanni² and Bernabucci Umberto²

¹ Department for Innovation in Biological Agro-Food and Forest Systems, University of Tuscia, Italy

² Department of Agriculture and Forests Sciences, University of Tuscia, Italy

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ABSTRACT

Buffalo breeding in Italy is becoming increasingly economically attractive, with milk production in the Lazio region growing by 15% since 2004. However, the optimal physical characteristics of total mixed rations (TMR) have not been thoroughly investigated. This lack of research suggests the need for greater attention to the physical characteristics of diet, which, combined with its chemical properties, may negatively impact milk yield (MY) and milk quality (MQ), as seen in dairy cows.

Therefore, the objective of this study was to assess the effect of diet composition, with a particular focus on the physical component, on MY and MQ, and apparent digestibility (AD) of dairy buffaloes. The study was conducted on 10 dairy buffalo farms in Amaseno valley (Italy). Monthly samples of TMR, faeces, and bulk milk were collected over a 12-month period, alongside interviews with farmers to gather information on MY and diet composition. The results revealed a high variability in the physical-chemical composition of diets across farms, attributable to differences in feeding management practices. Both chemical and physical component were found to be associated with MY, MQ and AD changes. A ration poor in roughage and rich in concentrate negatively affected MQ showing lower milk fat, and higher somatic cells count and milk urea, while a greater presence of coarse particles and high fiber were associated with lower MY. Nevertheless, coarse particles and aNDF were positively associated with milk clotting parameters and aNDF digestibility. Our study emphasized the high importance of fiber for improving MQ of dairy buffaloes.

Keywords: dairy buffalo, diets, physical particles, milk quality, digestibility

Highlights

1. Particle size distribution of dairy buffalo diet is affected by raw material and feeding management.
2. Dairy buffalo unbalanced diets (rich in fiber and low in starch) can negatively affect milk yield.
3. Physical characteristics of diets can positively affect milk clotting parameters of buffalo milk.

INTRODUCTION

Nutritional requirements of dairy buffaloes have long been studied and established, in relation to the physiological phases and production level (Bertoni, 1994; Zicarelli, 1999; Bartocci et al., 2002). Suggestions were provided concerning the chemical composition of the diet, but no recommendations were developed concerning its physical characteristics. The lack of research on physical characteristics of dairy buffalo diets can be attributed to the historically limited economic interest in this species until recently. It should by now be considered that genetic selection has made great strides on this species as well, having nowadays buffaloes with an average production of 9.18 kg/head/d (data referring to the Lazio region 2022 production, AIA, 2023) compared with 7.83 kg/head/d recorded in the same area for the year 2004. This means that it has become an animal that is no longer rustic. Consequently, to sustain the production level reached, it needs a specific physical-chemical composition of the diet, an accurate diet fine tuning and careful preparation of the TMR. Moreover, today dairy buffaloes farming is becoming more and more interesting in Italy, even because mozzarella cheese has become one of the key points of Italian exports. Notably, buffalo milk is used almost exclusively for cheese-making of mozzarella, a typical fresh and stringy-textured cheese certified with European Protected Designation of Origin (PDO) as “Mozzarella di Bufala Campana” (EC 103/2008). Additionally, the prevailing notion that buffaloes are capable of consuming substantial amounts of fiber and converting it into high-quality milk has further contributed to the oversight in investigating their specific dietary requirements (Bülbül, 2010).

Although buffalo milk yield and composition are influenced by a series of intrinsic and extrinsic factors, such as age, health status, lactation stage, milking technique, environmental conditions, and seasons, feeding represents one of the major managerial factors affecting productivity. It is important to note that a correct diet is crucial for supporting animal health, well-being, and production performance. Both the quality and the physical form of diet is essential to support normal rumen function and its health (Allen, 1997). The determination of the dietary physical characteristics can be performed using a sieve, as developed and modified by Heinrichs (2013) for dairy cows TMR. Sieve allows to classify particles into four fractions: long (> 19 mm), medium (< 19 and > 8 mm), short (< 8 and > 4 mm), and fine (< 4 mm). Another critical parameter to be considered is the physically effective neutral detergent fiber (peNDF), developed by Mertens (1997).

Mertens (1997) integrated the concept of sufficient chemical NDF with physical structure to establish peNDF as a metric capturing the physical attributes of fiber, considering both particle length and NDF content. Physically effective NDF plays a crucial role in supporting chewing and

facilitating the flow of salivary buffers to the rumen, maintaining a normal rumen environment (Mertens, 1997). As the average particle size decreases, peNDF also decreases, leading to a reduction in chewing time and rumen pH, primarily due to diminished saliva production and its buffering function (Grant et al., 1990; Simoni et al., 2021). Moreover, adequate NDF is crucial in dairy diets to support normal rumen activity, prevent milk fat depression, and address health problems associated with rumen acidosis (Caccamo et al., 2014). Numerous studies have investigated the effects of particle size distribution and peNDF on feed intake, digestibility, and performance in dairy cows (Righi et al., 2007; Henrichs, 2013; Caccamo et al., 2014; Fustini et al., 2016; Haselmann et al., 2019; Simoni et al., 2021). However, there is currently a lack of research regarding dairy buffalo.

The different physical characteristics of the diet could exert a substantial influence on buffalos' apparent digestibility, milk yield and milk quality. Consequently, the study aimed at examining the association between diet particle size distribution, as determined by the Penn State Particle Separator, apparent digestibility, milk yield and milk quality in typical buffalo dairy farms in Central Italy.

MATERIALS AND METHODS

The study lasted 12 months and 10 dairy buffalo farms, representative of the Amaseno valley in Lazio region (Figure 1S) were involved. This area falls within the production area of “Mozzarella di Bufala Campana (PDO)”. The farms were located within the same area and distribute over an area of approximately 7 km² (Figure 1S). The farms had an overall average size ($\pm$ SD) of 149 ($\pm$ 75) lactating buffaloes, with an overall average land size of 34.4 hectares ($\pm$ 14.83). The feeding management for all farms consisted of total mixed ration (TMR), prepared using a mixer wagon (MW) (refer to Table 1S for additional details on the farms participating in this study). To assess the physical-chemical characteristics of diets and obtain information on the apparent digestibility of diet, samples of TMR and faeces were collected during each visit in each farm. Moreover, bulk raw milk samples were collected during each visit to assess its quality. Additional details regarding milk yield, diet composition, and mixer wagon preparation were gathered through interviews with the farmers. The interviews were carried out by drafting a questionnaire.

Sampling procedures

Total mixed ration samples were collected once a month for each farm, immediately after morning distribution. Three samples were taken from the feed bunk (corresponding to the beginning, middle and end of the bunk). One aliquot was analysed for on-farm physical analyses. The rest was mixed

to produce a single TMR sample on which chemical analyses have been carried out. Faeces samples were collected after morning milking, directly from the rectal ampoule on a representative sample of animals, approximately 10% of the entire herd. All collected faeces samples were mixed and homogenized to create a single representative sample. From this, an aliquot of approximately 1.0 kg was taken for laboratory analyses. The pH of the faeces was determined immediately after collection with a portable pH meter (“XS Tester”, Giorgio Bormac, Italy). Both TMR and faeces samples were stored under refrigerated conditions (4°C) and transported to the laboratory for chemical analysis.

Physical characteristics and quality index of TMRs

Total mixed rations were analyzed for physical characteristics on farms using a 3-screen system (19.0, 8.0, and 4.0 mm) Penn State Particle Separator (PSPS), following the protocol outlined by Heinrichs (2013), utilizing approximately 400 g of material for sieving. In total, 360 TMR samples were sieved. To determine the average length of the TMR particles, 10 random particles were measured with a ruler for each sieve (for a total of 60 values for each sample). The average of these values represents the ration particle length (RPL) and was expressed in cm. Moreover, geometric mean particle length (GMPL) was estimated using the method described by Armentano and Taysom (2005) by using the following formula:

$$\text{GMPL (mm)} = 0.54 + 11.84 * (\text{US} + \text{MS}).$$

where US is the upper sieve and MS the middle sieve. US and MS were expressed as fraction of the unit.

Homogeneity index (HI) and sorting index (SI) (Serva et al., 2021) of TMR were determined on farm using a portable spectrophotometer (PoliSPEC^{NIR} diode array spectrometers, ITPhotonics, Italy) according to the protocol provided by the manufacturer. The procedure consists in directly scanning the TMR with PoliSPEC^{NIR} at a total of 9 points along the feeding line for HI, and 18 points (9 in front of the animals and 9 in front of the corridor) for SI. The HI scans were carried out immediately after the morning feed distribution, and the SI scans were carried out 2 hours after the morning TMR distribution.

Chemical analysis of TMRs, faeces and apparent digestibility

A total of 120 TMR and 120 faeces samples were analysed by wet chemistry. Dry matter (DM) was determined after oven drying at 65°C to constant weight. Total mixed rations and faeces samples were ground through a mill (Retsch Müller, Germany) to pass a 1 mm screen. Then,

samples were analyzed for crude protein (CP, AOAC, 2005), and total starch (AOAC, 2005) using a K-TSTA assay kit (Megazyme International, Bray, Ireland). Neutral detergent fiber (aNDF) and acid detergent lignin (ADL) were analyzed using an Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY) according to Van Soest et al. (1991). Chemical data are reported as percentages on DM basis. Physically effective NDF was estimated by multiplying the concentration of aNDF by the proportion of particles retained by the 19.0-, 8.0-, and the 4.0 mm sieves as proposed by Mertens (1997), and was expressed as a percentage.

Apparent digestibility was estimated for aNDF (dNDF), starch (dStarch) and crude protein (dProtein) following equation (1) by Fredin et al. (2014) using ADL as a marker and expressed as % on DM; apparent digestibility of DM (dDM) was estimated by equation (2).

$$(1) \text{ Apparent digestibility (\%)} = \left\{ 1 - \left[\frac{ADL \text{ TMR}}{ADL \text{ faeces}} \times \frac{\text{Analyte faeces}}{\text{Analyte TMR}} \right] \right\} \times 100$$

$$(2) \text{ Apparent digestibility DM (\%)} = \left\{ \left[\frac{ADL \text{ faeces} - AD \text{ TMR}}{ADL \text{ faeces}} \right] \right\} \times 100$$

Since the presence of green fodder (GF) was not constant in the diets, but only present in some farms in certain periods of the year (from February to September), we decided to subdivide the diets according to the GF presence: 91 diets did not contain GF (GF-free) and 29 had GF (GF-fed). The composition of two diets were reported in table 2S.

Milk yield and characteristics

Milk yield (MY) was recorded for each farm every month. Bulk raw milk samples were taken every month in all farms. The milk samples were taken directly from the tank and obtained from two milkings (evening + morning) and packaged in 500 mL sterile glass bottle. Samples were refrigerated (4°C) and analyzed within 24 to 36 h from collection. A total of 120 milk samples were analyzed. Fat, protein, lactose (%), and urea (mg/dL) were determined by I.R. spectrophotometry (MilkoScanTM7 RM, FOSS, Foss Allé 1 DK-3400 Hillerød, Denmark), somatic cells count (SCC) using a fluoro-opto-metric device (Fossomatic 5000 series; Foss Electric, Hillerød, Denmark); titratable acidity (°SH/100ml) and pH by potentiometric method. Milk clotting parameters [RCT (rennet coagulation time, min); k₂₀ (curd firming time, min); a₃₀ (curd firmness, mm, at 30 min)] were determined by lactodynamography (Mape System, Firenze, Italy).

Milk yield was converted into buffalo energy-corrected milk (ECM) using the following formula (Campanile et al., 1998):

$$Y = \{[(MF - 40) + (MP - 31)] \times 0.01155 + 1\} * MY$$

where Y is the amount (kg) of ECM equivalent to 1 kg of milk produced; MY is the milk yield; MF is the percentage of milk fat; MP is the percentage of milk protein.

To obtain a normal distribution of the SCC, somatic cell score (SCS) was calculated according to the following formula (Wiggans and Shook, 1987):

$$SCS = \log_2 (SCC/100,000) + 3$$

Statistical analyses

For dairy buffaloes, there is currently no indication of ideal physical composition of the diet, so it has not been possible to rely on reference data. The creation of the classes took place by evaluating the distribution of the observations in the US. The first sieve was chosen because it presents a high variability and better characterizes buffalo' diet.

Since the data did not follow a normal distribution, these were transformed into $\log_{10}$. To create three homogeneous classes, the data of the first sieve have been classified according to the following method for GF-free diets: diets with percentage of US ≥ 18.5 % goes to constitute the first class; diets with US ≤ 8 % goes to constitute the third class and the intermediate class consisted of US data between 18.4 - 8.1 %. For GF-fed diets: ≥ 32 % of US goes to constitute the first class; data ≤ 16 % go to constitute the third class and the intermediate class consisted of data between 31.9 - 16.1 %.

Statistical analyses were carried out using the software STATISTICA version 10.0. A first approach was about descriptive statistic regarding the characteristics of TMR, bulk milk, faeces and apparent digestibility for both groups of diets (GF-free and GF-fed).

To examine differences in physical and chemical compositions of the diets, MY, milk characteristics and apparent digestibility across particle size classes, the following mixed model with farm nested within class was applied:

$$Y_j = u_i + class_j + farm_k(class_j) + season_l + \varepsilon_{jkl}$$

where Y_j = mean of interest variables; u_i = overall mean for the item; $class_j$ = class based on upper sieve categories (1, 2 and 3); $farm_k(class_j)$ = farm nested in the particle size class (10 farms for GF-free and 6 for GF-fed diets) (random factor); $season_l$ (1, ..4); and ε_{jkl} = residual, random error.

To highlight the correlation among diets characteristics and MY, ECM, milk quality and apparent digestibility, a linear correlation model based on Pearson coefficient was used, for both diets (GF-free and GF-fed).

To test the differences between the diets for the overall means of all parameters a one-way analysis of variance (ANOVA) was carried out using “diet” (GF-free and GF-fed) as a factor. To test the differences in TMR composition among farms, a one-way analysis of variance (ANOVA) was carried out using “farm” as a factor. The significance was declared at $p < 0.05$.

RESULTS

Descriptive statistics

Table 1 show the means ($\pm$ SD) of TMR composition of GF-free and GF-fed diets. Results show a very high variability for all sieves including the bottom. GF-fed diets report a higher percentage of particles retained in the US (17.18 vs. 24.87 % for GF-free and GF-fed, respectively, $p < 0.05$). The GF used was not chopped, and this had a strong effect on US length variability. Consequently, GF-fed diets had less material in MS ($p < 0.01$) and had a much higher ($p < 0.00$) RPL than GF-free diets. The peNDF was slightly higher in GF-fed diets and presented lower variability. As expected, the moisture content of the GF-fed diets was on average 5 percentage points higher than the GF-free diets ($p < 0.00$). The starch, aNDF and protein were comparable even though GF-fed diets had less variability probably because they were numerically lower (29 vs. 91 observations for GF-fed and GF-free, respectively). Homogeneity index for both diets was found to be “sufficiently homogeneous” ($50 \leq HI \leq 65$) while the SI after 2 h, was classified for both the diets as “marked sorting” ($40 \leq SI \leq 60$). The average ($\pm$ SD) chemical-physical composition of the 10 farms is shown in table 2S.

Table 1. Physiochemical compositions of diets: GF-free and GF-fed diets (means±SD).

	GF-free n. 91	GF-fed n. 29
US (%)	17.18±14.16 ^b	24.87±13.27 ^a
MS (%)	24.12±8.90 ^A	19.02±4.60 ^B
LS (%)	32.74±9.41	31.13±6.66
Bottom (%)	26.03±5.87	24.98±3.92
peNDF (%)	33.62±6.04	34.00±3.55
RPL (cm)	4.74±0.94 ^B	5.78±1.11 ^A
GMPL (mm)	5.43±1.35	5.74±1.13
DM (%)	53.64±6.28 ^A	48.23±5.38 ^B
aNDF (%)	45.16±5.29	45.28±3.57
CP (%)	12.06±1.82	12.37±1.48
Starch (%)	13.95±2.94	14.87±3.27
HI	55.00±14.49	51.00±12.65
SI	44.36±16.89	45.06±17.58

GF = green fodder; US = upper sieve = % of diet retained by a sieve with holes of 19 mm; MS = middle sieve = % of the diet retained by a sieve of 8 mm; LS = lower sieve = % of the diet retained by a sieve of 4 mm; Bottom = % of diet with dimensions < 4 mm; peNDF = physically effective NDF; RPL = ration particle length; GMPL: geometric mean particle length; DM = dry matter; aNDF = neutral detergent fiber; CP = crude protein; HI = Homogeneity Index; SI = Sorting Index. ^{a, b} $p < 0.05$; ^{A, B} $p < 0.01$

Table 2 show the means (±SD) of MY, ECM and milk composition as well as rheological parameters of buffalo's milk for GF-free and GF-fed diets. Both MY and ECM were on average greater ($p < 0.01$ and $p < 0.05$, respectively) in GF-fed compared with GF-free diet. The other parameters are comparable between the 2 diets except for lactose which was higher ($p < 0.05$), and SCC, and consequently SCS, which were lower ($p < 0.05$) in GF-fed compared with GF-free diet. As regards the rheological parameters, the consistency of the curd was higher ($p < 0.05$) in the GF-fed diets (27.49 vs. 22.18 mm, for GF-free and GF-fed, respectively).

Table 2. Milk yield, ECM and milk characteristics (means $\pm$ SD).

	GF-free	GF-fed
MY (Kg/h/d)	8.09 $\pm$ 1.50 ^B	8.95 $\pm$ 0.84 ^A
ECM (Kg/h/d)	11.53 $\pm$ 4.68 ^b	13.58 $\pm$ 2.29 ^a
Fat (%)	8.16 $\pm$ 0.57	8.11 $\pm$ 0.43
Protein (%)	4.71 $\pm$ 0.17	4.74 $\pm$ 0.12
Lactose (%)	4.58 $\pm$ 0.09 ^b	4.63 $\pm$ 0.06 ^a
Urea (mg/dL)	40.44 $\pm$ 6.60	41.20 $\pm$ 6.27
Titrateable acidity ($^{\circ}$ SH/100ml)	7.21 $\pm$ 0.93	7.39 $\pm$ 0.82
pH	6.76 $\pm$ 0.13	6.72 $\pm$ 0.23
SCC (n/ml*)	305.15 $\pm$ 183.64 ^a	211.97 $\pm$ 122.21 ^b
SCS	3.94 $\pm$ 0.60 ^a	3.62 $\pm$ 0.49 ^b
RCT (min.)	22.22 $\pm$ 5.11	22.04 $\pm$ 4.45
k ₂₀ (min)	4.71 $\pm$ 3.06	4.00 $\pm$ 0.61
a ₃₀ (mm)	22.18 $\pm$ 9.22 ^b	27.49 $\pm$ 9.67 ^a

GF = green fodder; h = head; d = day; MY = milk yield; ECM = energy correct milk; SCC = Somatic Cell Count; SCS = somatic cell score; RCT = rennet coagulation time at 30 min; k₂₀ = curd firming time; a₃₀ = curd firmness. * x1000
^{a, b} p < 0.05; ^{A, B} p < 0.01

Table 3 shows the means ($\pm$ SD) of fecal characteristics and apparent digestibility for GF-free and GF-fed diets. The faeces chemical composition does not strongly differ between the two diets. There was evidence of a greater (p < 0.05) presence of lignin (ADL) in GF-free diets (14.84 vs. 13.79 %, for GF-free and GF-fed, respectively) probably because lignin is very low in diets with GF. The apparent digestibility of all parameters was not affected by the diet.

Table 3. Buffalo faeces chemical composition and apparent digestibility coefficient of DM, 166nd, starch and protein (means $\pm$ SD).

	GF-free	GF-fed
DM (%)	13.74 $\pm$ 1.40	13.71 $\pm$ 0.79
CP (%)	13.87 $\pm$ 1.29	14.04 $\pm$ 1.21
aNDF (%)	56.32 $\pm$ 3.52	55.80 $\pm$ 3.56
ADL (%)	14.84 $\pm$ 2.26 ^a	13.79 $\pm$ 1.58 ^b
Starch (%)	1.74 $\pm$ 1.01	2.00 $\pm$ 1.59
pH	6.44 $\pm$ 0.24	6.40 $\pm$ 0.26
dDM (%)	54.50 $\pm$ 10.00	53.21 $\pm$ 8.48
daNDF (%)	44.73 $\pm$ 10.91	42.44 $\pm$ 9.07
dStarch (%)	94.02 $\pm$ 4.20	93.92 $\pm$ 3.87
dProtein (%)	48.51 $\pm$ 11.81	46.03 $\pm$ 13.18

GF = green fodder; DM: dry matter; CP: crude protein; 166nd: neutral detergent fiber; ADL: acid detergent lignin; d = apparent digestibility coefficient; 166nd: neutral detergent fiber. ^{a, b} p < 0.05

Particle size classes

The partitioning of diets into classes based on the US is presented in table 4 for GF-free diets and in table 5 for GF-fed diets. Moreover, also MY, milk characteristics and apparent digestibility as a function of the 3 classes are reported. The three classes had an homogeneous number of observations, 30, 31 and 30 for GF-free diets and 10, 10 and 9 for GF-fed diets for 1st, 2nd and 3rd class, respectively.

For GF-free diets (Table 4), all physical and chemical parameters are very different between classes except for the content of CP and the RPL that did not change according to the class. Furthermore, the class did not affect the qualitative indices (HI and SI) of the TMR. In the 1st class (diets with a US content ≥ 18.5 %), there was lower percentage of particles retained in the remaining sieves (middle sieve (MS) and bottom in particular) and starch content, and there was the highest peNDF and aNDF contents. In addition, the 1st class had lower ($p < 0.00$) DM content compared with the other classes. Milk yield was not substantially affected by classes. The differences in the chemical composition of milk were in the content of SCC, SCS and a₃₀. a₃₀ values were higher in the 1st class. Somatic cell content and SCS were much lower ($p < 0.00$) in 1st class and tend to increase in 2nd and 3rd class. No differences were found between classes for the apparent digestibility. Farm affected several parameters such as bottom, peNDF, RPL, DM, aNDF, CP, MY, ECM, milk fat and protein content, SCC, SCS, a₃₀ and starch digestibility. The season affected multiple aspects of both the composition of the diet, MY, milk characteristics, and apparent digestibility.

Diets containing GF (Table 5) showed differences between US class for physical parameters, peNDF and DM. The 1st class had lower values of MS and LS and comparable values with the 2nd class for bottom and peNDF. Dry matter resulted greater ($p < 0.05$) in the 2nd class. Considering MY and milk characteristics only SCC and SCS were affected by US class. Somatic cells content was lower ($p < 0.01$) in the 1st class and higher ($p < 0.05$) in the 3rd class. No differences between US classes were found for the apparent digestibility. Farm, as opposed to GF-free diets, did not affect many parameters. The only affected parameters were DM, SCC, SCS and DM digestibility. The season was found to affect very few characteristics of both the composition of the diet, milk production and apparent digestibility.

Table 4. Least squares mean for physiochemical composition of diets, MY, milk characteristics, and apparent digestibility based on US 'particle sizes classes (PSCs) for the GF-free diets (91 samples).

Classes of US	1 ≥18.5 %	2 18.4-8.1%	3 ≤8%	SEM	PSCs	Farms	Season
<i>Physiochemical composition of diet</i>							
US (%)	33.41 ^A	13.90 ^B	4.33 ^C	1.48	***	ns	ns
MS (%)	17.05 ^B	28.61 ^A	26.53 ^A	0.93	***	ns	***
LS (%)	28.22 ^B	29.48 ^B	40.62 ^A	0.99	***	ns	***
Bottom (%)	21.31 ^B	27.99 ^A	28.73 ^A	0.62	***	***	ns
peNDF (%)	39.27 ^A	31.77 ^B	29.87 ^B	0.63	***	***	***
RPL (cm)	4.87	4.81	4.55	0.10	ns	*	*
GMPL (mm)	6.51 ^A	5.57 ^B	4.19 ^C	0.14	***	*	***
DM (%)	51.13 ^B	54.82 ^A	54.95 ^A	0.66	***	***	**
aNDF (%)	49.77 ^A	44.08 ^B	41.65 ^C	0.55	***	***	**
CP (%)	11.33	12.19	12.65	0.19	ns	***	*
Starch (%)	11.80 ^B	14.61 ^A	15.42 ^A	0.31	**	ns	*
HI	52.78	56.02	56.17	1.52	ns	ns	*
SI	44.09	42.86	46.17	1.77	ns	ns	*
<i>Milk yield and characteristics</i>							
MY (Kg/h/d)	7.87	7.90	8.52	0.16	ns	***	ns
ECM (Kg/h/d)	10.80	10.91	12.90	0.49	ns	***	ns
Fat (%)	8.12	8.30	8.05	0.06	ns	***	***
Protein (%)	4.67	4.75	4.71	0.02	ns	***	*
Lactose (%)	4.57	4.59	4.59	0.01	ns	ns	*
Urea (mg/dL)	40.84	40.81	39.65	0.69	ns	ns	ns
Titrateable acidity (°SH/100ml)	7.13	7.48	7.00	0.10	ns	ns	***
pH	6.76	6.78	6.74	0.01	ns	ns	*
SCC (n./ml)	213.37 ^C	312.61 ^B	389.23 ^A	19.25	***	***	ns
SCS	3.67 ^C	3.97 ^B	4.19 ^A	0.60	***	***	ns
RCT (min.)	20.83	22.73	23.13	0.54	ns	ns	*
k20 (min.)	5.40	4.18	4.33	0.39	ns	ns	ns
a ₃₀ (mm)	25.66 ^A	18.62 ^B	22.82 ^{AB}	1.01	**	*	ns
<i>Apparent digestibility</i>							
dDM (%)	53.53	55.77	54.17	1.05	ns	ns	***
dNDF (%)	47.12	46.54	40.14	1.18	ns	ns	***
dStarch (%)	93.82	93.47	94.79	0.44	ns	**	*
dProtein (%)	45.65	49.60	50.16	1.27	ns	ns	**

SEM= standard error of mean; US = upper sieve = % of diet retained by a sieve with holes of 19 mm; MS = middle sieve = % of the diet retained by a sieve of 8 mm; LS = lower sieve = % of the diet retained by a sieve of 4 mm; Bottom = % of diet with dimensions < 4 mm; peNDF = physically effective NDF; RPL = ration particle length; GMPL = geometric mean particle length; DM = dry matter; aNDF = neutral detergent fiber; CP = crude protein; HI = homogeneity Index; SI = Sorting Index; MY = milk yield; ECM = energy correct milk; SCC = Somatic Cell Count; SCS = Somatic Cell Score; RCT = rennet coagulation time at 30 min; k20 = curd firming time, min; a₃₀ = curd firmness; dDM = apparent digestibility of DM; dNDF = apparent digestibility of NDF; dStarch = apparent digestibility of Starch; dProtein = apparent digestibility of Protein. ns= not significant

a, b, c = p < 0.05; A, B, C = p < 0.01; *p < 0.05; **p < 0.01; ***p < 0.001

Table 5. Least squares mean for physiochemical composition of diets, MY, milk characteristics, and apparent digestibility based on US 'particle sizes classes (PSCs) for the GF-fed diets (29 samples).

Classes of US	1 ≥32 %	2 31.9- 16.1%	3 ≤16%	SEM	PSCs	Farms	Season
<i>Physiochemical composition of diet</i>							
US (%)	41.33 ^A	23.26 ^B	11.66 ^C	2.46	***	ns	ns
MS (%)	13.41 ^B	20.62 ^A	22.48 ^A	0.85	***	ns	ns
LS (%)	23.46 ^C	31.81 ^B	37.37 ^A	1.24	***	ns	ns
Bottom (%)	21.82 ^b	24.32 ^b	28.47 ^a	0.73	*	ns	*
peNDF (%)	36.34 ^a	34.40 ^{ab}	31.49 ^b	0.66	*	ns	ns
RPL (cm)	5.35	5.97	5.96	0.21	ns	ns	ns
GMPL (mm)	7.02 ^A	5.74 ^B	4.58 ^C	0.21	***	ns	ns
DM (%)	45.88 ^b	51.50 ^a	47.09 ^b	1.00	*	***	*
aNDF (%)	46.45	45.44	44.07	0.66	ns	ns	ns
CP (%)	13.38	11.49	12.33	0.28	ns	ns	ns
Starch (%)	14.09	15.10	15.35	0.61	ns	ns	ns
HI	46.83	51.61	54.14	2.35	ns	ns	ns
SI	47.83	51.24	36.39	3.27	ns	ns	ns
<i>Milk yield and characteristics</i>							
MY (Kg/h/d)	9.46	8.62	8.84	0.16	ns	ns	**
ECM (Kg/h/d)	15.06	12.58	13.23	0.42	ns	ns	**
Fat (%)	8.18	8.07	8.09	0.08	ns	ns	**
Protein (%)	4.69	4.73	4.79	0.02	ns	ns	***
Lactose (%)	4.62	4.65	4.61	0.01	ns	ns	**
Urea (mg/dL)	45.21	42.07	36.72	1.17	ns	ns	ns
Titrateable acidity (°SH/100ml)	7.34	7.51	7.32	0.15	ns	ns	*
pH	6.80	6.73	6.64	0.05	ns	ns	ns
SCC (n./ml)	156.78 ^A	211.90 ^B	261.70 ^B	22.69	**	***	ns
SCS	3.39 ^B	3.62 ^{AB}	3.84 ^A	0.09	**	***	ns
RCT (min.)	22.36	21.38	22.42	0.83	ns	ns	ns
k20 (min.)	3.90	4.04	4.04	0.12	ns	ns	ns
a ₃₀ (mm)	29.13	29.31	24.36	1.83	ns	ns	ns
<i>Apparent digestibility</i>							
dDM (%)	54.04	53.45	52.22	1.57	ns	**	***
dNDF (%)	44.15	42.35	40.98	1.68	ns	ns	***
dStarch (%)	92.94	94.06	94.66	0.72	ns	ns	ns
dProtein (%)	49.52	47.65	48.30	2.45	ns	ns	**

SEM= standard error of mean; US = upper sieve = % of diet retained by a sieve with holes of 19 mm; MS = middle sieve = % of the diet retained by a sieve of 8 mm; LS = lower sieve = % of the diet retained by a sieve of 4 mm; B= bottom = % of diet with dimensions < 4 mm; peNDF = physically effective NDF; RPL = ration particle length; GMPL = geometric mean particle length; DM = dry matter; aNDF = neutral detergent fiber; CP = crude protein; HI = homogeneity Index; SI = Sorting Index; MY = milk yield; ECM = energy correct milk; SCC = Somatic Cell Count; SCS = Somatic Cell Score; RCT = rennet coagulation time at 30 min; k20 = curd firming time, min; a₃₀ = curd firmness; dDM = apparent digestibility of DM; dNDF = apparent digestibility of NDF; dStarch = apparent digestibility of Starch; dProtein = apparent digestibility of Protein. ns= not significant
a, b, c = p < 0.05; A, B, C = p < 0.01; *p < 0.05; **p < 0.01; ***p < 0.001

Correlation between diet characteristics and milk production

Figure 1 shows the correlations between the characteristics of GF-free diets and MY, ECM and milk characteristics. Milk traits which were not correlated with diet characteristics (such as ECM, pH and k_{20}) are not reported.

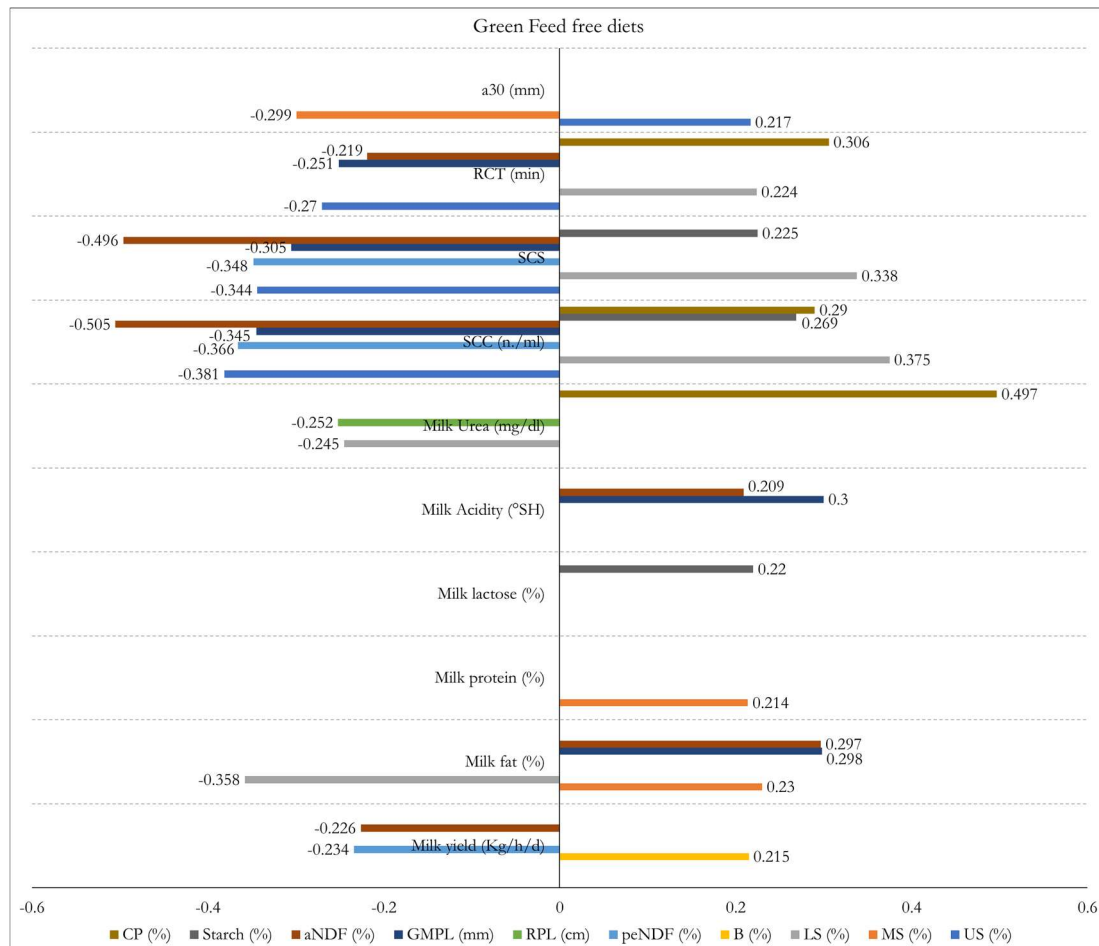


Figure 1. Direction and magnitude of Pearson correlation coefficients between physical and chemical characteristics of the diets and milk yield, ECM and milk characteristics for GF-free diets.

US = upper sieve = % of diet retained by a sieve with holes of 19 mm; MS = middle sieve = % of the diet retained by a sieve of 8 mm; LS = lower sieve = % of the diet retained by a sieve of 4 mm; B = bottom = % of diet with dimensions < 4 mm; peNDF = physically effective NDF; RPL = ration particle length; GMPL = geometric mean particle length; aNDF = neutral detergent fiber; CP = crude protein; SCC = Somatic Cell Count; SCS = Somatic Cells Score; RCT = rennet coagulation time at 30 min; a_{30} = curd firmness.

Milk yield showed negative correlations with aNDF ($r = -0.226$, $p < 0.001$) and with peNDF ($r = -0.234$, $p < 0.05$), and a positive correlation with bottom ($r = 0.215$, $p < 0.05$). Regarding milk characteristics, a positive correlation between milk fat and MS ($r = 0.230$, $p < 0.05$), aNDF ($r = 0.297$, $p < 0.001$) and GMPL ($r = 0.298$, $p < 0.01$) were observed. Milk fat was negatively

correlated with LS ($r = -0.358$, $p < 0.001$). Like milk fat, milk proteins were positively related to MS ($r = 0.214$, $p < 0.05$). Milk lactose showed a positive correlation only with starch content ($r = 0.220$, $p < 0.05$). Somatic cell content was associated with several parameters. It was strongly negatively related to US, aNDF, peNDF and GMPL ($r = -0.381$, $p < 0.001$; $r = -0.505$, $p < 0.001$; $r = -0.366$, $p < 0.001$; $r = -0.345$, $p < 0.01$ for US, aNDF, peNDF and GMPL, respectively). On the other side, SCC had positive correlation with LS, and content of protein and starch ($r = 0.375$, $p < 0.001$; $r = 0.290$, $p < 0.01$; $r = 0.269$, $p < 0.05$ for LS, protein, and starch, respectively). As expected, SCS showed the same correlations as for SCC: positive with LS and starch ($r = 0.338$, $p < 0.01$, $r = 0.225$, $p < 0.05$ respectively), negative with US, peNDF, aNDF and GMPL ($r = -0.344$, $p < 0.01$; $r = -0.348$, $p < 0.01$; $r = -0.496$, $p < 0.001$, $r = -0.305$, $p < 0.001$, respectively for US, peNDF, aNDF and GMPL). Milk urea was negatively correlated to LS and RPL ($r = -0.245$, $p < 0.05$ and $r = -0.252$, $p < 0.05$ for LS and RPL, respectively). Furthermore, milk urea was strongly influenced by the diet protein content ($r = 0.497$, $p < 0.001$). Milk pH did not show any correlation with physical and chemical characteristics of the diet. Milk titratable acidity showed a positive correlation with aNDF and GMPL ($r = 0.209$, $p < 0.05$ and $r = 0.300$, $p < 0.001$, respectively).

Noteworthy, the physical characteristics of GF-free diets was found to be correlated with MCP. Rennet coagulation time (RCT) was negatively correlated with US, aNDF and GMPL ($r = -0.270$, $p < 0.01$; $r = -0.219$, $p < 0.05$, $r = -0.251$, $p < 0.05$, respectively) and positively correlated with LS and protein content of the diet ($r = 0.224$, $p < 0.05$ and $r = 0.306$, $p < 0.05$, respectively). Curd firmness (a_{30}) showed a positive correlation with US ($r = 0.217$, $p < 0.05$) and a negative correlation with MS ($r = -0.299$, $p < 0.01$).

Figure 2 shows the correlations between the characteristics of GF-fed diets and MY, ECM and milk characteristics. Data for which no correlations have been found (such as pH, titratable acidity, SCC, SCS and k_{20}) are not reported.

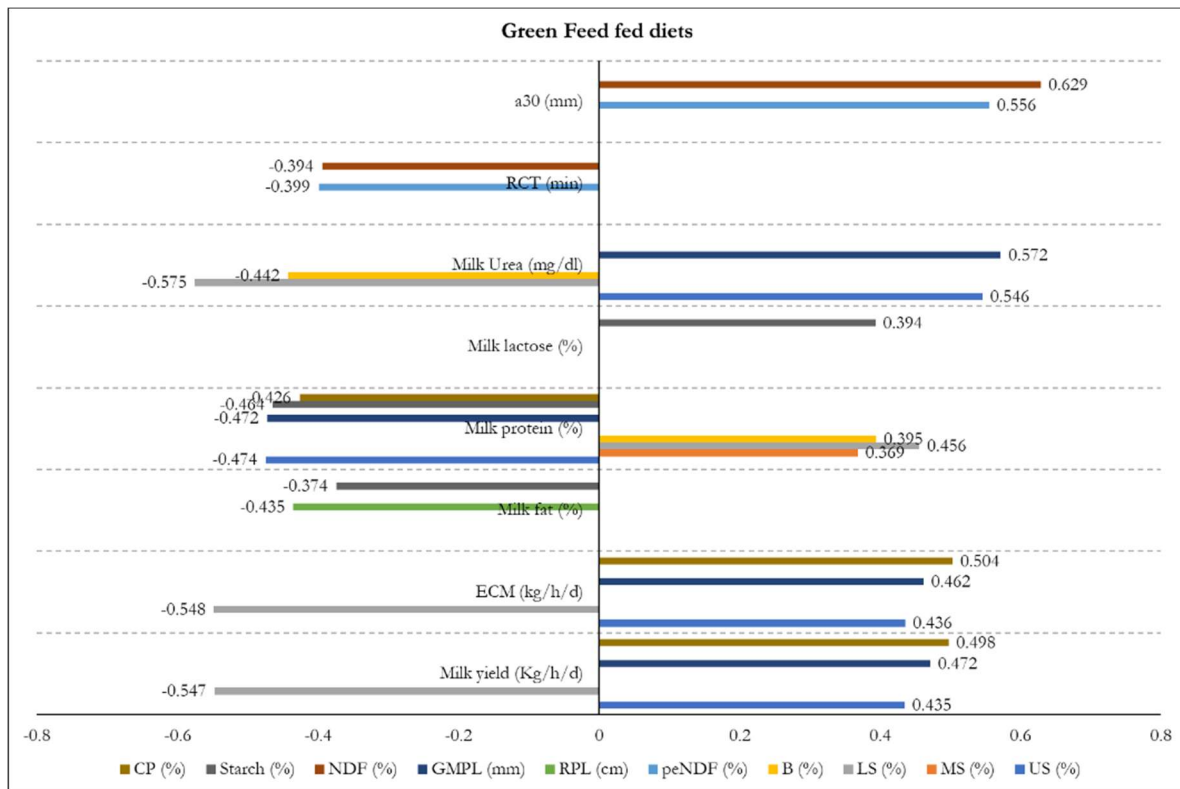


Figure 2. Direction and magnitude of Pearson correlations coefficients between physical and chemical characteristics of the diets and milk yield, ECM and milk characteristics for GF-fed diets.

US = upper sieve = % of diet retained by a sieve with holes of 19 mm; MS = middle sieve = % of the diet retained by a sieve of 8 mm; LS = lower sieve = % of the diet retained by a sieve of 4 mm; B = bottom = % of diet with dimensions < 4 mm; peNDF = physically effective NDF; RPL = ration particle length; GMPL = geometric mean particle length; aNDF = neutral detergent fiber; CP = crude protein; ECM = energy correct milk; RCT = rennet coagulation time at 30 min; a₃₀ = curd firmness.

Milk yield was positively correlated with US, protein content and GMPL ($r = 0.435$, $p < 0.05$, $r = 0.498$, $p < 0.05$ and $r = 0.472$, $p < 0.01$, respectively), and negatively correlated with LS ($r = -0.547$, $p < 0.001$). Energy corrected milk had the same correlation as MY ($r = -0.548$, $p < 0.01$; $r = 0.436$, $p < 0.05$; $r = 0.504$, $p < 0.05$, $r = 0.462$, $p < 0.01$, for LS, US, protein content and GMPL, respectively). Milk fat content showed negative correlation with RPL and starch content ($r = -0.435$, $p < 0.05$, $r = -0.374$, $p < 0.05$ for RPL and starch, respectively). Milk protein showed negative correlations with US and GMPL ($r = -0.474$, $p < 0.01$ and $r = -0.472$, $p < 0.01$, respectively) starch ($r = -0.464$, $p < 0.05$) and protein ($r = -0.426$, $p < 0.05$) and positive correlations with MS, LS and bottom ($r = 0.369$, $p < 0.01$; $r = 0.456$, $p < 0.05$; $r = 0.395$, $p < 0.05$ for MS, LS and bottom, respectively). Lactose content showed positive correlation with starch ($r = 0.394$, $p < 0.05$). Milk urea was positively correlated with US and GMPL ($r = 0.546$, $p < 0.01$ and $r = 0.572$, $p < 0.001$, respectively) and was negatively correlated with LS and bottom ($r = -0.575$, $p < 0.01$ and $r = -$

0.442, $p < 0.05$ for LS and bottom, respectively). Rennet coagulation time was negatively correlated with peNDF and aNDF ($r = -0.399$, $p < 0.05$; $r = -0.394$, $p < 0.05$, for peNDF and aNDF, respectively). Curd firmness was positively correlated with aNDF and peNDF ($r = 0.629$, $p < 0.01$; $r = 0.556$, $p < 0.01$, for aNDF and peNDF, respectively).

Correlation between diet characteristics and diet apparent digestibility

Figure 3 shows the correlations between physical chemical composition of GF-free diets with apparent digestibility.

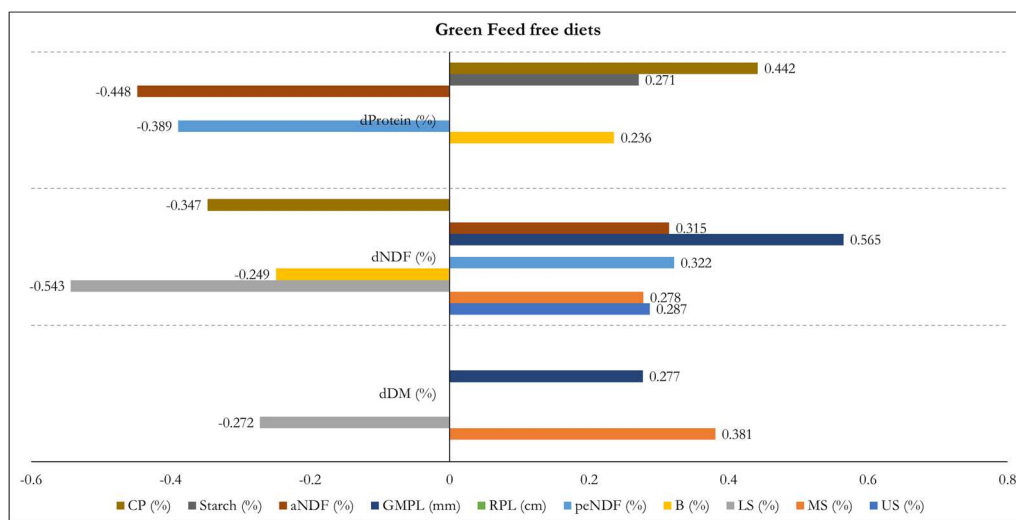


Figure 3. Direction and magnitude of Pearson correlations coefficients among diets physical and chemical characteristics and DM, NDF and protein apparent digestibility for GF-Free diets. US = upper sieve = % of diet retained by a sieve with holes of 19 mm; MS = middle sieve = % of the diet retained by a sieve of 8 mm; LS = lower sieve = % of the diet retained by a sieve of 4 mm; B= bottom = % of diet with dimensions < 4 mm; peNDF = physically effective NDF; RPL = ration particle length; GMPL = geometric mean particle length; aNDF = neutral detergent fiber; CP = crude protein; dDM = apparent digestibility of DM; dNDF = apparent digestibility of NDF; dProtein = apparent digestibility of Protein.

The qualitative indices of the TMR (HI and SI) did not show any correlation with the physical and chemical parameters of GF-free diets, as well as the dStarch. Dry matter digestibility had a negative correlation with LS ($r = -0.272$, $p < 0.05$). Furthermore, it was positively affected by particles with a size between 19 and 8 mm, *i.e.* those retained by MS ($r = 0.381$, $p < 0.001$) and by GMPL ($r = 0.277$, $p < 0.001$). The apparent digestibility of aNDF was positively correlated with US, MS, peNDF, dietary aNDF and GMPL ($r = 0.287$, $p < 0.01$; $r = 0.278$, $p < 0.01$; $r = 0.322$, $p < 0.01$; $r = 0.315$, $p < 0.01$, $r = 0.565$, $p < 0.001$, respectively), and was negatively correlated with the finest particles of the diet, with a diameter between 8 and 4 mm and those < 4 mm (LS and bottom) ($r = -0.543$, $p < 0.001$; $r = -0.249$, $p < 0.05$, respectively) and with the protein content of the diet ($r = -0.347$, $p < 0.05$). Crude protein digestibility was positively related to starch and

protein content and bottom ($r = 0.271$, $p < 0.05$; $r = 0.442$, $p < 0.001$; $r = 0.236$, $p < 0.05$ respectively) and negatively related to peNDF ($r = -0.389$, $p < 0.001$) and aNDF content ($r = -0.448$, $p < 0.001$).

Diets in which GF was present dDM was positively correlated with starch content ($r = 0.369$, $p < 0.05$). Apparent digestibility of aNDF showed negative correlations with bottom ($r = -0.399$, $p < 0.05$), and the dProtein was favoured by a greater protein content of the diet ($r = 0.631$, $p < 0.001$). Apparent digestibility of starch was not related to none of the parameters considered. Ration particle length showed a negative correlation with HI ($r = -0.460$, $p < 0.05$).

DISCUSSION

As expected, the diets fed to dairy buffaloes is ultimately physically different from that for dairy cows (Heinrichs, 2013). The overall average of physical characteristics of the present study were different from data reported in our previous study (Evangelista et al., 2022) carried out on a dairy buffalo farm in the Latina province, which is in the same production area. This confirms the high variability among dairy buffalo farms. The average RPL and GMPL found in this study, are closely associated to the presence of GF in the diets: diets with GF showed higher RPL and GMPL. Studies carried out on dairy cows (Caccamo et al., 2014; Andrighetto et al., 2023) reported higher GMPL (more than 6 mm) compared to the present study, probably because they have the second sieve much higher compared with MS of the present study.

The high variability in physical characteristics of the diets observed in the present study can be definitively attributed to the different feeding management among farms. Despite their proximity, the farms showed variations in diet composition and differences in the management of mixer wagon which, as well known, strongly affect diets physical characteristics (Mousa et al., 2018). The tendency to have a very high US in GF-fed diets compared to GF-free diets, can be explained by use a “cut and carry” green foraging technique. The overall average of aNDF supplied to dairy buffaloes falls within the guidelines provided by Bartocci et al. (2002) which indicated a content between 47 to 38 % of aNDF on DM basis to produce 7 or 12 kg/d of normalized milk, respectively. Furthermore, it agrees with other studies done on dairy buffaloes (Bertoni et al., 1994; Campanile et al., 1998). On average, peNDF content was higher compared with dairy cows’ diets (Mertens, 1997; Righi et al., 2007; Caccamo et al., 2014). This is due to the high aNDF content, and the high percentage of particles retained in the first three sieves (US, MS, and LS). The need for minimizing fiber levels is a common requirement in the diets of high-yielding dairy cows and beef, as underlined by Mertens (1997). However, this requirement does not extend to the dietary

considerations for dairy buffaloes. The DM content was lower on average, especially in GF-fed diets, compared with our previous study, where the DM content was 60% (Evangelista et al., 2022). The starch content was lower than those reported by Campanile et al. (1998), Di Francia et al. (2007) and Evangelista et al. (2022), where starch levels were documented to be among a range from 17 to 24% on DM basis. On the other hand, the protein content is closed to other studies which report a content around 12% (Campanile et al., 1998; Zicarelli et al., 2008; Evangelista et al., 2022).

The HI is comparable with our previous study (Evangelista et al., 2022) while SI was, on average, higher compared with the same study. Aside from our earlier research on buffaloes, the only available comparison is with data from dairy cattle (Andrighetto et al., 2023). Those authors obtained HI much higher than our study (greater than of 72), probably because, as we have observed in the present study, the diets for dairy buffaloes are physically different than those for dairy cows.

Overall average of MY falls below the data reported by the Italian farmers' association (AIA, 2023) for the same region in 2022 and within data from recent research on buffalo (Evangelista et al., 2022; Neglia et al., 2023) and was greater than findings by Manuelian et al. (2017). It's worth noting the high variability observed for this parameter as well. In line with the mentioned trends, even ECM was observed to be lower compared to findings of other studies (Evangelista et al., 2022; Neglia et al., 2023). In terms of fat and protein content, the levels were higher than AIA statistics for Lazio Region in 2022 (7.66% for fat and 4.68% for protein) and compared to the survey conducted by Pasquini et al. (2018). Milk fat results, however, align with the values reported by Di Francia (2007). On the other hand, the lactose content was lower compared to other studies (Di Francia et al., 2007; Pasquini et al., 2018). The pH values were similar to those observed by Di Francia et al. (2007) but higher than Manuelian et al. (2017) and Evangelista et al. (2022). Titratable acidity slightly exceeded the values reported by Manuelian et al. (2017). Somatic cells content was consistently higher on average than that found by Evangelista et al. (2022). Additionally, urea levels were higher compared to Di Francia et al. (2007). Clotting parameters, on average, were less favourable compared to other authors (Bartocci et al., 2002; Manuelian et al., 2017; Evangelista et al., 2022). The differences between results on milk composition of the present study and what was found by others can be traced back to the different composition of the diets, the genetic potential of animals, the stage of lactation, parity, and the period of the year in which the studies were carried out.

The chemical composition of faeces offers valuable insights into the digestive capacity of animals. A higher presence of potentially digestible component (*i.e.* starch) in faeces indicates poor digestibility. Ideally, faecal samples should reflect uniform digestion and utilization of feeds and nutrients provided to animals (Heinrichs et al., 2023). Dry matter content of faeces agrees with findings from previous studies (Bovera et al., 2007). However, other parameters such as CP and aNDF were higher, which could be attributed to variations in the diet composition and digestibility or depend on the physiological state of the animals or on the seasons in which the samples were taken (Bovera et al., 2007). Apparent digestibility of NDF and dStarch were consistent with other studies (Campanile et al., 2008), while dProtein was lower (Puppo et al., 2002; Campanile et al., 2008).

Particle size classes

The three classes created based on US content for both type of diets have a very different physical characteristic from each other. The physical modification among classes relates to their chemical composition (such as aNDF, starch and DM) as also seen by Caccamo et al. (2014) in dairy cow diets.

Subdivision into three classes based on the content of US showed interesting results: diets characterized by high content of long particles were associated with lower SCC content and relative SCS, and this was true both for GF-free and GF-fed diets. Diets with higher SCC and SCS (2nd and 3rd class for both type of ration) had lower US content and a higher proportion of particles < 19 mm (MS, LS and bottom). The association between physical characteristics and SCC is confirmed by the negative correlations between SCC and SCS with peNDF and aNDF, and positive correlations with the content of starch, protein, and LS for GF-free diets. Additionally, GF-free diets with higher US content showed improved curd compactness (a₃₀), likely due also to the lower SCC.

Correlation between diet characteristics and milk production

In GF-free diets, we observed a negative effect of peNDF and aNDF content on MY in agreement with findings by Righi et al. (2007) and Stojanovic et al. (2012) in dairy cows. Moreover, a positive correlation between MY and bottom has been observed in the present study in agreement with what was found in dairy buffaloes by some authors. Bartocci et al. (2006) demonstrated how MY was favoured by a low forage/concentrate ratio compared with a less concentrated one (high forage/concentrate value). Di Francia et al. (2007) observed that dairy buffalo diets with low energy density (starch) and high aNDF content negatively affected MY.

High aNDF content, in dairy cow, has been identified as the cause of the reduction in MY in the meta-analysis conducted by Tafaj et al. (2007). Several studies have attributed the greater milk production to the greater feed intake favoured by lower presence of aNDF which is responsible for the rumen filling effect (Allen, 2000). In this study, we assume that the positive correlation between MY and finest particles (bottom) is due to the high available energy for milk production. In GF-fed diets, MY (and ECM) showed different correlations in particular with LS (negative) and with the content of protein, GMPL and US (positive) probably influenced by the presence of grass as will be discussed later.

The positive correlation in GF-free rations between dietary aNDF and milk fat agrees with Mertens (1997), who found a reduction of milk fat content in diet characterized by low concentration of peNDF. Stojanovic et al. (2012) also reported reduced milk fat in dairy cows fed diets with shortened particles and lower peNDF. The meta-analysis carried out on 93 articles by Tafaj et al. (2007) reported that milk fat content increases proportionally with the aNDF content in the diet ($R^2 = 0.32$). In contrast, other studies have not found a significant link between diet physical characteristics and milk fat content (Righi et al., 2007; Haselmann et al., 2019). Fustini et al. (2016) observed a negative correlation between the diet content of pelleted mixed-feeds and milk fat percentage ($r = -0.41$) in diets of Parmigiano Reggiano area. In agreement with Fustini et al. (2016) the negative correlation observed between LS and milk fat percentage in GF-free diets, could be associated with high proportion of small particles such as, for example, pelleted feed and/or particles of corn which

have a high energy density. We observed that, among all farms involved, farm n. 10 (Table 3S) had a high content of particles retained in LS ($> 40\%$, on average), and fed the buffaloes with an average of 3.5 kg of pelleted mixed feed and had low average aNDF level ($39.73 \pm 2.61\%$) and high starch content ($> 15\%$). This farm showed the lowest average milk fat content ($7.70 \pm 0.40\%$) compared with their counterparts. This suggests that the negative relationship between LS/starch and milk fat may be due to the higher energy density of the diets, which lacks adequate fiber (aNDF) and long particles, potentially leading to ruminal imbalance and impaired milk fat synthesis. As noted by Allen (1997) excessive dietary concentration, particularly of fermentable carbohydrates, can reduce ruminal pH. This shift promotes non-cellulolytic microorganisms over cellulolytic (Van Soest, 1994), decreasing acetate and increasing propionate production, which can lower milk fat content.

Particles with a diameter between 19 and 8 mm (MS) (for both diets) and < 4 mm (bottom) in the GF-fed diets, positively affected milk protein content. In agreement with results of the present

study, Kononoff and Heinrichs (2003) reported some positive effect of smaller particle size on milk protein content and yield. Also in dairy buffaloes, Bartocci et al. (2006) observed an increase in milk protein content with high presence of concentrates in diet (high amount of starch). Increase of milk protein with decreased diet particle size is thought to be the result of increased starch digestibility (Clark and Armentano, 1999), as milk protein is positively related to dietary energy content (Grieve et al., 1986). This statement does not seem true for GF-fed diets which presented negative relationships between starch and milk protein.

Milk protein in GF-fed diets, was negatively correlated with starch, CP, GMPL and US. In these diets, US is directly linked to the presence of green fodder that is characterized by high content of ruminal degradable proteins. As already highlighted by several studies (Campanile et al., 1998; Roy et al., 2005; Tufarelli et al., 2008) the higher levels of ruminal degradable or soluble proteins, or excess of crude protein, do not enhance milk protein content but rather increase milk urea content. This could explain the negative relationship with milk protein and US in GF diets discussed previously and with CP content (in GF-free diets). Excess nitrogen supplied to the rumen, or an energy-deficient diet often results in elevated urea concentrations in blood and milk (Roy et al., 2005).

Physical characteristics of TMR were correlated with MCP. In GF-free diets US had positive impact on milk rheological properties. As the US increased, the RCT decreased, and the a_{30} increased. Additionally, the content of peNDF (only for GF-fed diets), GMPL (for GF-free diets) and aNDF (for both) were negatively correlated with RCT, indicating that greater peNDF, aNDF, and particle length improve milk coagulation properties. These results agree with findings by Bartocci et al. (2006), who obtained better coagulation properties with diets containing high forage/concentrate ratio. Diets with high presence of aNDF, GMPL, peNDF and US could be associated with a qualitatively better milk, which had a lower SCC content, higher titratable acidity, higher quantity of fat and protein which therefore allowed an improvement in the milk rheological parameters. For GF-diets, improvements in milk quality may also be attributed to the inclusion of green fodder, as noted in several studies (Hussain et al., 2010; Salzano et al., 2021; Neglia et al., 2023). Despite the benefits of green fodder, the key factor affecting both diets was the fiber content (aNDF). This suggests that proper fiber management enhances the chemical and rheological quality of buffalo milk.

Correlation between diet characteristics and diet apparent digestibility

In our study dDM is favoured by the presence of particles ranging between 19-8 mm (MS) and GMPL for GF-free diets, and the starch content for GF-fed diets. In dairy cow, Haselmann et al. (2019) observed an improving digestibility with shorter diets compared to longer ones. According to Kononoff and Heinrichs (2003) the reduction of particle size can increase dDM. In contrast, Mousa et al. (2018) observed reduced dDM when peNDF was reduced in buffalo heifers' diet. They concluded that reducing particle length increases escape rate from the rumen, giving to the particles too little time for proper digestion.

Apparent digestibility of starch was not affected by any parameter, while dProtein showed a positive correlation with starch and a negative correlation with peNDF and aNDF in GF-free diets. Therefore, in our study dProtein is linked to the chemical composition of the diet and not to the physical one, contrary to what has been reported in studies on dairy cows (Kononoff and Heinrichs, 2003; Stojanovic et al., 2012; Haselmann et al., 2019). Starch is crucial for promoting microbial growth in the rumen, which are responsible for proper digestion. Other studies on buffaloes (Puppo et al., 2002; Zicarelli et al., 2008), have also reported enhanced organic matter digestibility with diets rich in concentrates (starch).

Results of the present study indicate that smaller physical particle sizes in the diet, particularly particles less than 8 mm (LS and bottom) in GF-free diets, are associated with greater difficulty in digesting aNDF in buffaloes. Additionally, dNDF increased with higher GMPL, as indicated by the correlation analysis for GF-free diets. This is consistent with findings by Puppo et al. (2002), who reported a reduction in dNDF with increasing concentrate levels and a corresponding decrease in aNDF content in buffalo bulls. Mousa et al. (2018) found an increased dNDF when diets had a greater content of US and peNDF in heifer buffaloes. In contrast, studies on dairy cows have observed positive correlations between particle size reduction and dNDF (Simoni et al. 2021). The enhanced dNDF associated with coarse particles is likely due to increased saliva production, which can significantly affect rumen pH (Yang and Beauchemin, 2009; Mousa et al. 2018) and subsequently improve aNDF degradability. Positive correlations have been reported between ruminal pH and aNDF content by Tafaj et al. (2007). Longer forage particles increase rumination time, potentially enhancing digestibility (Tafaj et al., 2007; Mousa et al., 2018). In this study, farms with lower dNDF had a higher proportion of fine particles (bottom >30%), likely due to the extensive use of concentrates that accumulate in the bottom and third sieve (LS). Some authors have observed the reduction of dNDF in cows fed with low forage/concentrate ratio diet (Valadares et al., 2000). High-concentrate diets (rich in starch and sugars) lower rumen pH, which can impair

fiber digestibility and alter cellulolytic bacterial activity (Zicarelli et al., 2008). Thomas and Russel (2004) reported a reduction in *Fibrobacter succinogenes* with the increased soluble carbohydrates. The reduced activity of cellulolytic microbes is not solely the result of a low pH, but it can also be inhibited by increased starch fermentation and substrate competition (Van Soest, 1994). In dairy cattle fed with high-energetic diets, particles exceeding 19 mm are generally recognized as a primary factor influencing chewing activity (Kononoff and Heinrichs, 2003) that allows for better digestion of diet (Tafaj et al., 2007). Some researchers, (Beauchemin and Yang, 2005; Yang and Beauchemin, 2006), attributed this function to particles longer than 8 mm. Based on these assertions, it can be hypothesized that particles retained by the MS may have contributed to an enhanced digestibility of DM, and aNDF in GF-free diet.

Diet quality indices (HI and SI) did not show any correlation with physio-chemical parameters of the diets except for the HI which was negatively affected by RPL, but only in GF-fed diets. This is probably because in these diets, as seen before, the inclusion of GF tends to increase the length of the particles and obviously this can negatively affect HI.

This study has some limitations. Since there are not any information on physical characteristics of diet for dairy buffaloes, the study's experimental design was planned to add knowledge on physical characteristics of dairy buffalo's diets and evaluate the association of physical characteristics with milk production and diet digestibility. With this background, PSPS tool was used. PSPS tool has been developed and setup for determining physical characteristics of dairy cattle diets which are in general different in terms of forage content and fiber content than dairy buffaloes' diets. The use of PSPS tool was dictated by the fact that it is the only one available to date. However, we cannot exclude some limitations in the use of the PSPS tool in the evaluation of the physical characteristics of diets for lactating buffaloes.

CONCLUSIONS

This study highlighted the high variability of dairy buffalos' diets, despite being carried out in a small area. The division into classes based on the content of larger particles (>19 mm, US) showed a reduction in SCC and SCS in both GF-free and GF-fed diets with higher US content. Additionally, the correlation results suggest that an unbalanced diet, low in fiber and high in energy, may be associated with a deterioration in the chemical and technological characteristics of buffalo milk, as well as a tendency toward higher SCC. These findings imply that dairy buffaloes may benefit from a diet rich in fiber.

Moreover, the study of the correlations shows complex links among both the physical and chemical composition of the diets with milk yield, quality, and apparent diet digestibility. These results sometimes differ between the two types of diets (GF-free and GF-fed), suggesting a still unclear effect on the use of green fodder. This investigation conducted in a limited territory, although it was carried out over a long period of observation on a substantial number of farms, gives some general information on the importance of physical characteristics of the diets for dairy buffaloes, but does not allow us to fully understand the complex relationships with the production, quality, and diet digestibility. This first approach poses many questions to which it is necessary to give concrete answers due to the growing interest in this type of breeding in Italy.

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SUPPLEMENTARY FILES

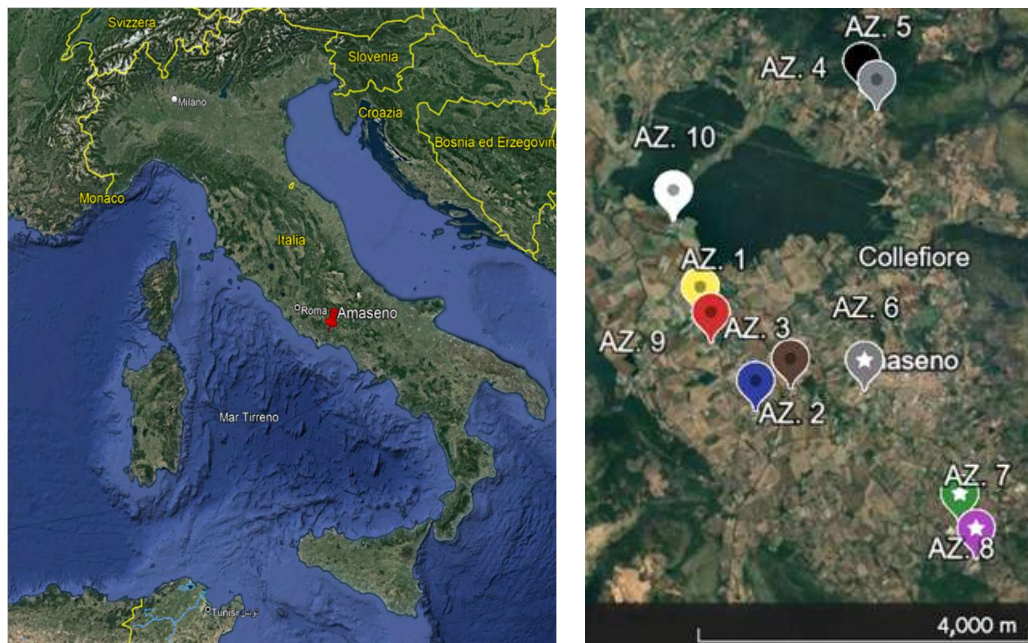


Figure 1S. Farms location (left); proximity between farms in the Amaseno Valley (right).
AZ. 1 = Farm 1; AZ. 2 = Farm 2; AZ. 3 = Farm 3; AZ. 4 = Farm 4; AZ. 5 = Farm 5; AZ. 6 = Farm 6;
AZ. 7 = Farm 7; AZ. 8 = Farm 8; AZ. 9 = Farm 9; AZ. 10 = Farm 10.

Table 1S. General information of the 10 dairy buffalos' farms (means $\pm$ SD).

Farms	Lactating Buffalo	Hectares	MY (L/h/d)	Mixing Time (min.)	Feed Loading sequence	Mixer Wagon type
F_1	176 ± 28	23	9.37 ± 1.16	7 ± 2	1	SMAX-240, towed
F_2	68 ± 9	17	7.61 ± 1.63	18 ± 23	2	ROTOMIX-4500, self-propelled
F_3	117 ± 11	30	8.14 ± 0.99	13 ± 5	1	FARESIN-1700, self-propelled
F_4	56 ± 7	50	6.50 ± 1.78	13 ± 6	2	ZAGO-king Feeder, towed
F_5	148 ± 33	25	8.58 ± 1.25	13 ± 8	2	ROTOMIX-5500, self-propelled
F_6	101 ± 17	15	8.35 ± 1.50	11 ± 3	1	ROTOMIX-5000, self-propelled
F_7	287 ± 84	60	9.15 ± 2.14	10 $\pm --$	2	ROTOMIX-6000, self-propelled
F_8	154 ± 19	45	8.37 ± 0.72	10 ± 4	2	ROTOMIX-5500, self-propelled
F_9	150 ± 25	50	8.03 ± 1.10	12 ± 3	2	ROTOMIX-5500, self-propelled
F_10	233 ± 31	29	7.84 ± 1.40	10 ± 1	2	ROTOMIX-5000, self-propelled
Overall	149	34.4	8.19	12	-	-
Mean	± 75	± 14.83	± 1.42	± 8		

Feed loading sequence = 1 Hay-concentrates-silages; 2 = Hay-silages-concentrates

Table 2S. Composition of diets (mean $\pm$ SD) divided by feed category, expressed on percentage on as it.

	GF-free	GF-fed
Silage	54.91 $\pm$ 8.55	40.55 $\pm$ 9.40
Hay	19.77 $\pm$ 6.25	16.21 $\pm$ 5.24
Green forage	-	15.84 $\pm$ 7.35
Corn mash	12.39 $\pm$ 2.95	8.11 $\pm$ 1.33
Brewer spent grain	20.69 $\pm$ 5.20	20.19 $\pm$ 5.18
Commercial mixed feed	13.56 $\pm$ 4.65	-
Concentrate	17.23 $\pm$ 6.92	17.27 $\pm$ 6.26
Supplements	1.43 $\pm$ 0.77	1.84 $\pm$ 1.67
F:C	75:25	72:28

Silage = corn, triticale; Hay = oat, alfalfa, straw, clover, ryegrass; green fodder = ryegrass, sorghum forage;

Concentrate = corn flour; barley flour; soybean meal; barley radicle; cotton seeds; horse-bean seeds; Supplements = yeasts, Linomix, vitamins, Calcium salts of palm fatty acids, CaCO₃; F:C = forage to concentrate ratio.

Table 3S. Chemical and physical characteristics of the 10 dairy buffaloes' farms' diet (means±SD).

Farms	US (%)	MS (%)	LS (%)	Bottom (%)	peNDF (%)	RPL (cm)	GMPL (mm)	DM (%)	aNDF (%)	CP (%)	Starch (%)
F_1	33.37 ^A ±13.18	16.54 ^B ±7.26	23.09 ^C ±4.40	26.99 ^{BC} ±5.96	33.22 ^{BCD} ±4.43	4.71 ^{ABC} ±0.68	6.45 ^A ±0.88	46.86 ^{FG} ±3.31	45.42 ^{AB} ±3.47	13.39 ^{AB} ±1.30	13.68 ^{AB} ±2.30
F_2	19.11 ^{ABCDE} ±13.99	26.38 ^{AB} ±9.16	32.34 ^{ABC} ±8.01	22.18 ^{CD} ±4.54	38.02 ^{AB} ±6.72	5.38 ^{AB} ±1.37	5.93 ^A ±1.22	51.52 ^{CDE} ±4.27	48.61 ^A ±6.22	9.98 ^D ±2.15	13.93 ^{AB} ±2.61
F_3	26.37 ^{ABC} ±11.58	18.48 ^{AB} ±8.70	29.23 ^{BC} ±5.69	25.90 ^{BC} ±4.75	35.82 ^{ABC} ±3.84	4.03 ^C ±0.63	5.85 ^{AB} ±1.00	48.73 ^{FG} ±5.34	48.30 ^A ±3.51	12.34 ^{ABC} ±1.26	13.57 ^{AB} ±3.20
F_4	32.52 ^{AB} ±17.96	17.32 ^B ±7.84	31.23 ^{BC} ±10.23	18.93 ^D ±3.46	40.42 ^A ±2.91	4.95 ^{ABC} ±1.36	6.44 ^A ±1.56	56.26 ^{ABC} ±2.72	49.82 ^A ±1.78	11.10 ^{CD} ±1.03	11.20 ^B ±1.87
F_5	8.50 ^E ±6.27	24.77 ^{AB} ±7.95	33.73 ^{AB} ±8.13	33.02 ^A ±2.52	26.80 ^E ±2.36	4.20 ^{BC} ±0.54	4.48 ^{BC} ±1.01	60.11 ^A ±5.79	39.98 ^C ±2.68	13.76 ^A ±0.87	16.35 ^A ±2.58
F_6	9.27 ^{DE} ±5.76	23.67 ^{AB} ±4.66	36.58 ^{AB} ±5.55	30.93 ^{AB} ±3.25	31.77 ^{CDE} ±2.99	5.58 ^A ±1.07	4.44 ^{BC} ±0.72	43.35 ^G ±2.07	45.65 ^{AB} ±2.91	12.49 ^{ABC} ±0.90	14.28 ^{AB} ±2.61
F_7	18.18 ^{BCDE} ±13.81	23.93 ^{AB} ±8.57	32.71 ^{ABC} ±8.43	25.16 ^C ±3.60	32.49 ^{CD} ±2.66	5.49 ^{AB} ±1.13	5.53 ^{ABC} ±1.26	55.60 ^{ABCD} ±6.02	43.41 ^{BC} ±2.51	11.96 ^{BC} ±1.04	15.91 ^A ±2.83
F_8	23.68 ^{ABCD} ±8.58	23.69 ^{AB} ±5.40	30.08 ^{BC} ±6.37	22.55 ^{CD} ±3.76	38.06 ^{AB} ±4.84	5.28 ^{ABC} ±0.67	6.15 ^A ±0.86	50.05 ^{DEF} ±3.65	49.00 ^A ±4.15	10.89 ^{CD} ±1.93	11.50 ^B ±2.79
F_9	14.01 ^{CDE} ±8.70	27.83 ^A ±7.19	32.24 ^{ABC} ±8.25	25.92 ^{BC} ±3.50	31.08 ^{CDE} ±2.72	5.37 ^{AB} ±1.12	5.49 ^{ABC} ±1.08	57.57 ^{AB} ±4.76	41.96 ^{BC} ±3.07	11.71 ^{BCD} ±0.99	15.88 ^A ±2.53
F_10	5.36 ^E ±2.79	26.24 ^{AB} ±9.10	42.28 ^A ±10.28	26.21 ^{BC} ±3.35	29.41 ^{DE} ±3.09	4.94 ^{ABC} ±0.86	4.28 ^C ±1.12	53.31 ^{BCD} ±3.60	39.73 ^C ±2.61	13.71 ^A ±1.00	15.44 ^A ±2.53
SEM	1.30	0.76	0.81	0.50	0.51	0.10	0.12	0.59	0.45	1.75	0.28
	*	*	*	*	*	*	*	*	*	*	*

US = upper sieve = % of diet retained by a sieve with holes of 19 mm; MS = middle sieve = % of the diet retained by a sieve of 8 mm; LS = lower sieve = % of the diet retained by a sieve of 4 mm; Bottom = % of diet with dimensions < 4 mm; peNDF = physically effective NDF; RPL = ration particle length; GMPL = geometric mean particle length; DM = dry matter; aNDF = neutral detergent fiber; CP = crude protein.

A, B, C, D, E = $p < 0.001$

* $p < 0.001$

Sub Chapter 4.3

Diet Composition shapes the metagenomic profile of the faecal microbiota in Italian Mediterranean Buffalo: Preliminary study

Evangelista Chiara^{1*}; Pietrucci Daniele^{1*}; Milanesi Marco¹; Gabbianelli Federica¹; Chillemi Giovanni¹; Failla Sebastiana²; Basiricò Loredana³; Bernabucci Umberto³

¹ Department for Innovation in Biological Agro-Food and Forest Systems, University of Tuscia, Italy

² CREA-Research Center for Animal Production and Aquaculture, 00015 Monterotondo (RM), Italy

³ Department of Agriculture and Forests Sciences, University of Tuscia, Italy

* These authors contributed equally to this work

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ABSTRACT

The gastrointestinal microbiome of ruminants is integral to health, influencing immune responses, nutrient metabolism, and environmental impact. While the ruminal microbiome has been extensively studied, the hindgut microbiome - particularly the faecal microbiome - remains less characterized. Dietary composition significantly shapes microbial communities, affecting digestion, metabolism, and methane production. Next-generation sequencing (NGS) has emerged as a powerful tool for microbiome analysis; however, no studies have characterized the faecal microbiota of Italian Mediterranean buffaloes in relation to diet. This study aims to address the gap by evaluating the bacterial composition and dietary influences on the faecal microbiota of buffalo. Over a six-month period, monthly group faecal samples (~10–15% of animals per group) were collected from lactating buffalo across 10 farms. Dietary data were recorded, and Total Mixed Ration (TMR) samples were analysed for their physicochemical properties and fatty acid content. A portion of the faecal samples was analysed in the laboratory, while another portion was stored in tubes with preservative for DNA extraction. DNA was extracted from faeces using the Quick-DNA™ kit, followed by 16S rRNA sequencing on an Illumina MiSeq System. Statistical analyses in R included alpha and beta diversity assessments, differential abundance testing, and one-way ANOVA ($p < 0.05$, with trends identified at $p < 0.10$). A total of 10 phyla, 13 classes, 26 orders, 47 families, 86 genera, and 120 species were identified, with *Firmicutes* being the most abundant phylum ($55.8 \pm 3.6\%$), followed by *Bacteroidota* ($37.7 \pm 3.4\%$). Among dietary variables, the forage-to-concentrate ratio (FC) and linseed (LS) inclusion had the strongest influence on faecal microbiota. A low FC ratio favoured phyla negatively associated with fiber digestibility and was linked to *Lachnospiraceae* and *Succinivibrionaceae*. Other studies in cows have confirmed the association between these families and high-concentrate diets rich in fine particles (<4 mm). LS inclusion reduced species abundance, and increased *Firmicutes*, *Spirochaetota*, and *Proteobacteria* - phylum including potentially pathogenic bacteria linked to ruminal dysbiosis. LS presence was also associated with reduced *Verrucomicrobiota*, essential for gut health and mucus layer regulation. This study provides the first characterization of the faecal microbiota in dairy buffaloes.

INTRODUCTION

Dairy ruminant farming is a cornerstone of the global agricultural economy, serving as a critical source of dairy products that are integral to human nutrition worldwide. Considering the growing demand for more sustainable and efficient agricultural practices, a comprehensive understanding of the ruminal microbial ecosystem has become indispensable for optimizing livestock health, enhancing productivity, and mitigating the environmental impact of farming.

The gastrointestinal tract (GIT) microbiome of ruminants is a highly intricate and dynamic ecosystem where the host, microbiota, and environmental factors engage in complex interactions that collectively shape the animal's health and performance. Achieving and maintaining a healthy gut is essential for preventing infectious diseases, as intestinal homeostasis and immune system balance are foundational to overall well-being. A well-functioning microbiota plays a pivotal role in modulating both local (mucosal) and systemic immune responses, offering protection against pathogen colonization and supporting the host's defences (Sharma et al., 2021; Jaramillo-Jaramillo et al., 2024). Beyond immune modulation, the gut microbiome is instrumental in producing critical nutrients, including amino acids, fatty acids, and vitamins, which influence metabolic regulation, energy homeostasis, and immune function (Zhao et al., 2022).

The composition of microbial communities within the GIT is shaped by a wide array of factors, including species, breed, age, nutrition, environmental conditions, rearing practices, stocking density, stress, antibiotic usage (Shehata et al., 2022), and reproductive status, such as oestrus (Li et al., 2020; Sharma et al., 2021). According to some authors (Callaway et al., 2010; Andrade et al., 2020), the rumen and faeces share a bacterial "core microbiome" at the family and/or genus level, which plays a crucial role in the GIT ecosystem. This overlap underscores the interconnection and interdependence between these microbial communities (Andrade et al., 2020).

Given the need for less invasive sampling methods to correlate microbial populations with functional traits (Mott et al., 2022) and to better understand the factors influencing their diversity and composition, the faecal microbiome has emerged as a valuable focus of study. Notably responsive to dietary changes (Callaway et al., 2010), the faecal microbiome is predominantly shaped by the animal's diet, with the forage to concentrate ratio (FC) serving as a key determinant of its structure and composition (Kim et al., 2014; Tang et al., 2017).

Ruminant nutrition significantly impacts the ruminal microbiome, which in turn influences the faecal microbiome. The ruminant diet primarily consists of plant-derived materials that undergo fermentation in the rumen. This fermentation process produces volatile fatty acids (VFA), carbon

dioxide (CO₂), and methane (CH₄). Additionally, the rectum functions as a complementary digestive organ by utilizing undigested but potentially digestible substrates to produce short-chain fatty acids (SCFA), a vital energy source for the host (Rivera-Chacon et al., 2024), with its role becoming particularly significant when transit rates are high or ruminal function is impaired, leading to an increased flow of substrates escaping ruminal degradation (Gressley et al., 2011). Diets low in forage and rich in degradable carbohydrates promote the growth of amylolytic bacteria, reducing CH₄ production (Haque et al., 2018). However, such diets increase the risk of ruminal acidosis, a metabolic disorder that can impair overall health and productivity. On the other hand, high-fiber diets - while less efficient for energy production - generate higher CH₄ emissions per kilogram of milk produced (Kim et al., 2017).

These dietary interactions underscore the intricate balance between optimizing energy production and mitigating the environmental impacts of ruminant systems. Understanding how nutrition influences microbial communities is essential for developing strategies that enhance animal health, productivity, and sustainability. In this context, studying the faecal microbiome offers a valuable approach not only for gaining insights into feed efficiency but also for monitoring CH₄ emissions and assessing animal welfare in a non-invasive manner (Cendron et al., 2020; Tong et al., 2022).

Despite extensive research on the ruminal microbiome, the hindgut microbiome, represented by the faecal microbiome, remains less characterised (Rivera-Chacon et al., 2024), particularly through advanced techniques like next-generation sequencing (NGS) (Noel et al., 2019). Next-generation sequencing is currently regarded as the most reliable method for assessing bacterial diversity in both the rumen and faeces of large ruminants (*e.g.* cattle, Kim et al., 2017). The 16S rRNA gene is commonly utilised as a reference for determining the composition of bacterial communities due to its phylogenetic variability (Tringe and Hugenholtz, 2008).

To date, no studies have characterised the microbiota composition of Italian Mediterranean buffalo faeces in relation to diet, nor have any utilised NGS for this purpose. Therefore, our objectives were twofold: to characterise the bacterial composition of dairy buffalo faeces and to evaluate the influence of diet on bacterial communities and their effect on animal performance.

MATERIALS AND METHODS

Experimental design

This study is part of the research conducted by Evangelista et al. (2024). Briefly, ten dairy buffalo farms representative of the Amaseno Valley area (Lazio, Italy) were selected and monitored

consecutively over six months, from June to November 2022. Each month, samples of total mixed ration (TMR) and group faeces were collected, along with information on the composition of the TMR.

Sampling procedure

The TMR were collected immediately after morning distribution, an aliquot was used for physical characterisation, another aliquot was stored at 4°C for laboratory chemical analyses. Faecal samples were collected approximately 2–3 hours after the distribution of the morning feed. Sampling was conducted on a representative group of animals, consisting of approximately 8–10 buffaloes per farm, corresponding to about 10–15% of the total lactating buffalo population on each farm, following the guidelines proposed by Pennsylvania Management (2024). Samples were obtained directly from the rectal ampoule to ensure accuracy and consistency. Buffaloes were visibly healthy, and no illnesses among these animals were reported subsequent to sample collection. The individual faecal samples were then combined and homogenised to create a composite sample, referred to as "group faeces". Then, the pH was immediately determined on the fresh group faeces using a portable pH meter ("XS Tester", Giorgio Bormac, Italy) which was calibrated using standard solutions of pH 4 and 7. An aliquot of faeces was stored at 4 °C for laboratory chemical analysis, and another aliquot was stored in triplicate in specific tubes (DNA/RNA Shield™ Lysis Tubes, Zymo Research) containing a liquid preservative to ensure sample stability during storage. The samples were refrigerated at 4 °C for a maximum of 24 hours before being stored at -80 °C as soon as possible, as recommended (Jaramillo-Jaramillo et al., 2024), until DNA extraction. In total, 60 TMR and 60 group faeces samples were collected.

Samples analyses

The particle size distribution (PSD) of the TMR was determined as-fed using the Penn State Particle Separator (PSPS), following the procedure outlined by Heinrichs (2013) and the methods described by Evangelista et al. (2024). The PSPS separates the sample into four fractions: Upper Sieve (US, >19 mm), Medium Sieve (MS, 8-19 mm), Lower Sieve (LS, 4-8 mm), and Bottom (<4 mm). Each fraction was weighed, and its percentage relative to the total sample weight was calculated.

Both TMR and faeces were chemically analysed as follows. Dry matter (DM) was measured after oven drying at 65°C to a constant weight. The samples were then ground using a mill (Retsch Müller, Germany) to pass through a 1 mm screen and stored in sealed polyethylene containers. The samples were analysed for crude protein (AOAC, 2005), ash (AOAC, 1990), ether extract

(AOAC, 2000), and starch (AOAC, 2005) using a K-TSTA assay kit (Megazyme International, Bray, Ireland). Additionally, crude fiber (AOAC, 2005), neutral detergent fiber (aNDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were analysed using an Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY) according to Van Soest et al. (1991). Chemical data are reported as percentages on a dry matter basis.

Fatty acid analysis of TMR was performed using 10 grams of dried TMR, following the procedure described by Folch (1957), using a chloroform mixture (2:1, v/v). The fat, dissolved in hexane, was methylated with methanolic KOH, and a 1 ml sample was injected into a gas chromatograph with a flame ionization detector (GC-FID) (Agilent Technologies, Santa Clara, CA, USA), equipped with a CP-Sil88 capillary column (100 m, 0.25 mm, 0.20 μ m, 100% cyanopropyl, Agilent Technologies). C19:0 was used as the internal standard. GC-FID conditions included: splitless inlet at 250°C; FID at 250°C; helium carrier gas at 1.2 mL/min. The oven temperature program started at 60°C (held for 4 minutes), then ramped up by 13°C/min to 175°C (held for 27 minutes), followed by a 4°C/min increase to 215°C (held for 25 minutes), and finally, a 2°C/min increase to 220°C (held for 5 minutes). Fatty acid peaks were identified by comparing them to the peaks in a Supelco Mix 37 standard (Sigma-Aldrich Merck, Darmstadt, Germany), which includes primary fatty acids such as myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1 cis-9), vaccenic acid (C18:1 cis-11), linoleic acid (C18:2 n-6), arachidic acid (C20:0), γ -linolenic acid (C18:3 n-6), α -linolenic acid (C18:3 n-3), and arachidonic acid (C20:4 n-6). Fatty acid methyl esters (FAMES) were expressed as a percentage of the total FAME content. The total amounts of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids omega-6 (PUFA n-6), and omega-3 (PUFA n-3) were calculated.

Calculated data

Physically effective NDF (peNDF) was calculate as follows (Mertens, 1997):

$$\text{peNDF} = [\text{US (\%)} + \text{MS (\%)} + \text{LS (\%)}] * \text{aNDF (\%)},$$

where US, MS and LS are the proportion of particles retained by the 19.0, 8.0, and the 4.0 mm screen, respectively.

Apparent digestibility of the nutrients was estimated using ADL as internal markers (Fredin et al., 2014):

$$\text{Apparent digestibility (\%)} = \left[1 - \left(\frac{\text{ADL marker conc.}}{\text{ADL marker conc.}} \times \frac{\text{Fecal nutrient conc.}}{\text{TMR nutrient conc.}} \right) \right] \times 100$$

$$DM \text{ Apparent digestibility (\%)} = \left[1 - \left(\frac{ADL \text{ marker conc.}}{ADL \text{ marker conc.}} \right) \right] \times 100$$

Sample Selection and selection of the variables to test

Of the 60 collected group faecal samples, a selection process was implemented to identify and exclude those with similar characteristics regarding diet composition in order to avoid including duplicate samples with the same diet composition (e.g., samples collected over two consecutive months in which the diet was not changed). This was done to ensure a diverse representation of the faecal microbiome composition. The selection process involved detailed data analysis and visualisation techniques. First, a heatmap (Figure S1) was used to display the similarity between samples based on their chemical composition. Next, a dendrogram (Figure S2) grouped the samples by similarity, revealing that adjacent samples and those on the same branches were more alike. Through these analyses, 19 samples identified as highly similar to others were subsequently excluded from DNA extraction, resulting in 41 faecal samples considered for further DNA extraction.

Based on previous studies carried out on dairy cows and goats (Kim et al., 2014; Tang et al., 2017; Guo et al., 2023) among the available variables, those considered potentially influential on the microbiota were selected. The variables selected were reported in table S1. To assess the existence of statistically significant differences in the bacterial community composition between groups of selected variables, PERMANOVA (Permutational Multivariate Analysis of Variance) was used. This test is particularly useful when comparing groups of data based on categorical variables (classes) rather than continuous variables. For this reason, the selected variables were subdivided into three categories, such as high (H), medium (M), and low (L), or yes/no for the presence or absence of a specific variable (e.g., presence of linseed in the diet – yes/no, presence of green forage – yes/no).

The comparisons were made between "yes" and "no", and between "H" and "L" in order to highlight any potential differences in the bacterial community. This is because, in faecal samples, it is quite difficult to detect minimal differences, choosing the extremes ensures a clearer identification of the potential differences.

Based on the results of PERMANOVA analyses (Table S1) and the observed correlations between similar variables and considering the challenges of detecting subtle microbiome differences in such a heterogeneous matrix, the focus was placed on:

1. the forage to concentrate ratio (FC_H vs FC_L)
2. inclusion of linseed (LS_no vs LS_yes)

These two factors demonstrated the greatest impact on modulating the composition of the faecal microbiome (Table S1).

Faecal DNA extraction

DNA was extracted from faecal samples using the Quick-DNA™ Faecal/Soil Microbe Miniprep Kit (Zymo Research Corporation, USA), following the manufacturer's protocol with minor modifications: 300 mg of sample was used instead of the recommended 150 mg. DNA quantification was performed using a DTX Multimode Detector 880 (Beckman, Austria) with the PicoGreen method (Quant-iT, Invitrogen), as per the manufacturer's instructions.

16S metagenomic sequencing libraries were prepared using the 16S V3-V5 Library Preparation Kit for Illumina (Norgen Biotek Corp., Canada), according to the manufacturer's guidelines. Input DNA underwent targeted PCR to amplify the V3-V4 region of the 16S rRNA-encoding DNA. The resulting PCR products were cleaned using Mag-Bind Total Pure NGS beads (Omega Bio-Tek, Georgia). Dual index primers were added during a limited-cycle PCR to generate indexed amplicons flanked by 5' and 3' barcoded adaptors. These amplicons were subsequently cleaned using Mag-Bind Total Pure NGS beads.

The libraries were quantified using a DTX Multimode Detector 880 with the PicoGreen method yielding an average DNA concentration of 25 ng/μl. Libraries were diluted to a concentration of 4 nM, denatured to 8 pM, and pooled using the "MiSeq Reagent Kit v3 (600-cycle)" (Illumina, USA). The pooled libraries were loaded onto the flow cell and sequenced on a MiSeq System.

Statistical analyses

The quality of raw sequencing data was assessed using FastQC (GitHub, 2024), followed by primer and adapter sequence trimming with Cutadapt. Processed reads were analysed using the QIIME 2 pipeline (Bolyen et al., 2019), incorporating chimera detection and clustering into amplicon sequence variants (ASVs) via the DEBLUR algorithm with default settings (Amir et al., 2017). Taxonomic classification of representative ASVs generated by DEBLUR was performed using the q2-feature-classifier plugin (Bokulich et al., 2018) with the Greengenes2 database (version 2022.10) (McDonald et al., 2024). Statistical analyses were conducted in the R 4.3.0 environment using the vegan version 2.6.4 (Oksanen et al., 2024) and phyloseq version 1.46.0 (McMurdie and Holmes, 2013) packages. Low-abundance ASVs, defined as those contributing

less than 0.005% of total reads across samples (Bokulich et al., 2013), were excluded. The remaining sequences were normalized via rarefaction, setting the seed to 1 and selecting an optimal sample size based on rarefaction curves generated with the `ggrare` function. Alpha diversity was quantified using the number of Observed Species, Shannon, Simpson, and Fisher indices, with statistical significance assessed via the Wilcoxon-Mann-Whitney. Beta diversity metrics, including Bray-Curtis distances and weighted generalized UniFrac, were calculated, and significance was tested using the PERMANOVA method with 9999 permutations. Differentially abundant taxa were identified using the DESeq2 algorithm (Love et al., 2014). Data visualisation, including relative abundance plots, boxplots for alpha diversity, and principal coordinates analysis (PCoA) for beta diversity, was performed using the `ggplot2` package.

To evaluate the differences between FC_H vs. FC_L (each consisting of 13 samples), and LS_no vs. LS_yes (consisting of 31 and 10 samples, respectively) a one-way ANOVA (Analysis of Variance) was performed. Significant differences were declared at $p < 0.05$, and trends were considered at $p < 0.10$.

RESULTS

Diet composition

The composition of the diets subdivided based on the 4 groups (2 for FC and 2 based on the presence of LS), is listed in table 1. The FC_L and FC_H diets had FC ratio of 65:35 and 85:15, respectively. For the LS_yes a linseed-based mixture (Linomix, Table 1), was used at a dosage of 0.5 kg per head per day, representing approximately 1.66% of the total diet on an as-fed basis.

The physicochemical composition of the diets is presented in table 2. The FC ratio had a greater influence in modifying the diet compared to the presence of LS, significantly impacting DM ($p < 0.01$), ash ($p < 0.05$), CP ($p < 0.01$), bottom ($p < 0.01$), and MFU ($p < 0.05$). In contrast, LS had, as expected, a greater influence on fatty acid composition of the TMR (Table 3). The presence of LS lead to a reduction ($p < 0.01$) of C14:0, C16:0, C16:1, C18:2n6c (LA), SFA, PUFA_{n6}, and the n6/n3 ratio, and simultaneously increased ($p < 0.01$) C18:3n3 (ALA), PUFA_{n3}, and PUFA_{tot} in TMR. Conversely, FC_H ratio reduced ($p < 0.05$) C20:1n9 and increased ($p < 0.05$) C24:0 and C20:0.

Table 1. Composition of the diets (mean $\pm$ SD).

Components (% on as it is)	FC_H (n.13)	FC_L (n.13)	LS_no (n.30)	LS_yes (n.11)
Silages	62.80 $\pm$ 6.92	41.36 $\pm$ 6.93	51.74 $\pm$ 8.14	43.90 $\pm$ 15.36
Hays	22.24 $\pm$ 6.87	17.75 $\pm$ 4.76	22.20 $\pm$ 8.79	25.40 $\pm$ 6.05
Green fodder	--	14.59 $\pm$ 5.14	8.40 $\pm$ 2.61	17.80 $\pm$ 4.04
Threshers	--	20.68 $\pm$ 4.55	20.65 $\pm$ 4.49	17.19 $\pm$ 3.83
Concentrates	14.23 $\pm$ 3.42	13.46 $\pm$ 1.87	19.21 $\pm$ 3.83	14.59 $\pm$ 3.53
Linomix mixture*	1.65 $\pm$ 0.10	1.62 $\pm$ 0.07	--	1.66 $\pm$ 0.33
Supplements	0.76 $\pm$ 0.61	0.85 $\pm$ 0.54	0.98 $\pm$ 1.07	0.44 $\pm$ 0.07
FC ratio	85:15	65:35	76:24	74:26

FC_H=high forage-to-concentrate ratio; FC_L=low forage-to-concentrate ratio; LS_no=diets without linseed; LS_yes=diets with linseed; The silages used included corn and triticale; Hay consisted of oat, alfalfa, straw, clover, and ryegrass; Green fodder was composed of sorghum (*Sorghum bicolor*) and/or Italian ryegrass (*Lolium italicum*); The concentrate portion included corn flour, barley flour, soybean meal, barley radicles, cottonseeds, and horse bean seeds; Supplements consisted of yeasts, vitamins, calcium salts of palm fatty acids, and calcium carbonate (CaCO₃); **Linomix mixture: Extruded linseeds, sugar beet pulp, corn, sodium chloride, calcium carbonate, wheat. Nutritional analysis: Crude protein 16%, crude fat 20%, crude fiber 13%, crude ash 8.5%, sodium 0.52%.

Table 2. Physiochemical composition of total mixed rations (Lsmean $\pm$ SE).

	FC_H (n.13)	FC_L (n.13)	LS_no (n.30)	LS_yes (n.11)
Dry matter (%)	54.57 $\pm$ 1.46 ^A	46.82 $\pm$ 1.43 ^B	52.19 $\pm$ 1.06	52.71 $\pm$ 2.28
Ashes (% DM ⁻¹)	6.93 $\pm$ 0.20	6.65 $\pm$ 0.20	7.09 $\pm$ 0.17 ^a	6.27 $\pm$ 0.17 ^b
Crude protein (% DM ⁻¹)	11.12 $\pm$ 0.46 ^A	12.46 $\pm$ 0.32 ^B	11.98 $\pm$ 0.39	12.23 $\pm$ 0.32
Ether extract (% DM ⁻¹)	2.28 $\pm$ 0.09	2.42 $\pm$ 0.11	2.53 $\pm$ 0.09	2.47 $\pm$ 0.07
Crude fiber (% DM ⁻¹)	28.84 $\pm$ 1.88	29.60 $\pm$ 1.92	29.02 $\pm$ 1.15	26.24 $\pm$ 1.87
aNDF (% DM ⁻¹)	48.28 $\pm$ 1.21	47.97 $\pm$ 1.12	46.94 $\pm$ 1.04	44.53 $\pm$ 1.07
ADF (% DM ⁻¹)	32.68 $\pm$ 0.84	31.17 $\pm$ 0.72	31.42 $\pm$ 0.78	30.32 $\pm$ 0.98
ADL (% DM ⁻¹)	6.31 $\pm$ 0.29	6.14 $\pm$ 0.18	6.27 $\pm$ 0.19	5.96 $\pm$ 0.32
Starch (% DM ⁻¹)	13.11 $\pm$ 0.68	12.76 $\pm$ 0.70	13.03 $\pm$ 0.49	14.42 $\pm$ 0.81
Upper Sieve (%)	25.22 $\pm$ 5.67	22.93 $\pm$ 3.95	20.09 $\pm$ 3.12	21.39 $\pm$ 3.75
Middle Sieve (%)	27.88 $\pm$ 3.47	20.79 $\pm$ 2.02	24.52 $\pm$ 1.72	25.72 $\pm$ 2.98
Lower Sieve (%)	26.04 $\pm$ 2.59	29.40 $\pm$ 1.76	30.33 $\pm$ 1.79	25.75 $\pm$ 1.96
Bottom (%)	20.84 $\pm$ 1.44 ^A	27.29 $\pm$ 1.83 ^B	24.41 $\pm$ 1.22	27.14 $\pm$ 1.63
peNDF (%)	38.36 $\pm$ 1.55	35.19 $\pm$ 1.44	35.81 $\pm$ 1.24	32.47 $\pm$ 1.21
MFU (kg DM ⁻¹)	0.82 $\pm$ 0.02 ^b	0.87 $\pm$ 0.01 ^a	0.85 $\pm$ 0.01	0.88 $\pm$ 0.01

FC_H=high forage-to-concentrate ratio (85:15); FC_L=low forage-to-concentrate ratio (65:35); LS_no=diets without linseed; LS_yes=diets with linseed; DM=Dry matter; aNDF= neutral detergent fiber; ADF=acid detergent fiber; ADL=lignin acid deterse, Upper Sieve=particles >19mm; Middle Sieve=particles >8mm; Lower Sieve=particles >4mm; Bottom=particles < 4mm; MFU=milk forage unit.

a,b = p<0.05; A,B= p<0.01

Table 3. Fatty acid composition of total mixed rations (Lsmean $\pm$ SE).

Fatty acid (g·100 ⁻¹ g)	FC_H (n. 13)	FC_L (n.13)	LS_no (n.30)	LS_yes (n.11)
C14:0	0.47 $\pm$ 0.03	0.45 $\pm$ 0.04	0.50 $\pm$ 0.02 ^A	0.35 $\pm$ 0.02 ^B
C16:0	16.43 $\pm$ 0.63	17.81 $\pm$ 0.59	18.12 $\pm$ 0.30 ^A	14.78 $\pm$ 0.50 ^B
C16:1	0.40 $\pm$ 0.04	0.47 $\pm$ 0.04	0.46 $\pm$ 0.02 ^A	0.36 $\pm$ 0.04 ^B
C18:0	3.25 $\pm$ 0.13	2.94 $\pm$ 0.15	2.89 $\pm$ 0.09	3.27 $\pm$ 0.08
C18:1n9/cis9	20.91 $\pm$ 0.46	19.37 $\pm$ 0.75	20.29 $\pm$ 0.41	20.76 $\pm$ 0.61
C18:1n7/cis11	1.07 $\pm$ 0.02	0.99 $\pm$ 0.02	1.00 $\pm$ 0.02	1.04 $\pm$ 0.02
C18:2n6c LA	43.03 $\pm$ 0.75	42.43 $\pm$ 1.28	45.45 $\pm$ 0.59 ^A	39.04 $\pm$ 0.88 ^B
C20:0	0.54 $\pm$ 0.02 ^a	0.48 $\pm$ 0.02 ^b	0.50 $\pm$ 0.02	0.49 $\pm$ 0.02
C18:3n6 GLA	2.03 $\pm$ 0.15	1.82 $\pm$ 0.16	1.82 $\pm$ 0.12	1.63 $\pm$ 0.17
C18:3n3 ALA	9.98 $\pm$ 1.31	11.11 $\pm$ 1.65	7.15 $\pm$ 0.57 ^B	16.40 $\pm$ 1.05 ^A
C20:1n9	0.41 $\pm$ 0.03 ^b	0.63 $\pm$ 0.04 ^a	0.44 $\pm$ 0.02	0.59 $\pm$ 0.07
C20:3 n6	0.38 $\pm$ 0.01	0.37 $\pm$ 0.01	0.36 $\pm$ 0.01	0.34 $\pm$ 0.01
C20:4 n6 AA	0.74 $\pm$ 0.10	0.78 $\pm$ 0.14	0.66 $\pm$ 0.07	0.63 $\pm$ 0.15
C20:5n3	0.02 $\pm$ 0.003	0.02 $\pm$ 0.003	0.02 $\pm$ 0.002	0.02 $\pm$ 0.002
C24:0	0.36 $\pm$ 0.01 ^a	0.35 $\pm$ 0.01 ^b	0.34 $\pm$ 0.01	0.34 $\pm$ 0.01
SFA	21.07 $\pm$ 0.69	22.02 $\pm$ 0.54	22.35 $\pm$ 0.31 ^A	19.23 $\pm$ 0.48 ^B
MUFA	22.76 $\pm$ 0.44	21.46 $\pm$ 0.77	22.20 $\pm$ 0.42	22.71 $\pm$ 0.57
PUFAn6	46.18 $\pm$ 0.89	45.40 $\pm$ 1.29	48.28 $\pm$ 0.50 ^A	41.65 $\pm$ 0.88 ^B
PUFAn3	10.00 $\pm$ 1.31	11.12 $\pm$ 1.65	7.17 $\pm$ 0.57 ^B	16.41 $\pm$ 1.05 ^A
PUFAtot	56.18 $\pm$ 0.50	56.52 $\pm$ 0.83	55.45 $\pm$ 0.36 ^B	58.06 $\pm$ 0.41 ^A
n6/n3	5.49 $\pm$ 0.59	5.41 $\pm$ 0.79	7.80 $\pm$ 0.57 ^A	2.67 $\pm$ 0.21 ^B

FC_H=high forage-to-concentrate ratio (85:15); FC_L=low forage-to-concentrate ratio (65:35); LS_no=diets without linseed; LS_yes=diets with linseed; SFA=Saturated Fatty Acids; MUFA=Monounsaturated Fatty Acids; PUFAn6=Polyunsaturated Fatty Acids omega-6; PUFAn3= Polyunsaturated Fatty Acids omega-3; PUFAtot=Total Polyunsaturated Fatty Acids; n6/n3=Omega-6/Omega-3 ratio. a,b = p<0.05; A,B= p<0.01

The composition of the faeces obtained from the different diets shows differences in nearly all the analysed parameters, except for ash content, which does not differ among any of the groups (Table 4). The FC ratio influenced ether extract (p < 0.01), which was higher in FC_L, while ADF (p < 0.01), ADL (p < 0.01), and pH (p < 0.01) were lower in FC_L. The presence of LS was the factor that had the most influence on the chemical composition of the faeces; its presence (LS yes) increased DM content (p < 0.01), crude protein (p < 0.05), ether extract (p < 0.01), and starch (p < 0.01), while reducing aNDF (p < 0.05), ADF (p < 0.01), and pH (p < 0.01). In contrast, the FC ratio had a greater impact on nutrient digestibility. The digestibility of DM (p < 0.01), aNDF (p < 0.05), starch (p < 0.01), and lipid (p < 0.01) was more affected by FC ratio.

Table 4. Chemical composition of faeces and diets' apparent digestibility coefficient (mean±SE).

	<i>FC_H</i> (<i>n.13</i>)	<i>FC_L</i> (<i>n.13</i>)	<i>LS_no</i> (<i>n.30</i>)	<i>LS_yes</i> (<i>n.11</i>)
<i>Chemical composition</i>				
Dry matter (%)	13.80±0.31	13.91±0.50	13.44±0.20 ^B	14.64±0.49 ^A
Ashes (% DM ⁻¹)	12.01±0.37	11.73±0.39	12.32±0.25	11.58±0.31
Crude protein (% DM ⁻¹)	14.07±0.44	14.02±0.31	13.75±0.20 ^b	14.63±0.42 ^a
Ether extract (% DM ⁻¹)	1.12±0.04 ^B	1.51±0.13 ^A	1.19±0.03 ^B	1.56±0.14 ^A
aNDF (% DM ⁻¹)	56.97±1.04	55.28±1.34	57.16±0.65 ^a	53.76±1.45 ^b
ADF (% DM ⁻¹)	45.91±0.72 ^A	41.09±0.81 ^B	44.29±0.53 ^A	41.26±1.11 ^B
ADL (% DM ⁻¹)	15.88±0.66 ^A	13.12±0.45 ^B	15.09±0.39	13.54±0.67
Starch (% DM ⁻¹)	1.30±0.19	1.84±0.39	1.24±0.08 ^B	2.27±0.43 ^A
pH	6.46±0.06 ^A	6.17±0.07 ^B	6.41±0.04 ^A	6.14±0.07 ^B
<i>Apparent digestibility coefficients</i>				
dDM (%)	59.87±1.78 ^A	52.90±1.45 ^B	57.82±1.40	54.78±3.64
dNDF (%)	52.19±2.78 ^a	45.78±1.49 ^b	48.30±1.81	46.07±3.40
dStarch (%)	96.17±0.38 ^A	93.15±1.48 ^B	95.83±0.34	92.07±2.00
dProtein (%)	48.62±2.76	46.64±2.33	51.91±2.03	46.01±4.21
dLipid (%)	79.99±1.24 ^A	69.84±3.25 ^B	79.36±1.11	70.39±4.42

FC_H=high forage-to-concentrate ratio (85:15); FC_L=low forage-to-concentrate ratio (65:35); LS_no=diets without linseed; LS_yes=diets with linseed; DM=Dry Matter; aNDF=neutral detergent fiber; ADF=acid detergent fiber; ADL=lignin acid deterse; dDM=apparent digestibility of DM; dNDF=apparent digestibility of NDF; dStarch=apparent digestibility of starch; dProtein=apparent digestibility of Protein; dLipid=apparent digestibility of Lipid. a,b = p<0.05; A,B= p<0.01

Sequence data analysis and overview of the microbiota composition

The sequencing of the 41 samples generated a total of 6,746,266 reads, with an average of 164,543±29,535 reads/sample. After performing clustering into ASVs and removing chimeric sequences with DEBLUR, the total number of reads was reduced to 2,566,663, with an average of 62,601±11,363 reads/sample. The processing of the number of reads at each step with DEBLUR is reported in table S2, while the distribution of the number of reads per sample following the DEBLUR procedure is shown in Figure S3A. From the ASVs table imported into R, low-frequency ASVs were removed. A total of 15 phyla, 22 classes, 49 orders, 86 families, 196 genera, and 278 species were identified. Considering the different sequencing depths of the samples, rarefaction to 10,000 sequences/sample was used for alpha and beta diversity analyses (Figure S3B). During the rarefaction procedure, no samples were eliminated, as all contained at least 10,000 sequences/sample. After rarefaction, the number of identified taxa was reduced to 10 phyla, 13 classes, 26 orders, 47 families, 86 genera, and 120 species. These 120 species were used to assess alpha and beta diversity. The average percentage abundances of the main taxa (phylum, family,

genus, species) identified across all samples were evaluated to provide a general characterization of the dairy buffalo microbiota. The most abundant phylum was *Firmicutes* ($55.8 \pm 3.6\%$, mean $\pm$ sd), followed by *Bacteroidota* ($37.7 \pm 3.4\%$), *Spirochaetota* ($4.1 \pm 1.5\%$), and *Proteobacteria* ($1.8 \pm 0.9\%$) (Figure 1A). At the family level, the most abundant taxa were *Oscillospiraceae* ($13.9 \pm 1.1\%$), *Lachnospiraceae* ($13.2 \pm 3.6\%$), *Rikenellaceae* ($11.2 \pm 1.3\%$), *Prevotellaceae* ($8.7 \pm 1.4\%$), *Bacteroidaceae* ($7.0 \pm 0.8\%$), *Spirochaetaceae* ($4.7 \pm 1.6\%$) (Figure 1B). Among the most frequently identified genera were an unclassified *Oscillospiraceae* ($12.3 \pm 1.5\%$), *Bacteroides* ($8.1 \pm 0.9\%$), *Alistipes* ($5.6 \pm 0.9\%$), an unclassified *Rikenellaceae* ($6.7 \pm 0.8\%$) and unclassified *Prevotellaceae* ($3.8 \pm 0.6\%$). A comprehensive list of all taxa identified in the samples is provided in table S3. Finally, the phylogenetic tree of the 120 species, shown in Figure S3C, was used for all beta-diversity analyses.

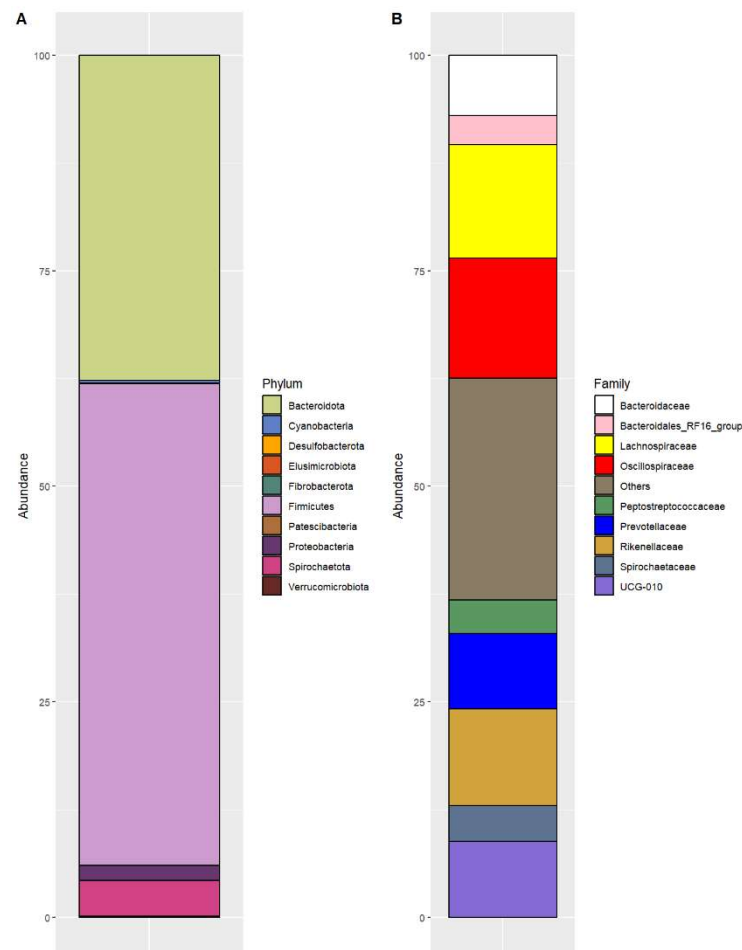


Figure 1. Microbiota characterization of dairy buffalo faeces, differential abundance: A) at the phylum level; B) at the family level.

Microbiota Characterization by FC ratio

Microbiota characterisation began with assessments of alpha and beta diversity by FC ratio are shown in figures 2A and 2B, respectively. Alpha diversity was assessed using several indices. The Observed Species index reflected the actual number of species, and no significant differences were found for the FC ratio (Wilcoxon-exact test p-value = 0.56). The Shannon, Simpson, and Fisher indices, which evaluate both richness and evenness, also showed no significant results for the FC ratio (Wilcoxon-exact test p-value for Shannon = 0.21, Simpson = 0.31, Fisher = 0.56). For beta diversity, the Bray-Curtis dissimilarity and Canberra distance were used to evaluate species composition. Bray-Curtis was significantly influenced by the FC ratio using the PERMANOVA test ($p = 0.01$), while Canberra was not statistically influenced by the FC ratio ($p = 0.12$). Additionally, UniFrac distances were used to consider the phylogenetic relationships among all species. Unweighted UniFrac (considering species presence/absence) and Weighted UniFrac (accounting for relative abundances) were employed. The Weighted UniFrac index was influenced by FC ratio ($p = 0.02$) as was the Unweighted UniFrac distance ($p = 0.005$). These results indicate that the FC ratio does not alter alpha diversity between the two categories but does modify microbiota structure through beta diversity (as highlighted by at least three significant metrics out of four).

The next step involved characterising differentially abundant taxa at the phylum, family, genus, and species levels. All results are reported in table S4 and were evaluated on the entire dataset normalised with DESeq2. A total of 10 phyla were identified (Figure 2C). The most abundant phylum was *Firmicutes* (55.0% vs. 56.8% in FC_H and FC_L, respectively), followed by *Bacteroidota* (39.3% vs. 37.1%), *Spirochaetota* (3.7% vs. 3.9%), *Proteobacteria* (1.2% vs. 1.7%), *Cyanobacteria* (0.5% vs. 0.3%), *Verrucomicrobiota* (0.2% vs. 0.1%), *Fibrobacterota* (0.05% vs. 0.03%), *Patescibacteria* (0.02% vs. 0.03%), *Desulfobacterota* (0.02% vs. 0.01%), and *Elusimicrobiota* (0.02% vs. 0.01%). There were no significant differences at the phylum level for FC ratio ($p > 0.05$).

Regarding the identified families, statistical differences were observed based on the FC ratio. Specifically, FC_H diets had a suppressive effect on *Lachnospiraceae* (10.9% vs. 14.7% $p < 0.01$, in FC_H and FC_L respectively), *Clostridiaceae* (1.2% vs. 2.3%, $p < 0.01$), *Selenomonadaceae* (0.06% vs. 0.13%, $p < 0.05$) and *Succinivibrionaceae* (0.8% vs. 1.3%, $p < 0.05$) while it enhanced [*Eubacterium*]*_coprostanoligenes_group* (3.2% vs. 2.5%, $p < 0.05$).

Finally, differential analysis at the genus level was conducted, with results showed in figure 2D. The FC ratio affected 10 genera. Specifically, FC_H diets increased the abundance of *[Eubacterium]_coprostanoligenes_group* (3.6% vs. 2.9% $p < 0.01$ in FC_H and FC_L, respectively), *Ruminococcaceae uncultured* (1.3% vs. 1.0%, $p < 0.05$), and *Butyricicoccaceae UCG-009* (1.2% vs. 0.9%, $p < 0.05$), *Victivallis* (0.05% vs. 0.02%, $p < 0.001$). In contrast, a reduction was found for *Clostridium_sensu_stricto_1* (1.3% vs. 2.3%, $p < 0.001$), *Cellulosolyticum* (0.7% vs. 1.4%, $p < 0.001$), *Clostridium_sensu_stricto_6* (0.08% vs. 0.45%, $p < 0.001$) and *Negativibacillus* (0.1% vs. 0.2%, $p < 0.05$).

Microbiota Characterization by LS presence

Alpha diversity (Figure 3A) showed differences between the two groups, with a reduction for all the metrics in LS_yes compared with LS_no. This reduction was significant with the Observed Species index (Wilcoxon-exact test $p = 0.05$), Shannon Index (Wilcoxon-exact test $p = 0.0004$), Simpson Index (Wilcoxon-exact test $p = 0.001$), and Fisher Index (Wilcoxon-exact test $p = 0.05$). Beta diversity (Figure 3B) also showed statistically significant differences across all metrics considered: Bray-Curtis ($p = 0.0001$), Canberra ($p = 0.0001$), Weighted UniFrac ($p = 0.0002$), and Unweighted UniFrac ($p = 0.0002$). Consistent with this, greater differences in differential abundance were identified for all analysed taxa (Table S5). For the identified phyla showed in figure 3C, seven were differentially abundant between the two conditions. Of these, four were reduced in LS_yes diets: *Verrucomicrobiota* (0.15% vs. 0.05%, $p < 0.001$ LS_no and LS_yes, respectively), *Bacteroidota* (38.1% vs. 36.6%, $p < 0.01$), *Elusimicrobiota* (0.01% vs. 0.07%, $p < 0.05$), and *Cyanobacteria* (0.4% vs. 0.2%, $p < 0.05$). The three phyla that increased in the presence of LS were *Spirochaetota* (3.9% vs. 4.6%, $p < 0.001$ LS_no and LS_yes, respectively), *Proteobacteria* (1.6% vs. 2.1%, $p < 0.001$), and *Firmicutes* (55.6% vs. 56.4%, $p < 0.01$).

This difference was also reflected at the family level, with 17 families differentially abundant between the two conditions, and at the genus level, with 34 taxa differentially abundant (Figure 3D). Specifically, in LS_yes diets an increased abundance was observed for *Oscillospiraceae* (13.7% vs. 14.5%, $p < 0.001$ in LS_no and LS_yes, respectively), *Prevotellaceae* (8.4% vs. 9.8%, $p < 0.001$), *Muribaculaceae* (1.8% vs. 2.3%, $p < 0.001$) and *Clostridiaceae* (1.5% vs. 2.5%, $p < 0.001$) and *Erysipelotrichaceae* (1.2% vs. 1.5%, $p < 0.001$). Meanwhile, the presence of LS favoured the reduction of *Uncultured Bacteroidota* (1.8% vs. 0.5%, $p < 0.001$), *p-2534-18B5_gut_group* (1.4% vs. 0.5%, $p < 0.001$), and *Victivallaceae* (0.2% vs. 0.05%, $p < 0.001$).

At genus level, LS_yes had a significantly greater abundance of *Prevotella* (0.3% vs. 0.8%, $p < 0.001$, in LS_no and LS_yes, respectively), *Ruminococcus* (0.1% vs. 0.5%, $p < 0.001$), *Clostridium_sensu_strictu* 1 (1.6% vs. 2.5%, $p < 0.001$), *Clostridium_sensu_strictu* 6 (0.1% vs. 0.5%, $p < 0.001$), *Alloprevotella* (2.4% vs. 3.5%, $p < 0.001$), *Lachnospira* (0.01% vs. 0.03%, $p < 0.001$), *Cellulosilyticum* (0.9% vs. 1.5%, $p < 0.001$) and *Ruminobacter* (1.6% vs. 1.1%, $p < 0.05$). Additionally, several unclassified bacteria from the *Prevotellaceae* family were found to be more abundant in the LS_yes diets. On the other hand, there was a reduction in the abundance of certain genera, such as *Dorea* (0.1% vs. 0.06%, $p < 0.05$), *Elusimicrobium* (0.03% vs. 0.008%, $p < 0.05$), and *Victivallis* (0.05% vs. 0.02%, $p < 0.05$) in the LS_yes.

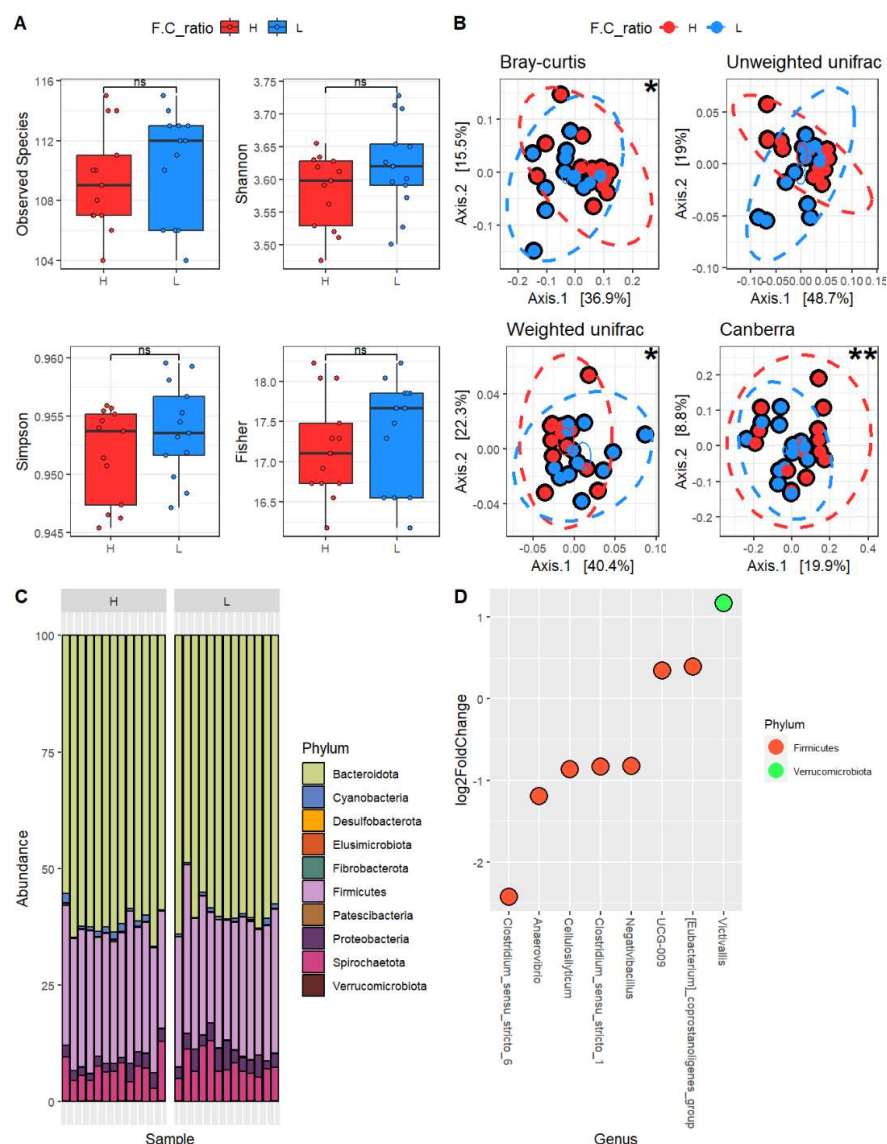


Figure 2. Microbiota characterization based on forage to concentrate (FC) ratio. A) Alpha diversity; B) Beta diversity; C) Abundance of identified phyla, reported for samples with “high” (H) or “low” (L) FC ratio; D) Differential abundance at the genus level, where a positive Fold Change indicates that the identified organism is more prevalent in samples with a high FC ratio.

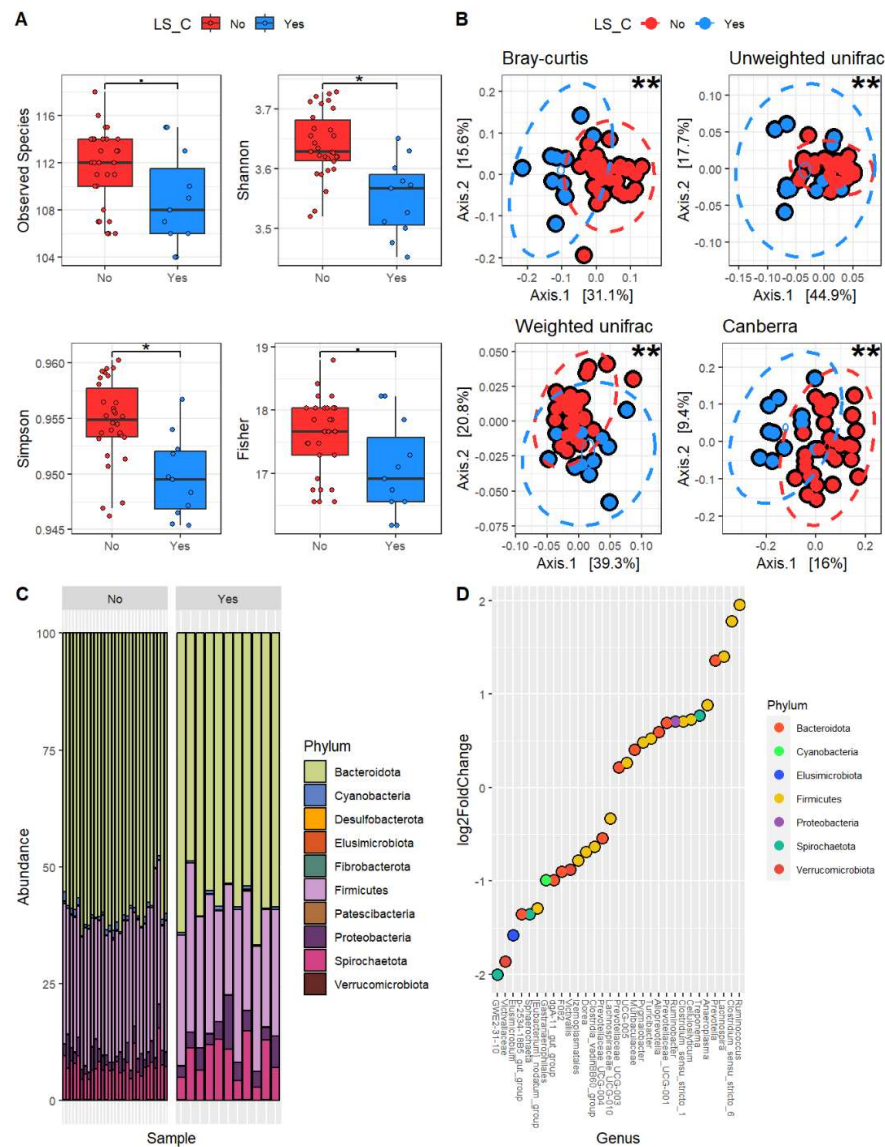


Figure 3. Microbiota characterization based on the presence of linseed (LS). A) Alpha diversity; B) Beta diversity; C) Abundance of identified phyla, reported based on the presence (yes) or absence (no) of Linseed; D) Differential abundance at the genus level, where a positive Fold Change indicates that the identified organism is more prevalent in samples where Linseed is present.

DISCUSSION

The aim of this study was to characterise the microbial composition of the faeces of Mediterranean dairy buffaloes and to identify which dietary factor had the greatest influence on the faecal microbiota. The findings revealed that the FC ratio and LS supplementation are the primary dietary factors affecting the microbiota composition.

The chemical composition of the diets was similar to that reported in recent studies on dairy buffaloes only in terms of DM and ash content (Evangelista et al., 2022; Masucci et al., 2024). Only fiber content was higher in this study compared with previous studies. Additionally, the physical composition varied compared to other studies (Evangelista et al., 2022; Masucci et al., 2024). Clearly, these differences can be attributed to the composition of the diets. While the similarities and differences between studies are noteworthy, it should be emphasised that Masucci et al. (2024) used a 2-sieve separator.

The composition of the diets differed between the considered groups. As we expected, FC_L resulted in a higher MFU and had a lower DM content, higher crude protein, and higher bottom compared with FC_H. This is attributed to the average presence of by-products (threshers) and the inclusion of green forage, which have high moisture and protein content. Meanwhile, the presence of LS does not affect the chemical composition of the diet, except for a decrease in ash content.

The FC ratio had minimal influence on the FAs composition of TMR. In contrast, LS significantly altered the FA profile. In LS_yes diets, there was a notable increase in ALA, while the content of LA decreased compared with LS_no, consistent with findings from previous studies (Iseberg et al., 2019; Ghedini et al., 2024). Moreover, the overall content of polyunsaturated fatty acids (PUFA) was elevated in LS_yes diets, indicating an improved FAs profile. Additionally, LS_yes diets led to a significant improvement in the n6 to n3 ratio, reflecting a more favourable balance of essential FAs. This modification is crucial, as PUFA are associated with various health benefits for animals.

The FC ratio influences apparent digestibility and faecal composition. As reported in the results, FC_L diets led to decreased digestibility across all parameters studied. Several authors (Mousa et al., 2018; Haselmann et al., 2019; Evangelista et al., 2024) have noted that particle size appears to play a critical role in feed digestion mechanisms. Studies on dairy cows (Haselmann et al., 2019) found that reducing TMR particle size from 52 mm to 7 mm improved digestive capacity. However, smaller particle size increases the passage rate and flow of fermentable nutrients from the rumen, potentially impacting hindgut fermentation (Castillo-Lopez et al., 2019). This finding aligns with the results of the present study, which showed a decline in the digestibility of most components in FC_L diets characterized by a higher proportion of fine particles (> 4 mm).

Faecal analysis also revealed lower pH levels in FC_L, suggesting increased fermentation in the gut. This result is consistent with reports indicating that high-concentrate diets, particularly those rich in starch and sugars, lower rumen pH and impair fiber digestibility while altering cellulolytic

bacterial activity (Valadares et al., 2000; Zicarelli et al., 2008). As discussed earlier, FC_L, with its higher fine material content, tends to have a reduced particle size. This reduction has been associated with an increased rate of particle escape from the rumen, leaving insufficient time for proper digestion (Mousa et al., 2018). Furthermore, the reduced activity of cellulolytic microbes is not only caused by a lower rumen pH but can also result from increased starch fermentation and substrate competition (Van Soest, 1994).

Alfa and beta diversity

Alpha diversity quantifies species richness and evenness within habitats, reflecting the variety and distribution of species in ecological communities (Thukral, 2017). Changes in nutrient supply and availability can shift community structure (Plaizier et al., 2017, 2018) and bacterial diversity (Tao et al., 2017). According to previous studies (Kim et al., 2014; Noel et al., 2019), diet significantly impacts the composition of bacterial communities by providing the necessary substrates for bacterial growth, thereby exerting selective pressure on these communities. In the present analysis, the alpha diversity indicated that neither the direct species count (Observed) nor the estimate of species richness (Fisher's α) showed significant differences related to the FC ratio or LS presence. This suggests that the samples do not exhibit a significant variation in species richness, or that any differences present are not substantial enough to affect overall diversity. However, diversity indices based on species abundance, such as Shannon and Simpson, were higher in the LS_no compared to the LS_yes, suggesting that the absence of LS contributes to greater overall evenness and diversity within those communities. These indices account for both the number of species and their distribution and relative abundance. The greater diversity observed in the LS_no may suggest a more equitable distribution of species, with a wider variety of species coexisting in similar abundances. This implies that no single species is dominating the resources, thereby contributing to increased diversity. The addition of LS in the diet appears to significantly modify the composition and structure of the faecal microbiome compared to the diet without LS. The Canberra index indicates differences in the relative abundances of microbial species between the two groups, suggesting that LS may promote some microbial groups while suppressing others. The significant result with Unweighted UniFrac shows differences in the presence or absence of species, indicating that the phylogenetic composition of the microbiome is significantly different between the two diets. Finally, the Weighted UniFrac index suggests that, in addition to the presence/absence of species, the relative abundance of species has also changed, altering the overall structure of the faecal bacterial community.

Diets with different FC ratios seem to primarily influence the composition and abundance of microbial species rather than their presence/absence. This suggests that dietary changes mainly affect the relative amounts of species in the microbiome, rather than introducing or removing specific species. Therefore, while the Unweighted UniFrac index does not detect significant differences in terms of species presence, other indices suggest that FC ratio may influence the structure and abundance of microbial species in the faeces. Research on dairy cows has shown that higher concentrate inclusion in their diet reduces microbial variability in faeces, typically accompanied by a decrease in faecal pH, which significantly impacts microbial communities (Plaizier et al., 2017).

In this study, faeces from the LS_no group were less concentrated, with lower levels of starch, ether extract, and crude protein, compared to those from the LS_yes group, and they also exhibited a higher pH. These differences in faecal concentration and pH may explain the greater microbial diversity observed in the LS_no group, as faecal starch has been identified as a key factor influencing microbial diversity (Shanks et al., 2011). Moreover, faecal pH is a critical determinant of microbial diversity, much like in the rumen, where lower pH levels are strongly associated with reduced species richness and diversity (Khafipour et al., 2009; Petri et al., 2013; Plaizier et al., 2017). However, starch and pH are not the only factors influencing microbial diversity. Linseed, which is rich in polyunsaturated fatty acids (PUFAs), may influence the gut microbiota profile either by promoting the lysis and solubilization of bacterial cell membranes, thereby exerting antimicrobial effects by destabilizing these membranes, inhibiting bacterial growth, and potentially reducing overall microbial diversity, or by serving as metabolic substrates for gut bacteria thus influencing the profile of the intestinal microbiota (Guo et al., 2023). Linseed has been shown to enhance the growth of bacteria associated with propionate and butyrate production, such as members of the *Prevotellaceae* family (Wang et al., 2019), as highlighted in this study and discussed further below. It has been observed that an increased abundance of *Prevotellaceae* may further reduce microbial diversity by inhibiting competing bacterial groups (Lyons et al., 2017). In addition to its nutrient composition, LS contains bioactive compounds, such as lignans and tannins (Imran et al., 2012; Ghedini et al., 2024), which can selectively modulate the microbiota. Tannins, for instance, have been shown to inhibit biohydrogenation bacteria, with sensitivity varying even within the same genus (Nelson et al., 1997). This may help explain the reduced microbial diversity observed in the LS_yes diets. Specifically, studies on goat rumen microbiota have reported a decline in bacterial diversity following dietary supplementation with LS (Cremonesi et al., 2018).

The richness and diversity of bacterial species are considered crucial indicators of gut microbiota health. A reduction in microbial richness and diversity can compromise the functionality and resilience of the microbiota, increasing its susceptibility to invasion by external microorganisms, including pathogens (Khafipour et al., 2016). However, while a less diverse microbiota may be more vulnerable in some contexts, it can also confer benefits, such as improved nutrient utilisation efficiency (Belanche et al., 2019).

Relative abundance

Our results reveal that *Firmicutes* and *Bacteroidetes* were the two most dominant phyla, together accounting for over 94% of the total abundance. These findings align with several other studies, including those by Zhao et al. (2024) on Murrah and Nili-Ravi buffalo faeces, and by Plaizier et al. (2017) and Liu et al. (2020) on dairy cow faeces. The FC ratio did not exhibit significant differences at the phylum level, which contrasts with findings reported in other studies in cow (e.g., Kim et al., 2014; Zhang et al., 2018).

In the present study, lower relative abundance of *Bacteroidota* and a higher relative abundance of *Firmicutes* and *Proteobacteria* in the FC_L diets was observed. However, these differences were not statistically significant, likely due to the inherent variability in group-level data. Previous studies (El Kaoutari et al., 2013) have shown that an increase in *Bacteroidota* is associated with improved digestibility, and we hypothesise that a similar trend might occur in the present study.

Populations of *Bacteroidota*, along with *Fibrobacterota* and *Cyanobacteria*, have been positively correlated with NDF and ADF levels in the diet (Martinez-Fernandez et al., 2020). This suggests a consistent pattern: the bacterial microbiota of ruminants consuming diets high in lignocellulose tends to be dominated by *Bacteroidetes*, in contrast to diets rich in more readily fermentable carbohydrates (Martinez-Fernandez et al., 2020). *Bacteroidetes* are crucial for maintaining gut homeostasis. Bacteria in this phylum encode numerous enzymes for polysaccharide degradation, complementing eukaryotic genomes by breaking down otherwise indigestible carbohydrates and modulating caloric absorption (Tang et al., 2017; Castillo-Lopez et al., 2020). They play a vital role in the gut ecosystem and are linked to host immunity. Dysbiosis of this phylum may contribute to infections, inflammatory bowel disease, or autoimmune disorders (Gibiino et al., 2018). Additionally, some studies (e.g., El Kaoutari et al., 2013; Plaizier et al., 2017) suggest that *Bacteroidota* are more efficient at degrading structural carbohydrates compared to *Firmicutes*. This efficiency stems from the higher number of glycoside hydrolase and polysaccharide lyase genes per genome in *Bacteroidota*, allowing them to act as key degraders of complex

polysaccharides in plant cell walls (Meale et al., 2016). This may partially explain the lower apparent digestibility observed in the FC_L diets. However, these effects were not clearly evident in the present study, likely because previous studies specifically increased the proportion of concentrates in the diet, while this study focused on characterising the microbiome under different dietary conditions.

The three most abundant bacterial families identified were *Oscillospiraceae*, *Lachnospiraceae*, and *Rikenellaceae*, with relative abundances ranging from 10% to 14.5%. The FC ratio significantly influenced the prevalence of both predominant and less abundant bacterial families. Specifically, *Lachnospiraceae*, known for their role in sugar fermentation (Russell, 2002), exhibited a marked response to diets rich in concentrates. This finding aligns with previous research, including Rivera-Chacon et al. (2024), who reported a higher abundance of *Lachnospiraceae* in high-concentrate diets. Similarly, Zhang et al. (2022) observed an increase in *Lachnospiraceae* when dietary grain inclusion rose from 40% to 70%. Furthermore, as reported by Khafipour et al. (2016) and Castillo-Lopez et al. (2019), both *Succiniovibrionaceae* and *Lachnospiraceae* are more prevalent in environments with higher levels of soluble carbohydrates and in the faeces of cows fed diets containing finer particles. Altering the particle size of forages can influence not only nutrient availability for the host and production performance but also the substrates and metabolic pathways available to microbes (Mertens, 1997), thereby modifying the microbial habitat (Castillo-Lopez, 2019). As discussed earlier, the FC_L diets in our study contained a higher proportion of fine particles. We hypothesize that these particles may have rapidly bypassed the rumen, creating a more acidic environment in the faeces and promoting the relative abundance of specific families, such as *Lachnospiraceae*. This aligns with the findings of Castillo-Lopez et al. (2019), who also reported lower faecal pH in diets with finer particle sizes. Higher inclusion of concentrate in the diet increases the starch content of the digesta, enhancing fermentation in the large intestine and altering the composition of the microbiota in the colon and faeces (Plaizier et al., 2017). A lower faecal pH, as observed in our study, suggests a corresponding decrease in pH within the large intestine. This could be associated with increased production of total SCFA, as reported by Rivera-Chacon et al. (2024), who emphasized the impact of high-concentrate diets on SCFA accumulation. Additionally, faecal pH has been proposed as a less invasive method to evaluate intestinal acidification in dairy cows fed high-concentrate diets (Rivera-Chacon et al., 2024). This underscores the potential of faecal pH as a valuable indicator for assessing the effects of dietary interventions on intestinal health and fermentation dynamics.

At the genus level, decreased abundance of *Victivallis*, *Eubacterium coprostanoligenes_group*, and *UCG-009* in the faeces of animals fed a FC_L diet was observed. This contrasts with previous studies that found *Victivallis* to be more abundant in diets with higher concentrate (Ali et al., 2020). *Clostridium sensu stricto 1*, which was found in higher amounts in the faeces of cows under heat stress, is believed to be a pathogen that can compromise intestinal health (Li et al., 2020). Metzler-Zebeli et al. (2013) reported that *Clostridium* was correlated with the presence of concentrates in the diet. This is consistent with what observed in this study and may support the hypothesis of impaired digestibility and intestinal issues resulting from excessive fermentation of non-structural carbohydrates in the intestine. Although there were similarities between the present study and others at the phylum and family levels, no clear associations were found at the genus level. This can be explained by the fact that microbiota composition is influenced by a variety of factors, including diet formulation, farm management, geographical location, and individual diversity, as reported by Tang et al. (2017).

The inclusion of LS in animal diets has shown to significantly influence the composition of the faecal microbiota, specifically at the phylum level, as demonstrated in the present study. These results align with previous studies in different animal models, including those on goat faeces (Liu et al., 2020; Guo et al., 2023) and the rumen (Cremonesi et al., 2018; Wang et al., 2019), which reported similar shifts in microbial populations when LS or its oil was incorporated into the diet. Notably, our findings revealed a higher relative abundance of *Firmicutes*, *Proteobacteria*, *Spirochaetota*, and *Elusimicrobiota*, with a concurrent reduction in *Bacteroidota*, *Cyanobacteria*, and *Verrucomicrobiota* in LS_yes diets. This shift is consistent with the results observed by Guo et al. (2023), who found that both LS seeds and its oil increased the abundance of *Firmicutes* and *Spirochaetota* in the rectum of cashmere goats, which supports our findings. The increased presence of *Proteobacteria* is particularly intriguing, as this phylum includes many Gram-negative bacteria, some of which are pathogenic and associated with ruminal dysbiosis (Liu et al., 2021; Guo et al., 2023). Guo et al. (2023) also reported an increase in *Proteobacteria* in goat with LS oil supplementation, suggesting that LS oil might increase the risk of pathogenic invasion or interfere with nutrient absorption. This is an important consideration when assessing the overall health implications of LS inclusion in the diet.

Another significant finding was the reduced abundance of *Verrucomicrobiota*, a group of predominantly anaerobic organisms essential for maintaining intestinal health, including the regulation of the mucus layer between the intestinal lumen and enterocytes (Arnold et al., 2020). The reduction of *Verrucomicrobiota* in LS_yes diets could potentially disrupt intestinal health,

particularly in the posterior intestine. Additionally, our observation of a lower faecal pH in the LS_{yes} suggests an acidic shift in the gastrointestinal tract, which is consistent with the onset of intestinal acidosis (Lyu et al., 2022). This decrease in pH could be attributed to the presence of easily fermentable substrates in the diet, such as starch, proteins, and fats, as discussed earlier, as well as bioactive components in linseed, like lignans and tannins. Such an acidic environment might inhibit the growth of certain bacterial groups, particularly those that prefer a more alkaline environment, while promoting bacteria that thrive in acidic conditions. This could explain the observed decrease in bacteria such as *Dorea*, *Victivallis*, and *Elusimicrobium*, which may be more sensitive pH changes.

The inclusion of LS significantly altered the relative abundance of certain bacterial families, including *Clostridiaceae*, *Oscillospiraceae*, *Prevotellaceae*. The increased presence of *Prevotellaceae* in LS_{yes} diets align with the findings of Wang et al. (2019), who reported similar microbial trends in the rumen of goats.

As mentioned earlier, LS contains lignans. Previous studies (Ghedini et al., 2024) have highlighted the ability of *Prevotella* to metabolize lignans, particularly secoisolariciresinol diglucoside, the most abundant lignan in LS, into bioactive compounds such as enterodiols and enterolactone. These metabolites have been linked to several health benefits, including potential anti-cancer properties (Ghedini et al., 2024).

In addition to these microbial shifts, LS has been shown to possess antimicrobial activity, likely due to the PUFAs it contains, as discussed earlier. The primary PUFAs, particularly ALA, have been reported to exert toxic effects on ruminal cellulolytic bacteria (Maia et al., 2007), which could further explain some of the microbial changes observed in the present study. This underscores the complex interplay between diet, gut microbiota, and the potential health implications of introducing LS or its derivatives into animal feeds. However, further research is needed to fully understand the mechanisms by which LS affects the gut microbiota and its long-term impacts on animal health and productivity.

CONCLUSIONS AND DIRECTIONS FOR FUTURE RESEARCH

These pioneering findings provide valuable insights into the structure of the faecal bacterial community in Italian Mediterranean dairy buffaloes, emphasising the significant impact of dietary composition on microbial diversity, as reported for other ruminants. The identification of key

microbial families serves as a foundation for future research into their functional roles and the development of strategies to optimise nutritional management. Such strategies have the potential to enhance animal health, productivity, and environmental sustainability in buffalo production systems. While faecal microbiota analysis remains a valuable tool for assessing bacterial composition linked to digestibility, our study found no statistically significant effects of bacterial populations on apparent fiber digestibility. This limitation may stem from the group-level sampling approach rather than individual-level analysis, as well as the descriptive focus of the study, which aimed to characterise the microbiome under varying dietary conditions rather than testing targeted feeding interventions.

In line with findings from goat and cow studies, LS appears to have partially negative effects on gut health, particularly by reducing microbial diversity. Although LS is typically included in diets to enrich products with polyunsaturated fatty acids, our results suggest potential negative effects on dairy buffaloes. However, there is no evidence of impaired digestibility, though further investigation is warranted to confirm these findings.

As dairy buffalo diets increasingly mirror those of dairy cows, with a focus on higher concentrate levels, it is essential to consider the implications of this shift on buffalo health and performance. Diets rich in concentrates and fine particles can lead to greater intestinal fermentation, potentially compromising gut health. Moreover, dairy buffaloes have a heightened requirement for coarse fiber compared to other large ruminants. Coarse fiber is critical for maintaining digestive efficiency, supporting gut health, and improving feed digestibility. Enhancing digestibility can not only boost productivity but also reduce the environmental impact of buffalo farming, a sector poised for substantial growth in the coming years.

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SUPPLEMENTARY FILES

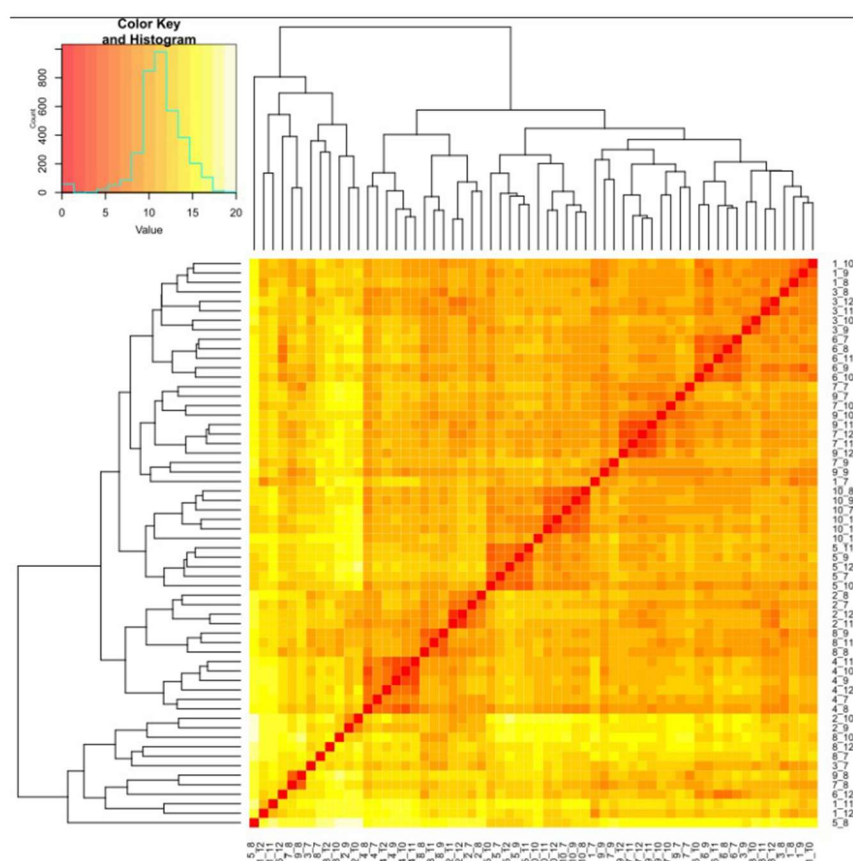


Figure S1. Heatmap of the 60 faeces samples.

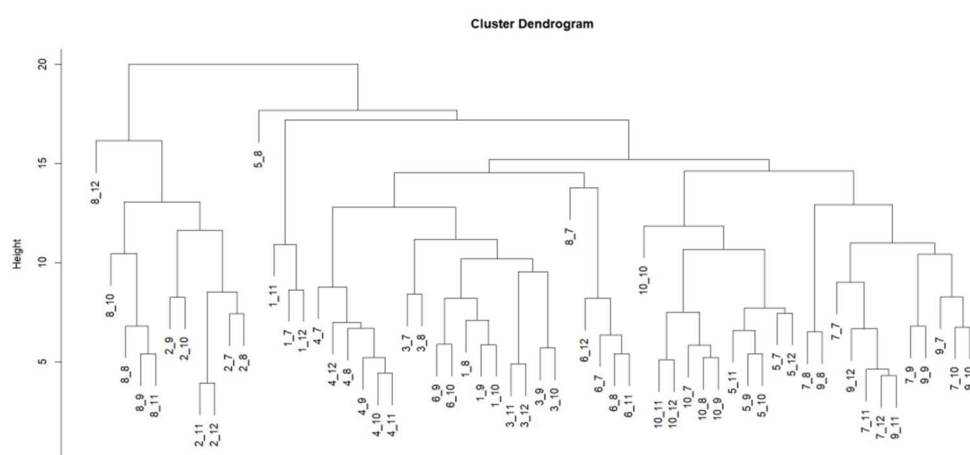


Figure S2. Dendrogram of the 60 faeces samples.

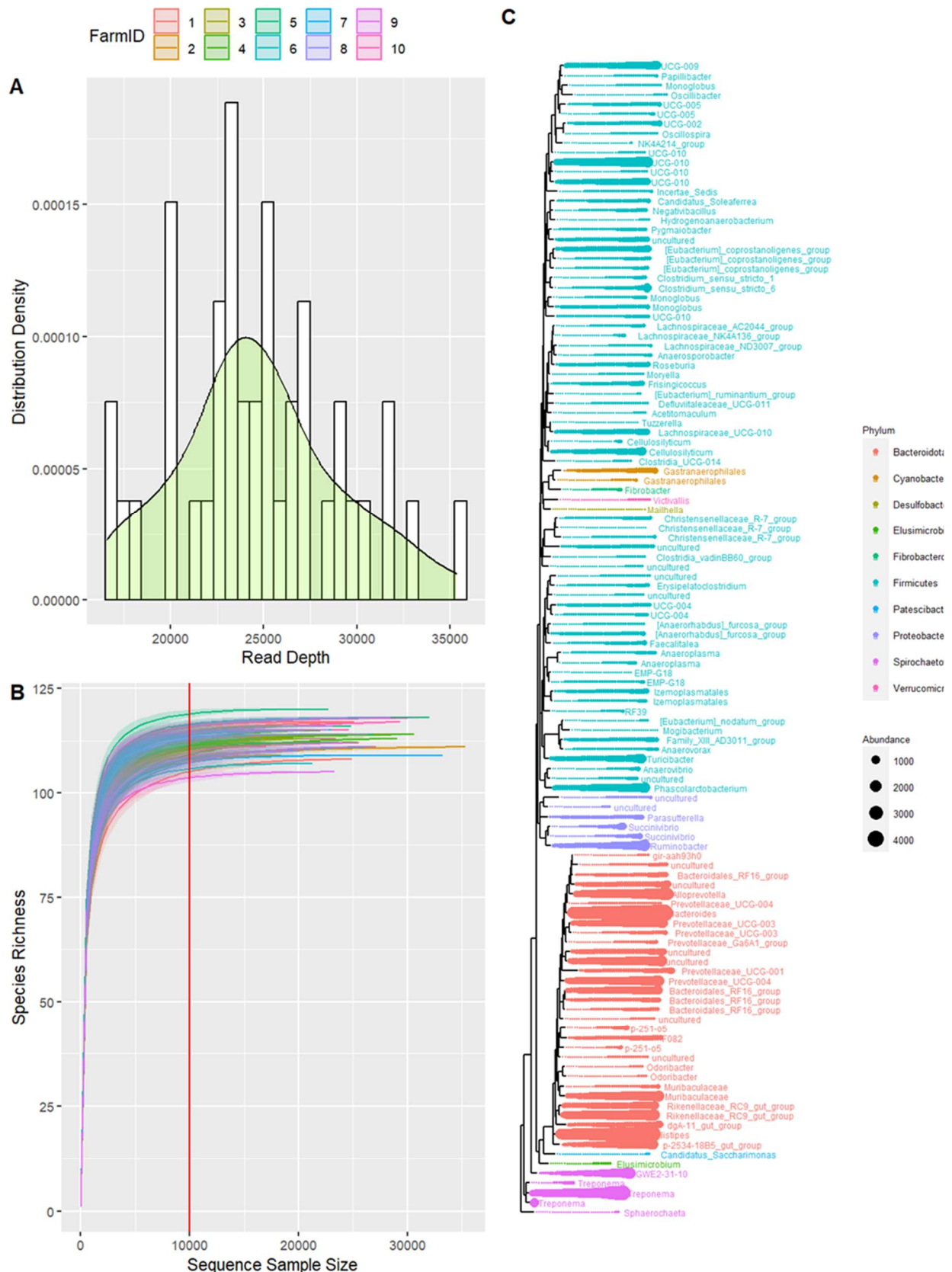


Figure S3. A) distribution of the number of reads per sample; B) distribution of sequences per sample; C) phylogenetic tree of the 120 species.

Table S1. List of the chemical-physical components of the diet and raw materials considered in the analysis.

Variables	P value PERMANOVA
MFU	0.0154
CP	0.1634
EE	0.2287
aNDF	0.1494
Starch	0.1438
Upper sieve	0.2833
bottom	0.0424
peNDF	0.0814
Green Forage	0.3864
Corn Silage	0.0746
LS	0.0001
FC ratio	0.0058

MFU = milk forage unit; CP = Crude Protein; EE = Ether Extract; aNDF = neutral detergent fiber; Upper Sieve = particles >19mm; Bottom = particles < 4mm; peNDF = physical effective NDF; LS = linseed presences; FC ratio=forage-to-concentrate ratio.



Chapter 5

Chapter 5

Summary and conclusions

Livestock play a crucial role in providing essential nutrition and supporting the livelihoods of families and communities worldwide. However, the growing demand for animal products, driven by population growth and rising global incomes, has led to a surge in GHG emissions from the livestock sector. As previously mentioned, according to the FAO (2023), livestock and agrifood systems account for 12% of all anthropogenic GHG emissions, among all the gases produced by the sector, methane is the most significant, contributing to more than 50% of total emissions. Furthermore, methane has a much greater impact on global warming compared to CO₂, with a warming effect approximately 28 times more potent (Grossi et al., 2019). The latest assessment from the International Panel on Climate Change (IPCC) has emphasized the urgent need to reduce atmospheric methane concentrations to combat the rise in global temperatures. Consequently, to meet international GHG mitigation targets and policies, there is a pressing need for the rapid implementation of methane abatement strategies within the global livestock sector.

In response to this increasing demand and its associated environmental impact, addressing these emissions through effective mitigation strategies has become crucial. One of the most effective approaches to achieving this is through the proper management of livestock feeding. As highlighted in the literature presented in **Chapter 2**, diet emerges as the third most important factor when discussing enteric methane emissions in the livestock sector (Evangelista et al., 2024). The first two terms were milk and cow, with a clear reference to cows, which emit more methane compared to other species.

Moreover, the modulation of the diet can also have a direct impact on the animal's performance. Adjusting the feed can improve productivity while simultaneously reducing methane emissions. This creates a dual benefit: optimizing both the environmental sustainability of livestock farming and the economic performance of livestock operations. Therefore, improving feeding practices and focusing on precision feeding is a vital step towards mitigating methane emissions from the sector and addressing the broader challenge of climate change. Precision feeding, which focuses on providing an optimal diet tailored to the specific needs of the animals, is a key area of research.

To improve nutrition and reduce waste, more efficient and rapid analysis systems, such as NIR spectroscopy, have been employed in recent years. The most recent innovations involve the introduction of portable devices for on-farm use. However, these tools do not demonstrate unquestionable predictive capabilities, as current food analysis protocols are still based on standard methodologies. Despite this, the results obtained allow for improved feed management and increased farm income, as highlighted by Piccioli-Cappelli et al. (2019), who evaluated the effect of NIRS technology on the distribution of TMR, particularly in terms of accurate DM determination. Their study demonstrates that integrating such systems leads to a more consistent ration, closely resembling the formulated diet, resulting in a reduction of DM usage by 1.35% while simultaneously increasing total daily milk production by 2.4%.

The calibrations made for TMR were based on rations formulated for dairy cows, characterized by a high proportion of concentrates (at least 40%) compared to those intended for buffaloes. For this reason, in **subchapter 3.2**, attempts were made to assess both benchtop and portable devices for predicting the chemical composition of TMR for buffaloes and, pioneeringly, the fatty acid content of TMR. However, some issues emerged, partly due to the limited number of samples analysed and partly due to the influence of water present in the samples, which affected the accuracy of the results.

The study highlighted several challenges in applying NIR spectroscopy to TMR analysis, especially when using a portable device under field conditions. One of the main issues was the limited chemical information obtained from the spectral data, despite preprocessing efforts. This is due to the complexity of the TMR matrix, which, especially in fresh, unground samples, made it difficult to differentiate the individual chemical components spectrally.

Another important aspect is related to the R^2 values for cross-validation and prediction, which remained below 0.9 for most components, despite the use of advanced techniques such as outlier detection and variable selection. Only a few models, particularly those based on benchtop NIRS, showed better performance. Additionally, the risk of overfitting was a significant concern due to the high number of latent variables used. Although stepwise variable selection was employed to reduce spectral noise, the prediction of fatty acids was particularly inaccurate, further confirming the inherent challenges of using NIR spectroscopy for their determination in complex matrices like TMR. A key point concerns the operational limitations of portable NIRS, which, despite being promising, demonstrated inferior performance when compared to benchtop NIRS when applied directly in the field.

However, the development and adoption of portable instruments are still hindered by their high cost, which remains too expensive to date. For this reason, in **subchapter 3.2**, machine learning techniques were employed to select the specific wavelengths for analysing the fibrous components (NDF, ADF, ADL) and DM of the TMR. The goal was to obtain a spectral range that could be applied in a portable, user-friendly device that would also reduce the production costs of such tools.

Although understanding the chemical composition of the diet is considered a fundamental practice for managing and mitigating the risk of animals not receiving a diet tailored to meet their specific needs, the preparation and distribution of TMR can compromise this objective. For this reason, in **subchapter 3.3**, we decided to investigate the key factors that influence the preparation of a balanced diet and its even distribution along the entire feeding lane for dairy buffaloes. The aim was to provide practical guidance to farmers who participated in evaluating the homogeneity of distribution within their operations.

From this study, it emerged that farmers do not pay attention to this aspect, and moreover, most of them do not use standardized methods in the routine preparation of the mixer wagon (e.g., mixing times). Our study revealed that the loading sequence of feeds, which affects the adhesion properties of particles, significantly impacts distribution homogeneity. The type of mixer wagon and the dry matter content were also identified as influential factors. Our study serves as an initial exploration into the factors influencing homogeneity of distribution, but it is limited in scope and does not offer a comprehensive understanding of all the variables and their modes of influence. Therefore, future research should expand to include a greater number of farms and factors to provide more comprehensive insights. Understanding these factors will enable farmers to manage them effectively to improve the homogeneity of the ration.

Precision feeding focuses not only on providing macronutrients but also on supplying essential micronutrients such as vitamins, minerals, and antioxidants. This approach is particularly important during periods of stress (e.g., transition periods or heat stress), as deficiencies in these nutrients can compromise animal health and well-being. Initially, a lack of these nutrients can lead to a reduction in productive and reproductive efficiency, causing economic losses for the farm. In **subchapter 4.1**, we tested the effect of supplementing antioxidants, zinc, and selenium on the productive response of lactating buffaloes. The results showed that the treatment positively

improved milk yield (+3.1%) and milk coagulation properties, with no significant differences in other milk parameters. While it is not easy to explain the improvement in milk coagulation properties, the benefits of proper antioxidant supplementation to enhance the cheese-making properties of milk are particularly relevant to farmers, as 100% of buffalo milk is used for cheese production. Further research is needed to explore the effects of Zn, Se, and SOD supplementation on milk coagulation properties, considering other parameters closely linked to the milk's coagulation ability.

The study on the physical characterization of buffalo diets presented in **subchapter 4.2** revealed significant variability in the diets of dairy buffaloes, despite being conducted in a relatively small area. This variability also resulted in considerable fluctuations in the physical component of the diets, particularly in the first sieve (particles greater than 19mm), where values ranged from a minimum average of 4.33% to a maximum average of 41.33%. Furthermore, significant correlations were observed between the particle size distribution and the amount of milk produced, as well as between the quality of milk and the apparent digestibility of the feed. Several factors, including the negative correlation between somatic cell count and the first sieve, warrant further investigation.

Although the study was conducted in a limited area, it involved 10 farms over the course of a full year, including periods when animals were fed fresh forage. Therefore, it can be concluded that the study is representative of the feeding management practices of Italian buffaloes, and it also confirms the need for standardization of the physical component of the diet. This is especially relevant considering that the Italian buffalo farming sector has experienced significant growth in recent years, with an increase in production per animal. For this reason, the intensification of the sector calls for the adoption of feeding management protocols that take into account not only the chemical components but also the physical aspects of the diet.

Finally, the faecal microbiota of Italian Mediterranean buffaloes was characterized for the first time, as presented in **subchapter 4.3**. This preliminary study was carried out using samples collected in the previous study, during which faecal samples were taken from groups of buffaloes from 10 different farms. Thanks to the observed variability, it was possible to identify various dietary variables and determine those that had a statistically significant influence on shaping the faecal microbiota of the buffaloes involved in the study.

The study revealed an association at the phylum and family levels with other studies conducted on cows and goats, and it clarified the dynamics of the more abundant presence of certain phyla/families in groups that were fed a more concentrated ration or that included, for example, linseed in the diet. Interesting information also emerged regarding the association between particle size distribution and the abundance of certain families, confirming what had already been observed by Castillo-Lopez et al. (2019) in dairy cows. However, the association with other studies on genera was not detected, likely because the data compared were from cows and goats, as well as differences in feeding management and farming practices among the studies. Additionally, even though only slight associations were observed between the composition of the faecal microbiota and the apparent digestibility of the animals, these results are promising, as non-invasive methods for assessing feed conversion efficiency are being sought. Using the faecal microbiota of ruminants could represent a potential solution to this issue.

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Chapter 6

Chapter 6

Lists of the PhD activities done during the course

List of publications:

1. **Evangelista, C.**; Bernabucci, U.; Basiricò, L. 2022. Effect of Antioxidant Supplementation on Milk Yield and Quality in Italian Mediterranean Lactating Buffaloes. *Animals*, 12, 1903. <https://doi.org/10.3390/ani12151903>
2. Basiricò, L.; Morera, P.; **Evangelista, C.**; Galaverna, G.; Sforza, S.; Prandi, B.; Bernabucci, U.; Nardone, A. 2022. Effect of Parmigiano Reggiano Consumption on Blood Pressure of Spontaneous Hypertensive Rats. *Dairy*, 3, 364–376. <https://doi.org/10.3390/dairy3020028>
3. Ortenzi, L.; Violino, S.; Costa, C.; Figorilli, S.; Vasta, S.; Tocci, F.; Moscovini, L.; Basiricò, L.; **Evangelista, C.**; Pallottino, F.; Bernabucci, U. 2023. An innovative technique for faecal score classification based on RGB images and artificial intelligence algorithms. *The Journal of Agricultural Science* 1 – 6. <https://doi.org/10.1017/S0021859623000114>
4. Petrocchi Jasinski, F.; **Evangelista, C.**; Basiricò, L.; Bernabucci, U. 2023. Responses of Dairy Buffalo to Heat Stress Conditions and Mitigation Strategies: A Review. *Animals*, 13, 1260. <https://doi.org/10.3390/ani13071260>
5. Rossi, C.; Bernabucci, U.; Grossi, G.; Cesarini, F.; Lacetera, N.; **Evangelista, C.**; Turriziani, G.; Vitali, A. 2023. Cradle-to-grave life cycle assessment of buffalo mozzarella cheese supply chain in central Italy. *Journal of Agriculture and Food Research*. 14, 100871. Doi: <https://doi.org/10.1016/j.jafr.2023.100871>
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7. Giannuzzi, D.; **Evangelista, C.**; Costa, A.; Conte, G.; Neglia, G.; Bernabucci, U.; Schiavon, S.; Mele, M.; Cecchinato, A. 2024. Milk phenomics: leveraging biological bonds

- with blood and infrared technologies for evaluating animal nutritional and health status, Italian Journal of Animal Science, 23:1, 780-801, DOI: 10.1080/1828051X.2024.2353226
8. Castellani, S.; **Evangelista, C.**; Lepore, M.; Portaccio, M.; Basiricò, L.; Bernabucci, U.; Delfino, I. 2024. Insights on early response to acute heat shock of bovine mammary epithelial cells through a multimethod approach, Animal, 18(9), 101264. doi: <https://doi.org/10.1016/j.animal.2024.101264>
 9. Mecocci, S.; Pietrucci, D.; Milanesi, M.; Capomaccio, S.; Pascucci, L.; **Evangelista, C.**; Basiricò, L.; Bernabucci, U.; Chillemi, G.; Cappelli, K. 2024. Comparison of colostrum and milk extracellular vesicles small RNA cargo in water buffalo. Sci Rep 14, 17991. <https://doi.org/10.1038/s41598-024-67249-6>
 10. **Evangelista, C.**; Basiricò, L.; Bernabucci, U. 2024. Investigation the factors affecting homogeneity index of total mixed ration in dairy buffalo herds. 11th European Conference on Precision Livestock Farming, pp. 1610–1619. Conference Paper.
 11. **Evangelista, C.**; Petrocchi Jasinski, F.; Basiricò, L.; Turriziani, G.; Bernabucci, U. 2024. Particle size distribution of total mixed rations fed to Italian Mediterranean buffaloes measured by the PSPS: impact on milk quality and digestibility, Italian Journal of Animal Science, 23:1, 1535-1550, DOI: 10.1080/1828051X.2024.2417714
 12. **Evangelista, C.**; Milanesi, M.; Pietrucci, D.; Chillemi, G.; Bernabucci, U. 2024. The enteric methane emission in livestock sector: bibliometric research from 1986 to 2024 with text mining and topic analysis approach by machine learning algorithms. Animals, 14, 3158. <https://doi.org/10.3390/ani14213158>
 13. Ferrari, C.; **Evangelista, C.**; Basiricò, L.; Castellani, S.; Biffani, S.; Bernabucci, U. 2024. Application of a Generalized Additive Mixed Model (GAMM) in Timeseries Study of Dairy Cows' Behavior Under Hot Summer Conditions. J. Dairy Sci. 108(2), 1554-1572. <https://doi.org/10.3168/jds.2024-25001>
 14. Primi, R.; Bernabucci, G.; **Evangelista, C.**; Viola, P.; Girotti, P.; Spina, R.; Compagnucci, S.; Ronchi, B. 2025. Ecosystem Services Linked to Extensive Sheep and Goat Farming in Mountain Areas: A Global Literature Analysis Using Text Mining and Topic Analysis. Animals 2025, 15, 350. <https://doi.org/10.3390/ani15030350>

List of submitter paper:

1. Bernabucci, G.; **Evangelista, C.**; Girotti, P.; Viola, P.; Spina, R., Ronchi, B.; Bernabucci, U.; Basiricò, L.; Turini, L.; Mantino, A.; Mele, M.; Primi, R. Precision Livestock Farming: an overview on the application in extensive systems. Italian Journal of Animal Science. Submission ID 249902507 (16-10-2024).
2. **Evangelista, C.**; Contò, M.; Renzi, G.; Basiricò, L.; Bernabucci, U.; Failla, S. Assessment of the Predictive Performance of a Benchtop and Portable NIR Instrument for Analysing the Chemical Composition and Fatty Acid Profile of Dairy Buffalo's Total Mixed Ration. Sensors. Submission ID 3381734 B (04-12-2024).
3. Petrocchi Jasinski, F., **Evangelista, C.**, Basiricò, L., Iacurto, M., Steri, R., Meo Zilio, D., U. Bernabucci. Evaluation of the effects of a nutritional supplement in Italian Mediterranean Buffalo exposed to hot weather conditions. Italian Journal of Animal Science. Submission ID 250164413 (16-01-2025).

Articles Published on Web Journals:

1. **Evangelista, C.** Granulometria della razione: ecco come influisce su qualità e digeribilità del latte bufalino. Ruminantia web (Available at: <https://www.ruminantia.it/granulometria-della-razione-ecco-come-influisce-su-qualita-e-digeribilita-del-latte-bufalino/>)
2. **Evangelista, C.** Bufale, anche le caratteristiche della razione contano. Allevatori.TOP. Anno 8, n.12, dicembre 2024. P. 72-75

List of oral presentations and poster communications,

1. **Evangelista, C.** Controllo dell'alimentazione tramite sistemi NIRS". Convegno INNOVALAT- Tecnologie innovative per l'alimentazione del bovino da latte ai fini di garantire benessere animale e qualità delle produzioni dal titolo. Aula Magna dell'Università degli studi della Tuscia- Viterbo (VT), 25/03/22. [Oral presentation]
2. Bernabucci, U; **Evangelista, C.**; Basiricò, L. Characterization of dairy buffaloes diet by NIRS technology. Pre-conference AIEAA "Sfide ed opportunità per un settore agroalimentare digitale e climate smart"- Viterbo June 16th 2022. [Poster presentation]
3. **Evangelista, C.** Monitoraggio della razione con tecnologia NIRS. Convegno TecBufala - Tecnologie innovative per la gestione della bufala da latte ai fini della caratterizzazione,

- della qualità e della sostenibilità delle produzioni. Sala Parrocchiale – Amaseno (FR), 14/07/2022. [Oral presentation]
4. **Evangelista, C.**; Milanesi M.; Pietrucci, D.; Chillemi, G.; Bernabucci, U. Evaluation of fed fiber components using portable and low-cost NIRS instrument to improve feed efficiency, product quality and reduce environmental impact. 26th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology Asti, 19-21 September 2022. [Poster presentation]
 5. Milanesi, M.; Pietrucci, D.; Serva, L.; Renzi, F.; Vignali, G.; **Evangelista, C.**; Marchesini, G.; Andrighetto, I.; Bernabucci, U.; Valentini, R.; Chillemi, G. Machine Learning NIR Wavelength Selection: Application for a Low-Cost Portable Instrument for Livestock Feed Management. In proceedings of IEEE International Workshop on Measurements and Applications in Veterinary and Animal Sciences (MeAVeAS). Napoli 28-30 aprile 2023 IEEE Catalogue Number: CFP23CX4-USB, ISBN: 979-8-3503-3461-6. [Oral presentation]
 6. **Evangelista, C.**; Basiricò, L.; Galli, T.; Bruni, G.; Bernabucci, U. Relationship between feeding management and milk characteristics in dairy buffalo. In proceedings of ASPA 25th Congress Book of Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI: 10.1080/1828051X.2023.2210877. [Oral presentation]
 7. Grossi, G.; Vitali, A.; **Evangelista, C.**; Rossi, C.; Cesarini, F.; Basiricò, L.; Turriziani, G.; Lacetera, N.; Bernabucci, U. Implementation of water footprint assessment methodology on buffalo mozzarella cheese. U. In proceedings of ASPA 25th Congress Book of Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI: 10.1080/1828051X.2023.2210877. [Oral presentation]
 8. Vitali, A.; Grossi, G.; Rossi, C.; Cesarini, F.; **Evangelista, C.**; Basiricò, L.; Turriziani, G.; Lacetera, N.; Bernabucci, U. Cradle-to-grave life cycle assessment of buffalo mozzarella cheese. In proceedings of ASPA 25th Congress Book of Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI: 10.1080/1828051X.2023.2210877. [Oral presentation]
 9. Ferrari, C.; **Evangelista, C.**; Basiricò, L.; Luziatelli, F.; Ruzzi, M.; Bernabucci, U. Effects of environmental factors on milk microbiome. In proceedings of ASPA 25th Congress Book of Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI: 10.1080/1828051X.2023.2210877. [Oral presentation]
 10. Franceschi, P.; Malacarne, M.; Cipolat-Gotet, C.; **Evangelista, C.**; Basiricò, L.; Bernabucci, U.; Summer, A. Distribution of Zn, Fe and Cu and their relationships with milk quality in individual cow milk samples. In proceedings of ASPA 25th Congress Book of

- Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI: 10.1080/1828051X.2023.2210877. [Poster presentation]
11. Malacarne, M.; Franceschi, P.; Cipolat-Gotet, C.; **Evangelista, C.**; Basiricò, L.; Bernabucci, U.; Summer, A. Influence of content and distribution of Ca, P and Mg on rennet coagulation properties in individual cow milk samples. In proceedings of ASPA 25th Congress Book of Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI: 10.1080/1828051X.2023.2210877. [Poster presentation]
12. Mecocci, S.; Pietrucci, D.; Milanese, M.; Capomaccio, S.; **Evangelista, C.**; Basiricò, L.; Bernabucci, U.; Chillemi, G.; Cappelli, K. Transcriptomic characterization of water buffalo's Extracellular Vesicles from colostrum and milk for their Immunomodulatory Potential. In proceedings of ASPA 25th Congress Book of Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI: 10.1080/1828051X.2023.2210877. [Poster presentation]
13. **Evangelista, C.**; Basiricò, L.; Colantoni, A.; Bernabucci, U. Use of NIRS to predict diet characteristics and digestibility in dairy cows. In proceedings of ASPA 25th Congress Book of Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI: 10.1080/1828051X.2023.2210877. [Poster presentation]
14. **Evangelista, C.**; Ferrari, C.; Basiricò, L.; Garcia, R.; Bernabucci, U. Effects of an electrolyte, antioxidant and osmolyte blend supplement on milk yield and cheesemaking properties in Holstein lactating cows during warm season. In proceedings of ASPA 25th Congress Book of Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI: 10.1080/1828051X.2023.2210877. [Poster presentation]
15. Franceschi, P.; Malacarne, M.; Cipolat-Gotet, C.; **Evangelista, C.**; Basiricò, L.; Bernabucci, U.; Summer, A. Effect of environmental factors on content and distribution of Ca, P and Mg in bovine milk. In proceedings of ASPA 25th Congress Book of Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI: 10.1080/1828051X.2023.2210877. [Poster presentation]
16. Contò, M.; Ferrari, C.; Renzi, G.; **Evangelista, C.**; Basiricò, L.; Bernabucci, U.; Failla, S. Comparison of sialic acids content in cow and buffalo milk in different season. In proceedings of ASPA 25th Congress Book of Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI: 10.1080/1828051X.2023.2210877. [Poster presentation]
17. Vetturini, T.; Basiricò, L.; **Evangelista, C.**; Contò, M.; Renzi, G.; Bernabucci, U. Effect of different feeding management on the fatty acid profile of buffalo milk. In proceedings of

- ASPA 25th Congress Book of Abstract, Italian Journal of Animal Science, 22:sup1, 1-320, DOI: 10.1080/1828051X.2023.2210877. [Poster presentation]
18. **Evangelista, C.** Gestione della alimentazione e qualità del latte. Convegno finale TecBufala - Tecnologie innovative per la gestione della bufala da latte ai fini della caratterizzazione, della qualità e della sostenibilità delle produzioni. Ristorante le fontanelle - Amaseno (FR), 29/06/2023. [Oral presentation]
19. **Evangelista, C.**; Milanese, M.; Pietrucci, D.; Gabbianelli, F.; Chillemi, G. Development of strategies for the adaptation of the livestock sector to the new climate regime with machine learning and artificial intelligence methods. 27th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology Portici, 13-15 September 2023. [Poster presentation]
20. Failla, S.; Palocci G.; Contò, M.; Renzi, G.; **Evangelista, C.**; Basiricò, L.; Bernabucci, U. Effect of different diets on the fatty acid composition of buffalo bulk milk. *Revista Científica, FCV-LUZ* / Vol. XXXIII, Supl. Esp., 7 - 24, 2023. <https://doi.org/10.52973/rcfcv-wbc149>. [Poster presentation]
21. Manganello, F.; Bernabucci, G.; Danieli, PP.; Bernabucci, U.; **Evangelista, C.** Comparisons of Two Portable NIR Spectrometers in the Prediction of the Chemical Composition of Total Mixed Rations for Buffaloes and Dairy Cows. In proceedings of IEEE International Workshop on Measurements and Applications in Veterinary and Animal Sciences (MeAVeAS) Turin 22-24 April 2024. [Oral presentation]
22. Basiricò, L.; Bonini, P.; **Evangelista, C.**; Bernabucci, U.; Ruzzi, M. Effect of seasonality and farm management on the buffalo's milk lipidome. In proceedings of 75th European Federation of Animal Science (EAAP) Florence, 1-4 September 2024. [Poster presentation]
23. Meo Zilio, D.; Bianconi, G.; Petricca, C.; Negretti, P.; Petrocchi Jasinski, F.; **Evangelista, C.**; Steri R. Accuracy of a low-cost device based on 2D image analysis in predicting weight and body condition in Mediterranean buffaloes. In proceedings of 75th European Federation of Animal Science (EAAP) Florence, 1-4 September 2024. [Poster presentation]
24. Pietrucci, D.; Milanese, M.; **Evangelista, C.**; Spina, R.; Ronchi, B.; Bernabucci, U.; Lacetera, N.; Primi, P.; Chillemi, G. Remote monitoring of pasture and animals in extensive farming is functional to genetic improvement. In proceedings of 75th European Federation of Animal Science (EAAP) Florence, 1-4 September 2024. [Oral presentation]
25. **Evangelista, C.**; Pietrucci, D.; Milanese, M.; Bernabucci, U.; Basiricò, L.; Gabbianelli, F.; Chillemi, G. Characterization of buffalo faecal microbiome depending on ration

- concentrate level. In proceedings of 75th European Federation of Animal Science (EAAP) Florence, 1-4 September 2024. [Oral presentation]
26. Petrocchi Jasinski, F.; **Evangelista, C.**; Steri, R.; Iacurto, M.; Basiricò, L.; Meo Zilio, D.; Bernabucci, U. Effects of a nutritional supplement in Italian Mediterranean Buffalo exposed to heat stress. In proceedings of 75th European Federation of Animal Science (EAAP) Florence, 1-4 September 2024. [Oral presentation]
27. Mecocci, S.; Pietrucci, D.; Milanesi, M.; Capomaccio, S.; Pascucci, L.; **Evangelista, C.**; Basiricò, L.; Bernabucci, U.; Lacetera, N.; Chillemi, G.; Cappelli, K. Transcriptomic characterization of buffalo extracellular vesicles from colostrum and milk: cargo comparison for potential applications. In proceedings of 75th European Federation of Animal Science (EAAP) Florence, 1-4 September 2024. [Oral presentation]
28. Petrocchi Jasinski, F.; **Evangelista, C.**; Basiricò, L.; Iacurto, M.; Steri, R.; Meo Zilio, D.; Bernabucci, U. Analysis of ear tag sensors' data through GAMM in lacting buffaloes under heat stress conditions. 11th European Conference on Precision Livestock Farming (ECPLF) Bologna, 9-12 September 2024. [Poster presentation]
29. Petrocchi Jasinski F.; **Evangelista, C.**; Basiricò, L.; Iacurto, M.; Steri, R.; Meo Zilio D.; Bernabucci, U. A nutritional supplement administered to Italian Mediterranean Buffalo exposed to heat stress: effects on milk and on enteric methane emission. 11th European Conference on Precision Livestock Farming (ECPLF) Bologna, 9-12 September 2024. [Poster presentation]
30. Benelli, A.; **Evangelista, C.**; Spina, R.; Primi, R.; Bernabucci, U.; Ronchi, B.; Moscetti, R. Near-Infrared Spectroscopy to Predict Nutritional Factors of Green Forage. 11th European Conference on Precision Livestock Farming (ECPLF) Bologna, 9-12 September 2024. [Poster presentation]
31. **Evangelista, C.**; Basiricò, L.; Bernabucci, U. Investigation the factors affecting homogeneity index of total mixed ration in dairy buffalo herds. In proceedings of 11th European Conference on Precision Livestock Farming (ECPLF) Bologna, 9-12 September 2024. [Poster presentation]
32. Contò, M.; Failla, S.; Renzi, G.; Basiricò, L.; **Evangelista, C.**; Bernabucci, U. Effect of linseed and fresh forage diet inclusion on mozzarella fatty acid profile. In proceedings of 1st International Conference on buffalo mozzarella & milk products (BMMP) Naples, 24-25 September 2024. [Poster presentation]
33. Benelli, A.; **Evangelista, C.**; Spina, R.; Primi, R.; Pietrucci, D.; Milanesi, M.; Chillemi, G.; Bernabucci, U.; Moscetti, R. Near-Infrared Spectroscopy to Predict Nutritional Factors

of Green Forage. In proceedings of IEEE International Workshop on Metrology for Agriculture and Forestry (MetroAgriFor) Padua, 29-31 October 2024. [Oral presentation]

List of conferences and workshop participations

1. 26th Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology
 - Location: Asti (TO)
 - Date: 19-21 September 2022
2. 25th Congress of Animal Production Science: Innovations and Sustainability for Future Generations (ASPA)
 - Location: Bari (BA)
 - Date: 13-16 June 2023
3. 27th Workshop on the Developments in the Italian PhD Research on Food Science, Technology and Biotechnology
 - Location: Portici (NA)
 - Date: 13-15 September 2023
4. The International Dairy Nutrition Symposium “Feed Efficiency in Dairy Cattle”
 - Location: Wageningen
 - Date: 10 October 2023
5. 75th European Federation of Animal Science (EAAP) Annual Meeting
 - Location: Florence (FI)
 - Date: 1-5 September 2024
6. 11th European Conference on Precision Livestock Farming (ECPL)
 - Location: Bologna (BO)
 - Date: 9-12 September 2024

7. 3rd PLF Student's Travel Workshop

- Location: Villa Motta (MO)
- Date: 12-14 September 2024

List of Seminars Held

- **10/03/2022**

Speaker for the seminar titled *"Tecnologie digitali per la valutazione del benessere animale"*.

Held as part of the course *Biotechnologie e nutraceutica delle produzioni animali* of the Master's Degree in *Biotechnologie agrarie* (LM-9), taught by Prof. Loredana Basiricò.

- **26/04/2022**

Speaker for the seminar titled *"Precision Livestock Framing & Precision Feeding"*.

Held as part of the course *Scienza e tecnica dell'alimentazione nei sistemi zootecnici* of the Master's degree in *Scienze Agrarie ed Ambientali* (LM-69), taught by Prof. Umberto Bernabucci.

- **03/05/2022**

Speaker for the seminar titled *"Precision Livestock Farming & Precision Feeding"*.

Held as part of the course *Nutrizione ed alimentazione animale* of the Bachelor's Degree in *Scienze Agrarie ed Ambientali* (L-25), taught by Prof. Umberto Bernabucci.

- **26/04/2023**

Speaker for the seminar titled *"Precision Livestock Farming & Precision Feeding"*.

Held as part of the course *Scienza e tecnica dell'alimentazione nei sistemi zootecnici* of the Master's degree in *Scienze Agrarie ed Ambientali* (LM-69), taught by Prof. Umberto Bernabucci

- **08/04/2024**

Speaker for the seminar titled *"L'utilizzo della spettroscopia NIRS nell'alimentazione di precisione"*.

Held as part of the course *Zootecnia di precisione* (Rieti) taught by Prof. Loredana Basiricò.

- **06/05/2024**

Speaker for the seminar titled "*Alimentazione e razionamento: Bufala da latte*". Held as part of the course *Scienza e tecnica dell'alimentazione nei sistemi zootecnici* of the Master's degree in Scienze Agrarie ed Ambientali (LM-69), taught by Prof. Umberto Bernabucci

- **13/05/2024**

Speaker for the seminar titled "*Introduction to Precision Feeding (PF)*".

Duration: 2 hours.

Held as part of the course *Extensive and Sustainable Precision Livestock Farming* (AFS) taught by Dott. Raffaello Spina.

- **21/05/2024**

Speaker for the seminar titled "*Automation feeding and watering system - ruminants*".

Duration: 2 hours.

Held as part of the course *Extensive and Sustainable Precision Livestock Farming* (AFS) taught by Dott. Raffaello Spina.

- **06/06/2024**

Speaker for the seminar titled "*Automation feeding and watering system - monogastric and aquaculture*".

Duration: 2 hours.

Held as part of the course *Extensive and Sustainable Precision Livestock Farming* (AFS) taught by Dott. Raffaello Spina.

- **27/06/2024**

Speaker for the seminar titled "*Practical examples of the use of PF sensors in cattle farms*".

Duration: 2 hours.

Held as part of the course *Extensive and Sustainable Precision Livestock Farming* (AFS) taught by Dott. Raffaello Spina.

Tutorship Activities

Below is a list of tutorship activities conducted by Dr. Chiara Evangelista as co-supervisor:

1. Lo stress ossidativo nella bovina da latte

- Academic Year: 2021-2022
- Candidate: Alessia Guasconi
- Supervisor: Prof.ssa Loredana Basiricò
- Co-supervisor: Dr. Chiara Evangelista

2. Le malattie podaliche nella bovina da latte: ricerca bibliometrica tramite approccio text mining e topic analysis

- Academic Year: 2023-2024
- Candidate: Elena Boccialoni
- Supervisor: Prof. Umberto Bernabucci
- Co-supervisor: Dr. Chiara Evangelista

Master Thesis

3. Effetti di un integratore a base di una miscela di elettroliti, antiossidanti e osmoliti sulla produzione di latte e sulle proprietà casearie in vacche Holstein in lattazione durante la stagione calda

- Academic Year: 2022-2023
- Candidate: Andrea Pellegrini
- Supervisor: Prof. Umberto Bernabucci
- Co-supervisor: Dr. Chiara Evangelista

4. Effetto della supplementazione di antiossidanti sulla produzione e sulla qualità del latte in bufale mediterranee italiane in lattazione

- Academic Year: 2022-2023
- Candidate: Gianluca Feudo
- Supervisor: Prof. Umberto Bernabucci
- Co-supervisor: Dr. Chiara Evangelista

- 5. Valutazione del contenuto di lipidi bioattivi nel latte e nella mozzarella di bufala mediterranea italiana: impatto dell'alimentazione e delle variazioni stagionali**
 - Academic Year: 2022-2023
 - Candidate: Beatrice Menichelli
 - Supervisor: Prof.ssa Loredana Basiricò
 - Co-supervisor: Dr. Chiara Evangelista

- 6. Risposta alle condizioni di stress da caldo in bufale mediterranee italiane in lattazione: effetti del mangime minerale RuFO sull'ingestione di alimento, sulla digeribilità della razione unifeed, sulla produzione e la qualità del latte**
 - Academic Year: 2022-2023
 - Candidate: Andrea Musetti
 - Supervisor: Prof. Umberto Bernabucci
 - Co-supervisor: Dr. Chiara Evangelista

- 7. Composizione acidica del latte di bufala: effetto della gestione, dell'alimentazione e della stagione**
 - Academic Year: 2022-2023
 - Candidate: Maurizio Stromieri
 - Supervisor: Prof. Umberto Bernabucci
 - Co-supervisor: Dr. Chiara Evangelista

- 8. Utilizzo del NIRS per la valutazione delle caratteristiche chimico-fisiche della dieta e della digeribilità apparente della razione nelle vacche da latte**
 - Academic Year: 2022-2023
 - Candidate: Gloria Bernabucci
 - Supervisor: Prof.ssa Loredana Basiricò
 - Co-supervisor: Dr. Chiara Evangelista

9. Gestione dell'alimentazione della bufala mediterranea italiana e qualità tecnologica del latte

- Academic Year: 2022-2023
- Candidate: Francesca Cellitti
- Supervisor: Prof. Umberto Bernabucci
- Co-supervisor: Dr. Chiara Evangelista

Abroad Internships

Animal Nutrition Group, Wageningen University and Research (WUR)

Research group: André Bannink, Rudy de Mol, and Sanne van Gastelen

Supervised by: Roselinde Goselink

Duration: From October, 2023, to December, 2023

List of courses participations

Mandatory courses:

Course: Applied Statistics – R Statistical Software (72 hours)

Course: Intellectual Property, Academic Technology Transfer, and Soft Skills (24 hours)

Course: Technical English (32 hours)

Course: Python for Computational Sciences (16 hours)

Course: Sustainability Assessment of Bioeconomic Systems (32 hours)

Course: Introduction to High Performance and Data Intensive Computing (32 hours)

Extra activities:

Course: Analisi statistica multivariata, Sassari (SS), 18–22 September 2023

Course: Big Data and Digital Tools Applied to Livestock Production, Padova (PD), 25–29 September 2023

Course: Statistical Learning and Deep Learning: dalla teoria alla pratica, Institute of Biology and Agricultural Biotechnology (IBBA-CNR), Bari (BA), 11–14 June 2024

Winter School: Machine Learning Applications to Marine and Terrestrial Monitoring and Livestock Farming, Council for Agricultural Research and Analysis of Agricultural Economics (CREA), Monterotondo (RM), 12–16 February 2024

Course: National Legislation and Ethics (Level 1), Modules 1 and 2 (DM 5 August 2021), online

Course: Use of Ungulates in Research – Bovines and Equines, Modules 3.1, 4, 5, and 7 (DM 5 August 2021), online

Course: Use of Ungulates in Research – Swine, Small Ruminants, and Camelids Modules 3.1, 4, 5, and 7 (DM 5 August 2021), online

Acknowledgements

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