UNIONE EUROPEA
Fondo Sociale Europeo

UNIVERSITÀ DEGLI STUDI DI TORINO

DIPARTIMENTO DI SCIENZE AGRARIE, FORESTALI E ALIMENTARI

DOTTORATO DI RICERCA IN SCIENZE AGRARIE, FORESTALI E ALIMENTARI

CICLO: 37°

TITOLO DELLA TESI: **FROM POLLUTION TO SOLUTION: MICROPLASTIC CONTAMINATION AND GREENHOUSE GAS EMISSIONS IN RUMINANT LIVESTOCK**

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ANNI ACCADEMICI: 2022; 2023; 2024

SETTORE SCIENTIFICO-DISCIPLINARE DI AFFERENZA*: AGRI-09/B Nutrizione e alimentazione animale

(*N.B. Nel caso di più settori disciplinari interessati, deve essere indicato quello più presente nella trattazione della tesi).

La borsa di dottorato è stata cofinanziata con risorse del **Programma Operativo Nazionale Ricerca e Innovazione 2014-2020 (CCI2014IT16M2OP005), risorse FSE REACT-EU, Azione IV.5 "Dottorati su tematiche Green"**

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Chapter 1: Introduction

1.1 Environmental Challenges of Ruminant Livestock

Ruminant livestock contribute significantly to human food systems and the livelihoods of farmers. Ruminants provide a diverse range of products, such as meat, milk, hides, wool, heat and energy (Gerber et al., 2013; Place, 2024; Pulina et al., 2017). They have the unique ability to convert human-inedible biomass into high-quality animal proteins (Cheng et al., 2022). Approximately one-third of global protein and 17% of total caloric intake are derived from animal sources, with a major portion of this contribution originating from ruminants (FAO, 2018). With the global population projected to grow from 8.2 billion in 2025 to 9.7 billion by 2050, the demand for ruminant products is anticipated to rise proportionally (United Nations, 2019; Worldometer, 2025). According to FAO data, global meat consumption is estimated to increase by over 50% between 2000 and 2050, rising from 274 Mt to 557 Mt. Specifically, consumption of beef and buffalo meat is forecast to grow by 44%, from 59 Mt to 106 Mt over the same period (Fig. 1a) (FAO, 2023a).

Global cow milk production, which accounts for approximately 85% of total milk output, is likely to reach between 810 and nearly 1,000 Mt by 2050 - an increase of 40% to 51% compared to the 489 Mt produced in 2000 (FAOSTAT, 2025; Pulina et al., 2020).

China is the largest meat producer (79 Mt), followed by United States (47 Mt), Brazil (29 Mt), and Russia (11 Mt) (Fig. 1b). In milk production, India

ranks first (210 Mt), followed by United States (101 Mt), Pakistan (58 Mt), and China (39 Mt) (Fig. 1c) (FAOSTAT, 2020).

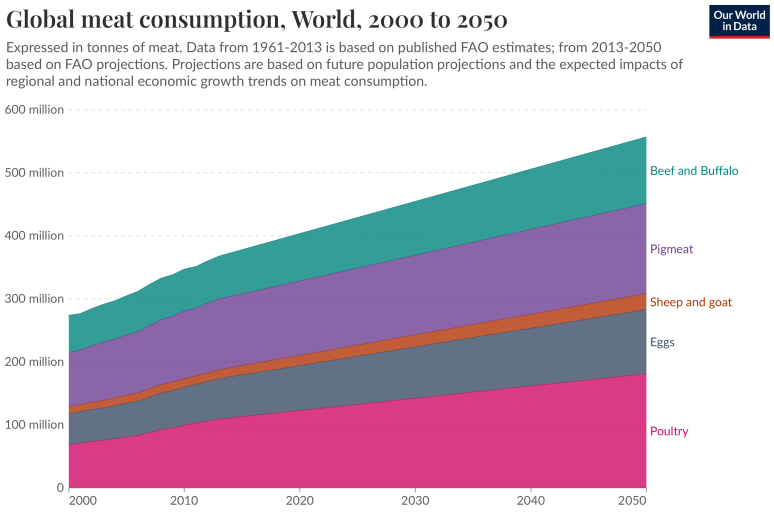


Fig. 1a. Global meat consumption (2000-2050) in tonnes, based on FAO estimates and projections. Graph sourced from Our World in Data (https://ourworldindata.org).

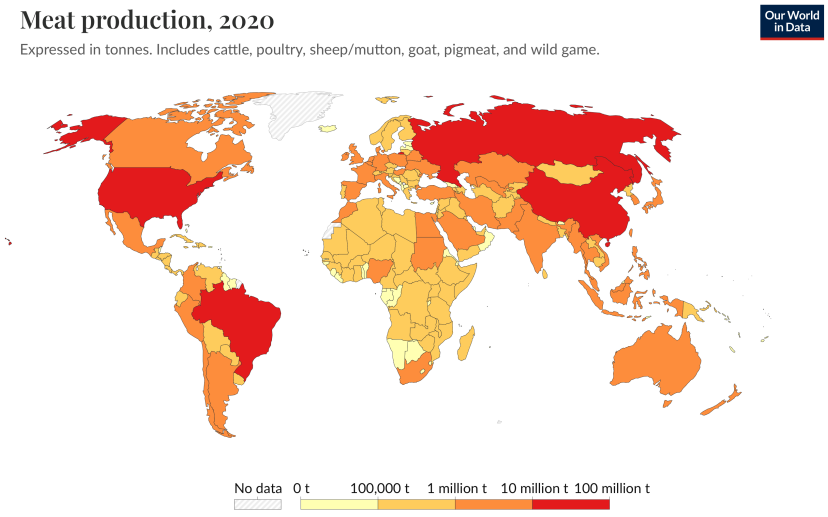


Fig. 1b. Meat production in 2020 (including cattle, poultry, sheep/mutton, goat, pigmeat, and wild game) in tonnes, based on FAOSTAT data. Graph sourced from Our World in Data (https://ourworldindata.org).

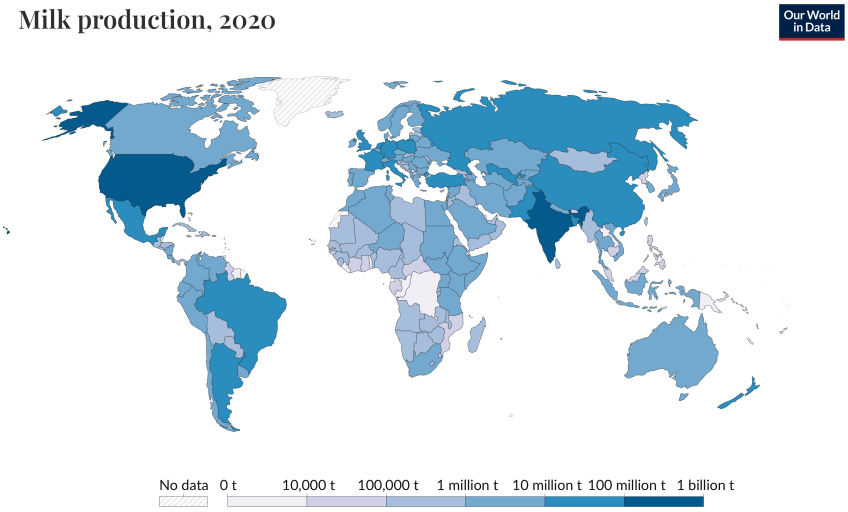


Fig. 1c. Milk production in 2020 expressed in tonnes, based on FAOSTAT data. Graph sourced from Our World in Data (https://ourworldindata.org).

However, ruminant livestock systems are connected with environmental issues. Therefore, meeting the booming demand for ruminant products while reducing their environmental impacts poses a fundamental challenge for sustainable food systems and human well-being (Du et al., 2022). Greenhouse gas (GHG) emissions represent the most widely recognized and extensively studied environmental challenge associated with ruminant livestock production (Llonch et al., 2017). In contrast, microplastics (MPs) have received significantly less attention in livestock, despite their potential to affect animal health, food safety, and environmental sustainability (Corte Pause et al., 2024; Su et al., 2025). This knowledge gap underscores the need for further research on MPs in ruminant livestock systems.

1.2 Microplastic Contamination focusing on Ruminants

The word “Microplastic” was coined 21 years ago by Thompson et al. (2004) and defined as microscopic plastic debris. Nowdays according to International Organization for Standardization (ISO, 2023), MPs are solid plastic insoluble in water with dimension between 1 µm to 5 mm.

Based on their origin, MPs can be divided in primary and secondary (Fig. 2). Primary MPs are manufactured on purpose directly as MPs for their applications in different products, including personal care products, paints, cleaning products, fertilizers, etc. Secondary MPs are generated through fragmentation of larger plastics due to physical and chemical processes, including ultraviolet photodegradation, mechanical abrasion, oxidative degradation, and abiotic weathering processes (Diao et al., 2023; Klimasz and Grobelak, 2024; Kolluru and Raja, 2024; Sharma et al., 2023).

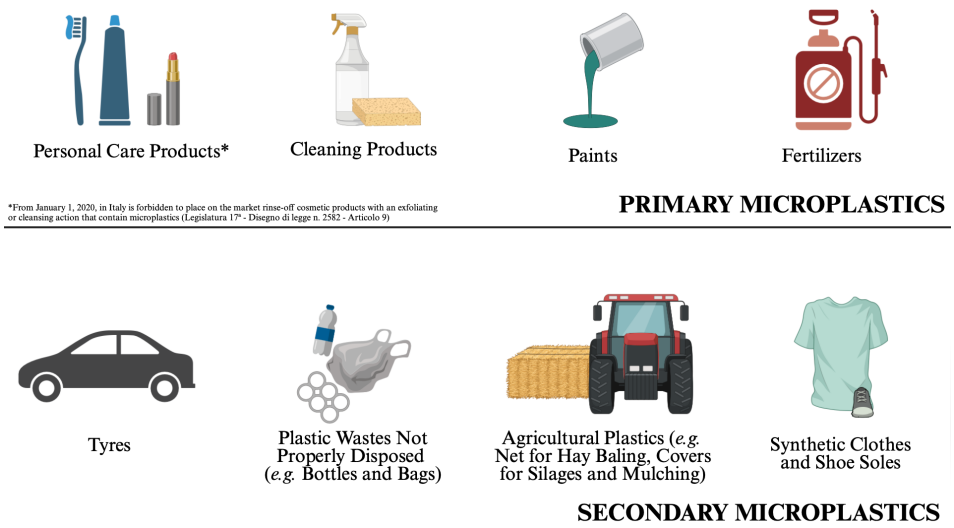


Fig. 2. Example sources of primary and secondary microplastics (created with BioRender.com).

Unfortunately, MPs are widespread, contaminating all the environments from the deepest point of the ocean (Mariana Trench), where MPs were found in deep-sea amphipods at 10890 m below the sea's surface (Jamieson et al., 2019), to the balcony of Mount Everest, where MPs were found at 8440 m of elevation (Napper et al., 2020). European Union estimated that between 200 and 600 olympic-size swimming pools of MPs are unintentionally released into the environment every year in Europe (European Commission, 2023).

MPs are an emerging environmental pollutant that could act as carrier for inorganic and organic contaminants, including additives, heavy metals, persistent organic pollutants (POPs), antibiotics, pesticides, and fungicides (Kinigopoulou et al., 2022; Okeke et al., 2022; Silva et al., 2018). MPs are potentially responsible of interconnected risks to environmental, human and animal health (Prata et al., 2021).

Up to now, the majority of studies on MP pollution focused on the marine environment, while fewer addressed terrestrial ecosystems (Lamichhane et al., 2023). Focusing on farmed animals, particularly ruminants, little attention has been given to their role in MP contamination, despite their potential as key players in MPs transfer to humans through animal products such as meat and milk, as well as their return to the environment via their excreta (Corte Pause et al., 2024; Lackner and Branka, 2024; Su et al., 2025).

Fig. 3 illustrates the number of published articles on MPs from 2004 - the year in which MPs term was coined - to 2024. The total number of studies on MPs has grown exponentially, with significantly greater attention given

to the marine environment compared to the terrestrial one. Research on MPs in ruminants began in 2016 and remains relatively scarce, while studies on MPs in ruminant feeds are even more limited, with the first publications appearing only in 2022. The reaserch was conducted using Scopus (https://www.scopus.com).

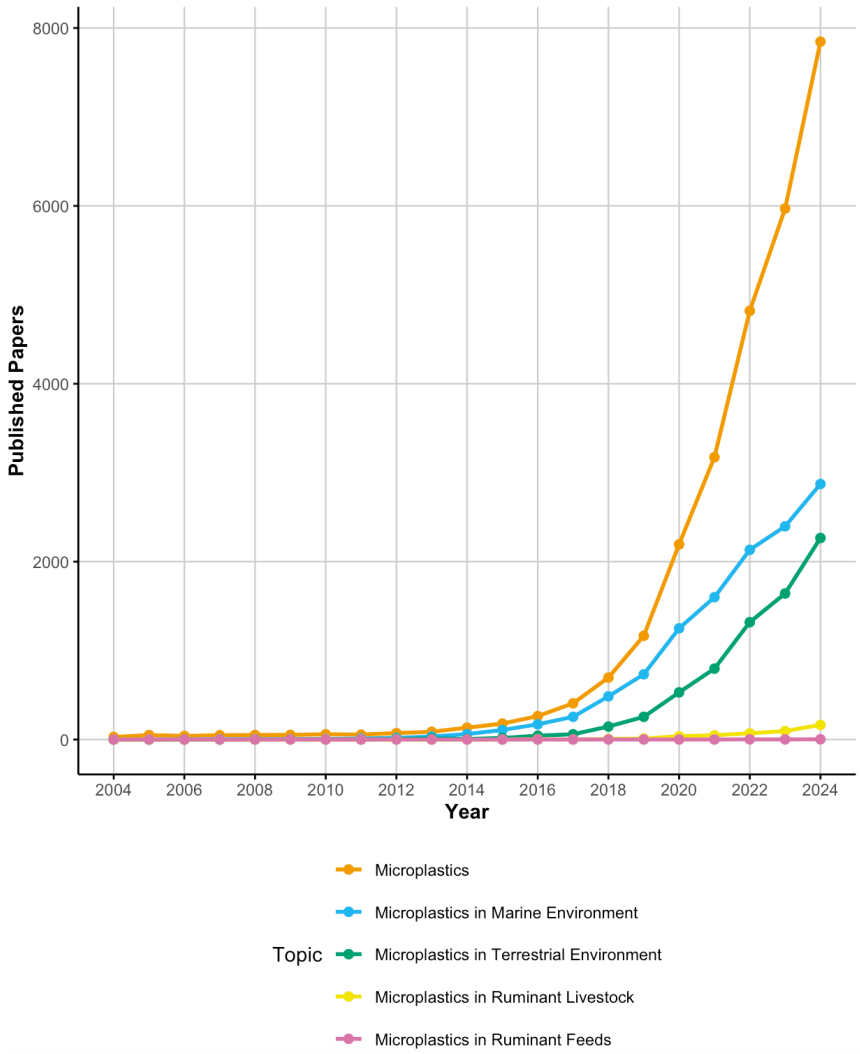


Fig. 3. Yearly published papers (2004 - 2024) for Microplastics, Microplastics in Marine Environment, Microplastics in Terrestrial Environemnt, Microplastics in Ruminant Livestock, Microplastics in Ruminant Feeds according to Scopus database.

Research queries used on Scopus: ("microplastic" OR "micro plastic"); ("microplastic*" OR "micro plastic*") AND ("marine" OR "marine environment" OR "marine ecosystem*" OR "sea*" OR "ocean" OR "coastal waters" OR "estuaries" OR "marine pollution" OR "marine sediment*" OR "sea sediment*" OR "sea food*" OR "marine organism*" OR "marine microorganism*" OR " marine bacteria" OR " marine fungi" OR "plankton" OR "phytoplankton" OR "fish" OR "bivalve*" OR "crustacean*" OR "marine mammalian" OR "marine fauna" OR "marine flora" OR "marine biodiversity" OR "marine health"); ("microplastic" OR "micro plastic") AND ("terrestrial" OR "terrestrial environment" OR "terrestrial ecosystem" OR "soil" OR " soil pollution" OR "agricultural soil*" OR "urban soil*" OR "land pollution" OR "soil contamination" OR "terrestrial organism*" OR "soil microorganism*" OR "soil bacteria" OR "soil fungi" OR "earthworm*" OR "terrestrial invertebrate" OR "ruminant*" OR "cattle" OR "cow*" OR "sheep" OR "goat*" OR "bovine" OR "livestock" OR "farm animal*" OR "agriculture" OR "pasture*" OR "grazing land" OR "animal product*" OR "meat*" OR "milk*" OR "feed*"); ("microplastic" OR "micro plastic*") AND ("ruminant*" OR "cattle" OR "cow*" OR "sheep" OR "dairy" OR "goat*" OR "bovine" OR "livestock" OR "pasture" OR "grazing land" OR "ruminant product*" OR "meat" OR "milk*" OR "ruminant feed*"); ("microplastic*" OR "micro plastic*") AND ("feed*") AND ("ruminant*" OR "dairy" OR "cow*" OR "dairy cow*", "cattle*" OR "beef" OR "bovine" OR "goat*" OR "sheep*").

Returning to ruminants, they are potentially exposed to MPs through both indirect and direct ways (Fig. 4). Indirect exposure occurs through the ingestion of improperly discarded plastic debris larger than 5 mm. Various ruminant species (cattle, buffaloes, sheep, goats, dromedary camels, and dairy cows) have been found to retain plastic materials in their rumen and reticulum (Corte Pause et al., 2024; Khan et al., 2024; Yemelyanenko et al., 2021). Approximately 72% of foreign materials in the rumen of domestic cattle consist of plastic-based materials, with plastic bags (65.5%) and plastic ropes (6.3%) being the most common (Mushonga et al., 2015). During digestion process, ingested plastics could undergo fragmentation into MPs (Dong et al., 2023; Khan et al., 2024).

Ruminants can also be directly exposed to MPs through three main sources: inhalation of contaminated air, ingestion of polluted water, and consumption of contaminated feeds (Khan et al., 2024). In addition,

dermal contact with MPs present in air and water is another potential route of exposure in ruminants. However, the dense hair covering the surface of their bodies offer protection by reducing direct contact between MPs and the skin (Su et al., 2025). MPs can reach air, water and feeds through both internal and external farm sources: internal farm sources include the breakdown of plastic materials within farms due to physical and chemical degradation processes, such as ultraviolet (UV) photodegradation, mechanical abrasion, oxidative degradation, and abiotic weathering (Klimasz and Grobelak, 2024). These processes affect plastics commonly used in feed storage such as silage covers, hay bale wraps (Borreani and Tabacco, 2017; Urli et al., 2023) and crop cultivation as mulch films, irrigation systems, and improperly managed plastic waste (Urli et al., 2023). External farm sources involve the transport of MPs by atmospheric agents such as wind, rainfall, and snowfall, which carry MPs from urban and industrial areas to agricultural and remote rural regions, including farms (Ramachandraiah et al., 2022; Roy et al., 2023).

Although air, water, and ruminant feeds are the main sources of MPs, very little research has been conducted on their detection. In particular, ruminant feeds could be a crucial link, as they are cultivated and stored with plastic and represent a complex matrix for MP extraction compared to air and water. To date, no study has developed a specific method for extracting MPs from ruminant feeds.

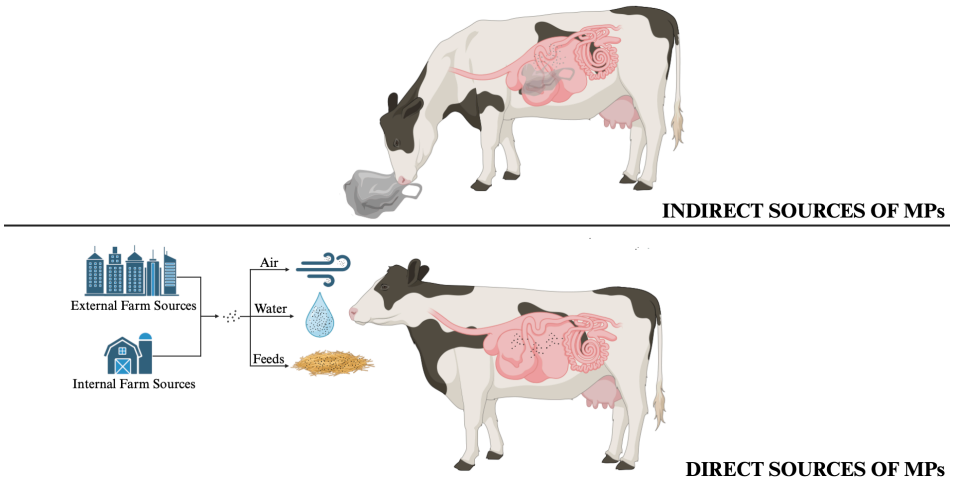


Fig. 4. Scheme illustrating the direct and indirect sources of microplastics (MPs) exposure in ruminants. (created with BioRender.com).

Once inside ruminant's body, MPs can follow two possible paths: i) MPs greater than 150 µm can be excreted; ii) MPs smaller than 150 µm can cross the intestinal mucosal barrier enter the bloodstream, and reach organs (Khan et al., 2024). In fact, MPs have been detected in several biological matrices of ruminants, including feces (Beriot et al., 2021; Wu et al., 2024), blood (van der Veen et al., 2022), meat (van der Veen et al., 2022) follicular fluid (Grechi et al., 2023), and epididymal sperm (Grechi et al., 2024). In addition, MPs can transfer from ruminants to humans through animal products such as meat and milk (Bahrani et al., 2024; Da Costa Filho et al., 2021; van der Veen et al., 2022; Visentin et al., 2024).

MPs exposure is an important cause of potential and long-term harm to ruminant health (Su et al., 2025). Recent studies have proved that MP exposure in ruminants can affect:

i) production performance: MPs can cause digestive system disorders in ruminants, thus affecting their normal growth and

reduced the quality and nutritional value of meat (Chang et al., 2024; Su et al., 2025);

ii) immune fuction: MPs can alter the immune system of ruminants inducing oxidative stress, endoplasmatic reticulum stress and apoptosis. Moreover, MPs can adsorb pollutants which can stimulate the immune system after release in the body (Wang et al., 2024; Su et al., 2025);

iii) reproductive system: MPs negatively affect the fertility of ruminants by impairing the maturation of bovine oocytes (Grechi et al., 2023) and reducing the motility of bovine epididymal sperm (Grechi et al., 2024).

iv) rumen microbiota: a study by Chang et al. (2024) in lambs reported an increased relative abundance of Bacteroidetes and Actinomycetes, alongside a reduction in key microbial groups involved in cellulose degradation and aromatic amino acid synthesis. MPs may also impair microbial fermentation by adsorbing nutrients and limiting substrate availability, resulting in decreased volatile fatty acid production and reduced energy intake in ruminants (Su et al., 2025). Conversely, rumen microbiota may exhibit potential for MP biodegradation, a topic that will be further explored in Section 1.3 *Microplastic Biodegradation by Microorganisms.*

1.3 Microplastic Biodegradation by Microorganisms

As discussed in the Section *1.2 Microplastic Contamination focusing on Ruminants*, MPs are potentially responsible of interconnected risks to environmental, human and animal health (Prata et al., 2021). These tiny plastics are persistent, highly mobile, and, once released, are extremely difficult to remove from the environment (European Commission, 2023). In 2021, the European Commission adopted the EU Action Plan: "*Towards a Zero Pollution for Air, Water, and Soil*," which set a target to reduce MP releases into the environment by 30% by 2030 (European Commission, 2021). While this initiative aims to curb future releases, effective solutions for removing MPs already present in the environment remain limited.

One potential solution to reduce MPs already present in the environemnt is the degradation of MPs by microorganisms, a process called MP biodegradation (Thakur et al., 2023). MP biodegradation involves different steps:

i) biofilm formation: microorganisms attach to the MPs surface by excreting exopolysaccharides, which enhance adhesion and facilitate colonization. This biofilm reduces the plastic's buoyancy and hydrophobicity, promoting further microbial activity;
ii) biodeterioration: affects the surface of plastics and changes their chemical, physical and mechanical properties;
iii) biofragmentation: involves the enzymatic action on plastic polymers, passing from polymers to monomers. In this stage, enzymes such as hydrolases and oxidoreductases hydrolyze the polymeric structure of MPs;

iv) assimilation: monomers that are formed in biofragmentation enter in the microbial cells through cell membranes. The entered monomers endure several metabolic pathways to produce adenosine triphosphate, metabolites, vesicles, and biomass. These metabolites are discharged into the extracellular surrounding. Intracellular metabolites hydrolyzed completely reach mineralization;

v) mineralization: it is the last step of biodegradation in which MPs are completely mineralized into CO_2 and water in aerobic condition and CO_2, water and methane (CH_4) in anaerobic condition (Bacha et al., 2023; Mohanan et al., 2020; Thakur et al., 2023; Zeenat et al., 2021) (Fig 5).

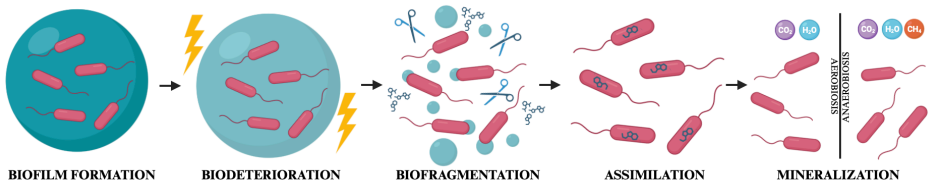


Fig. 5. The main steps of microplastic (light blue circles) biodegradation by microorganisms (created with BioRender.com).

MP biodegradability is affected by several intrinsic factors as: functional group availability, structural complexity (linear chains are generally easier to break down compared to branched structures), bond type (ester > ether > amide > urethane), physical form (smaller MPs with higher surface areas facilitate microbial access and enhance biodegradation); polymer density and molecular weight (lower density and lower molecular weight are generally more biodegradable); toughness (softer MPs degrade more quickly as they allow easier penetration by microbial enzymes, whereas

harder and tougher polymers resist microbial attack and persist longer in the environment); additives addition (increase the difficulty of degradation) (Liu et al., 2022; Zeenat et al., 2021).

Studies on MP biodegradation can be categorized into two main approaches:

i) pure cultures of microorganisms (including bacteria, fungi, or microalgae), which are the most extensively studied due to their controlled conditions (Gao et al., 2024). The bestknow microorganism reported is *Ideonella sakainensis* for the biodegradation of polyethylene terephthalate (PET) (Yoshida et al., 2016);
ii) natural consortia of microorganisms, which involve microbial communities working together to degrade MPs in complex environmental settings (Gao et al., 2024). One promising but underexplored consortium is the rumen microbiota. The rumen hosts a complex microbial ecosystem, including: bacteria (10^{10} – 10^{11} cells/mL), methanogenic archaea (10^{6} to 10^{8} cells/mL), ciliate protozoa (10^{4} – 10^{6} cells/mL), fungi (10^{3} – 10^{6} zoospores/mL), and a largely uncharacterized viruses, especially phages (Matthews et al., 2019; Waters et al., 2025). These microorganisms are essential for breaking down plant fibers through ruminal fermentation, a process that enables ruminants to digest materials the human body cannot. As metabolic by-products, a large proportion of volatile fatty acids (VFAs) are produced which are subsequently metabolised by the ruminant host, accounting for 70-80% of the energy requirements of ruminant livestock (Beckett et al., 2021; Morgavi et al., 2010). Alongside VFAs,

CO_2 and H_2 are also released during rumen fermentation. Ruminal fermentation involves the use of fibrolytic enzymes that enable the degradation of plant materials such as cellulose, hemicellulose, and cutin. These enzymes may also have the potential to break down plastics and MPs (Quartinello et al., 2021; Sanjorjo et al., 2023). So far, research on this promising ability has primarily focused on biodegradable and bio-based plastics and MPs (Quartinello et al., 2021; Galyon et al., 2022, 2023a, 2023b), leaving a significant gap in understanding the rumen microbiota potential to degrade synthetic polymers - the most widely used plastics on farms and major contributors to environmental pollution (Borreani and Tabacco, 2017; Khan et al., 2024).

1.4 Objectives and Thesis Structure

As described in Chapter 1. *Introduction*, meeting the booming demand for ruminant products while reducing their environmental impacts poses a fundamental challenge for sustainable food systems and human well-being (Du et al., 2022; Su et al., 2025). This research investigates a critical and largely understudied environmental issue within the ruminant livestock sector: MP contamination (Corte Pause et al., 2024; Khan et al., 2024). The goal is to transition from pollution to solution, enhancing the eco-sustainability of ruminant production.

The study is structured around four key objectives, each developed in a dedicated chapter:

1. **Development of MP Extraction Protocol for Ruminant Feeds (Chapter 2) – published paper (Glorio Patrucco et al., 2024)**: MPs in ruminant feeds are a crucial link in MP contamination being one of the main potential sources for ruminants (Khan et al., 2024). However, no standardized protocol currently exists for extracting MPs from these complex feed matrices. This chapter focuses on optimizing a specific extraction protocol tailored to the complex composition of ruminant feeds, ensuring accurate detection and quantification of MPs (this part was done in collaboration with a dairy farm cooperative located in Piedmont).

2. **Assessment of MP Contamination in Ruminant Feeds (Chapter 3) - prepared paper:** the MP extraction protocol optimized for ruminant feeds (Chapter 2), was applied to investigate the presence of MPs in

feeds from northwestern Italian farms. The results of this chapter give a first indication of the pollution level in ruminant feeds (this part was done in collaboration with a dairy farm cooperative located in Piedmont).

3. **Investigation of MP Effects on Ruminant Digestion (Chapter 4) - published paper (Tassone et al., 2024):** MPs entering ruminant bodies through air, water, and feeds pose a potential threat to their health (Corte Pause et al., 2024; Khan et al., 2024). However, research on the effects of MPs on ruminant health remains limited (Chang et al., 2024; Grechi et al., 2023, 2024; Wang et al., 2024). Chapter 4 presents a pioneering investigation into the impact of polyethylene terephthalate (PET) MPs on ruminal-gastrointestinal system ability to degrade and digest hay.

4. **Study Rumen Microbiota Ability to Biodegrade MPs (Chapter 5):** Rumen microbiota could potentially degrade plastics and MPs due to their fibrolytic enzymes (Quartinello et al., 2021; Sanjorjo et al., 2023). However, research on this ability remains limited, with the few studies available focusing on biodegradable and bio-based plastics (Galyon et al., 2022, 2023a, 2023b; Quartinello et al., 2021). Chapter 5 investigates rumen microbiota ability to biodegrade synthetic MPs offering insights into a potential biomechanism for MP reduction.

Moreover, following the Conclusions chapter, the **Appendix** titled "**From Pollution to Solution: Greenhouse Gas Emissions in Ruminant Livestock**" is presented. This appendix summarizes the results of the mandatory PhD period at Nutristar S.p.A. It includes a brief introduction to greenhouse

gas (GHG) emissions from ruminants and nutritional strategies aimed at reducing them, followed by the section "**Effects of Essential Oils on Rumen Fermentation and GHG Emissions**", which presents a published paper (Glorio Patrucco et al., 2025) resulting from research conducted within the Research and Development unit at Nutristar.

Chapter 2: Development of MP Extraction Protocol for Ruminant Feeds

Published paper on Science of the Total Environment (ELSEVIER)

https://doi.org/10.1016/j.scitotenv.2024.174493

Science of the Total Environment 947 (2024) 174493

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Development and application of a novel extraction protocol for the monitoring of microplastic contamination in widely consumed ruminant feeds

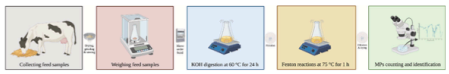
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HIGHLIGHTS

- Sources of MPs exposure in ruminants but lack of protocols for feeds monitoring.
- MPs first non-destructive protocol for MPs extraction from 4 most used ruminant feeds.
- Optimal combination of KOH/Fenton oxidation for the removal of interfering matter.
- First visual characterization of MPs in ruminant feeds from N—W Italian farm.
- MPs contamination in feeds from 8.3 ± 3.51 to 39.3 ± 7.02 MPs/g.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Microplastics
Ruminants
Feeds
Extraction protocol optimization
Identification
Pyr-GC/MS

ABSTRACT

Plastics and, in particular, microplastics (MPs) (< 5 mm) are emerging environmental pollutants responsible for interconnected risks to environmental, human, and animal health. The livestock sector is highly affected by these contaminants, with 50–60 % of the foreign bodies found in slaughtered domestic cattle being recognized as plastic-based materials. Additionally, microplastics were recently detected inside ruminant bodies and in their feces. MPs presence in ruminants could be explained by the intensive usage of plastic materials on farms, in particular to store feeds (i.e. to cover horizontal silos and to wrap hay bales). Although feed could be one of the main sources of plastics, especially of microplastics, a specific protocol to detect them in ruminant feeds is not actually present. Hence, the aim of this study was to optimize a specific protocol for the extraction, quantification, and identification of five microplastic polymers (high-density polyethylene, low-density polyethylene, polyamide fibers/particles, polyethylene terephthalate and polystyrene) from feeds typically used in ruminant diets (corn silage, hay, high protein feedstuff and total mixed ration). Several combinations of Fenton reactions and KOH digestion were tested. The final extraction protocol involved a KOH digestion (60 °C for 24 h), followed by two/three cycles of Fenton reactions. The extraction recoveries were of 100 % for high-density, low-density polyethylene, polyamide particles, and polystyrene and higher than 85 % for polyethylene terephthalate and polyamide fibers. Finally, the optimized protocol was successfully applied in the extraction of microplastics from real feed samples. All the feeds contained microplastics, particularly polyethylene, thus confirming the exposure of ruminants to MPs.

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https://doi.org/10.1016/j.scitotenv.2024.174493
Received 6 November 2023; Received in revised form 21 May 2024; Accepted 2 July 2024
Available online 3 July 2024
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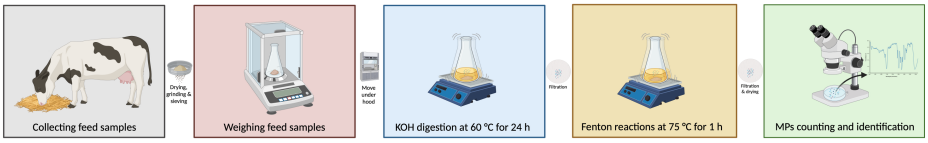
Development and Application of a Novel Extraction Protocol for the Monitoring of Microplastic Contamination in Widely Consumed Ruminant Feeds

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Graphical Abstract



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feeds is not actually present. Hence, the aim of this study was to optimize a specific protocol for the extraction, quantification, and identification of five microplastic polymers (high-density polyethylene, low-density polyethylene, polyamide fibers/particles, polyethylene terephthalate and polystyrene) from feeds typically used in ruminant diets (corn silage, hay, high protein feedstuff and total mixed ration). Several combinations of Fenton reactions and KOH digestion were tested. The final extraction protocol involved a KOH digestion (60 °C for 24 h), followed by two/three cycles of Fenton reactions. The extraction recoveries were of 100% for high-density, low-density polyethylene, polyamide particles, and polystyrene and higher than 85% for polyethylene terephthalate and polyamide fibers. Finally, the optimized protocol was successfully applied in the extraction of microplastics from real feed samples. All the feeds contained microplastics, particularly polyethylene, thus confirming the exposure of ruminants to MPs.

Highlights

- Sources of MPs exposure in ruminants but lack of protocols for feeds monitoring.
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- Optimal combination of KOH/Fenton oxidation for the removal of interfering matter.
- First visual characterization of MPs in ruminant feeds from N-W Italian farm.
- MP contamination in feeds from 8.3 ± 3.51 to 39.3 ± 7.02 MPs/g.

1. Introduction

Plastic pollution is one of the most pressing and growing problems that humanity should face in the 21st century (Du and Wang, 2021; Golwala et al., 2021), which also affects the livestock sector (Borreani and Tabacco, 2017; Urli et al., 2023). In fact, 50 to 60% of the foreign bodies found in slaughtered domestic cattle are plastic-based materials (Galyon et al., 2023a, Galyon et al., 2023b).

The limited and inappropriate recovery of plastic wastes has led to a visible accumulation of polymer debris in the environment, where they suffer from systematic fragmentation, generating different sizes of plastics: megaplastics, macroplastics, mesoplastics and microplastics (Du and Wang, 2021; Souza Machado et al., 2018; Yang et al., 2021). Among them, microplastics (MPs), classified as emerging ubiquitous pollutants (Ivleva, 2021), are commonly defined as particles smaller than 5 mm (Souza Machado et al., 2018). Indeed, MPs could act as carriers for inorganic and organic contaminants (*e.g.* heavy metals, persistent organic pollutants, antibiotics, pesticides, and fungicides) (Kinigopoulou et al., 2022; Okeke et al., 2022; Silva et al., 2018), being potentially responsible for interconnected risks to environmental, human, and animal health (Prata et al., 2021).

In the livestock sector, the presence of MPs was recently detected in sheep feces (Beriot et al., 2021), cow follicular fluid (Grechi et al., 2023), cow milk (Da Costa Filho et al., 2021; van der Veen et al., 2022) and cow blood and meat (van der Veen et al., 2022), thus confirming the exposure of ruminants to MPs. One potential source of MPs for ruminants could be their feeds. In fact, plastic materials are extensively used as storage

medium, for dairy cow, pig, and beef farms, with the formers consuming the largest quantities. As an example, in the northern part of Italy, an average of 10.3 kg of plastic per cow is used annually, with 3.9 kg per cow used specifically for covering bunker silages (Borreani and Tabacco, 2015). Plastic products mainly used in dairy farms are silage bags, bale wraps, and plastic bunker silo covers. They are typically composed of low-density polyethylene (LDPE) or LDPE coextruded with polyamide (LDPE-PA). Moreover, veterinary drugs are typically packed in high-density polyethylene (HDPE) supports (Borreani and Tabacco, 2015, Borreani and Tabacco, 2017). Finally, atmospheric transport of MPs could also account for MP contamination in feeds (Evangeliou et al., 2020).

It was estimated that 0.07% of polyethylene-based plastics is contained in ruminant diets (Pizol et al., 2017). When these materials enter the reticulum, they aggregate to form large spherical masses that press against the rumen epithelium during rumination. With prolonged presence, they could reduce rumen functionality (Akraiem and Abd Al-Galil, 2016), lead to erosion and ulcerations of ruminal papillae (Otsyina et al., 2017), and promote heavy metal leaching (Mahadappa et al., 2020).

Considering that feeds can be a major source of MPs for ruminants - due to both the extensive use of plastic on farms and external contamination from atmospheric agents (Borreani and Tabacco, 2015; Evangeliou et al., 2020) - there is an urgent need for protocols capable of quantifying MPs in feed, despite the significant challenges involved (European Food Safety Authority, 2021). Indeed, feeds are complex matrices, as they contain a wide variety of components such as grains, protein sources, fibers, minerals, vitamins, and additives (Bueno et al., 2020), all of which pose

challenges to the extraction and identification of MPs, particularly the fibrous component. In addition, feed composition varies greatly according to animal characteristics such as breed, body size, sex, life stage, and digestive capacity (McGrath et al., 2018; Tedeschi et al., 2019).

Hence, the development of a specific MPs extraction protocol requires a careful optimization of several operational steps to remove the interfering inorganic and organic compounds in the matrix, avoiding, at the same time, degradation, and loss of MPs.

Unlike other environmental and food matrices (He et al., 2021; Hurley et al., 2018; Sridhar et al., 2022), currently specific non-destructive protocol for the extraction, visual evaluation, and quantitation of MPs in ruminant feeds is not available yet. The only work devoted to MP investigation in feeds is a pilot study from van der Veen et al. (2022), in which the presence of MPs was investigated in cow and pig feeds (pellets, fresh and shredded). The target of van der Veen et al. (2022) work was the quantitation of several polymers in feeds using pyrolytic GC–MS, without focusing on the development of an effective protocol for their selective extraction and singular characterization. MPs were extracted by dissolution in tetrahydrofuran, thus not preserving their physical integrity, which is essential for carrying out further visual, and spectroscopic investigations (*e.g.* stereomicroscope or μ-FTIR).

Based on the above-mentioned premises, the aim of this work was the optimization of an effective protocol for the extraction, quantification, and identification of five types of MPs from feeds typically used in ruminant diets, in particular those of dairy cows.

The optimization of extraction conditions was performed directly on real feed samples, previously fortified with MP standards, using extraction recoveries as the analytical response. Finally, the optimized protocol was applied for the screening of selected MPs in real feed samples from local farmers. To the best of our knowledge, this work represents the first study reporting MP contamination in dairy cow feeds.

2. Materials and Methods

2.1 Feed Samples

As summarized in Table 1, four dairy cow feeds were investigated, namely: corn silage (CS), hay (H), high protein feedstuff (HPF) and total mixed ration (TMR). All these matrices were sampled from an Italian dairy farm located in Piedmont. Plastic materials were avoided during sampling procedures with all clothing including lab coats and gloves made of cotton to minimize contamination. CS, HPF and TMR were sampled using a stainless-steel trowel which was thoroughly cleaned with disposable paper sheets between samples to prevent cross-contamination. H samples were collected by hand, using cotton gloves that were carefully cleaned in the same way as stainless-steel trowel. CS, H, HPF and TMR samples were stored in natural fiber bags. Each bag was labelled with the sample name, tied with a cotton string, and immediately transported to the laboratory for analysis. Each feed sample was dried at 60 °C overnight, grinded (5 mm sieved - Restch GmbH Knife cutting mill, type SM1, Germany) and conserved in a glass bottle. For MP extractions, 1 g of dry matter of each feed was collected by thoroughly mixing the contents of the bottle and sampling from multiple points to ensure representativeness. Feed samples were then fortified with MPs to

optimize the extraction protocol (Section 2.3) and extracted using the optimized protocol (section 2.4).

Table 1
Feed sample, acronym, composition, and picture of chosen dairy cow feeds.

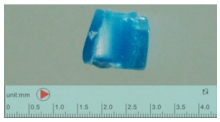
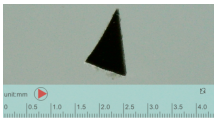
Feed	Acronym	Composition	Picture
Corn Silage	CS	Whole corn plant: stover (stalk, leaves, husk that covers the cob, cob that holds the maize grain) and maize grain.	
Hay	H	Ryegrass hay.	
High Protein Feedstuff	HPF	Corn, dehulled soybeans, wheat bran, partially dehulled sunflower, beet pulp, corn flour, soybean husks, sugarcane molasses, inactivated yeast pool, minerals.	
Total Mixed Ration	TMR	Main ingredients: silages (mostly corn silage), ryegrass hay, high protein feedstuff.	

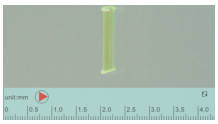
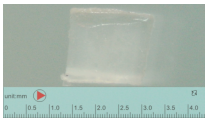
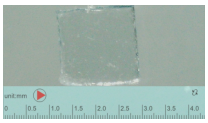
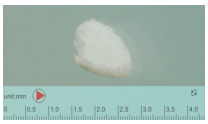
2.2 MP Standards and Reagents

2.2.1 MP Standards

Five widespread types of MPs (Ajith et al., 2020; Khalid et al., 2021) were used to optimize the protocol, namely: high-density polyethylene (HDPE), low-density polyethylene (LDPE), polyamide (PA), polyethylene terephthalate (PET) and polystyrene (PS). In addition, to differentiate the MP shape, the protocol was customized for fibers (cylindrical shape and length to diameter ratio > 3) and particles (non-cylindrical shape and length to diameter ratio < 3) (ECHA, 2019), choosing PA as model polymer (hereafter called PA fibers and PA part., respectively). The list of each MP, together with its density (Harper, 2002), origin, length range and picture are reported in Table 2. All the MP standards were produced in the laboratory from real samples (bags, bottles, etc.) using a stainless-steel grinder and were subsequently sieved by 5 mm mesh-sieves to discard MPs larger than 5 mm.

Table 2
List of MPs chosen for the protocol optimization, together with their density, origin, length range (mm) and their visual aspect.

MP	Density (g/cm^3)	Origin	Length range (mm)	Picture
HDPE	0.95	Falcon tube cup	1.147-3.483	
LDPE	0.92	Rubbish bag	0.682-3.436	

PA fibers	1.13	Fishing net	0.541-3.174	
PA part.	1.13	Ties	1.188-2.524	
PET	1.56	Bottle	1.590-3.286	
PS	1.05	Food packaging	0.579-3.396	

HDPE = high density polyethylene; LDPE = low density polyethylene; PA fibers = polyamide fibers; PA part. = polyamide particles; PET = polyethylene terephthalate; PS = polystyrene

2.2.2 MP Reagents

Throughout this work, ultrapure water (18.2 MΩcm at 25 °C) obtained with a Milli-Q Academic system (Millipore, Billerica, MA, USA) was used. In addition, the following reagents were used: 30% hydrogen peroxide (H_2O_2) (VWR Chemicals, Milan, Italy); iron sulphate heptahydrate ($FeSO_4$ $7H_2O$) (VWR Chemicals, Milan, Italy); 95-97% sulfuric acid (H_2SO_4) (Honeywell, Charlotte, North Carolina, USA) and potassium hydroxide (KOH) (Honeywell, Charlotte, North Carolina, USA).

2.2.3 MP Background Contamination: Quality Assurance

Contamination from laboratory equipment or airborne particles during sample transfers should be addressed as the main MP background sources. Currently, in the field of microplastic research, there are no

standardized protocols to account for background contamination. Hence, in the context of this study, the following precautions were adopted:

i) when possible, avoiding the use of plastic materials;
ii) the operators using cotton clothes, coats and nitril gloves;
iii) preventing equipment contamination by thoroughly rinsing all glassware and containers with 0.45 µm filtered ultrapure water prior to use and covering them with aluminum foil;
iv) conducting extraction processes within a chemical hood to limit exposure to unfiltered air;
v) running two blanks (no feed sample, only reagents) in parallel with each extraction to monitor contamination and subtracting the MPs found in blanks from the sample results.

2.3 Optimization of MP Extraction Protocol

To achieve a quantitative extraction of MPs from feed samples, several oxidation protocols were tested, as hereafter described.

2.3.1 Degradation of Organic Matter (OM)

2.3.1.1 Fenton Reactions

The protocol of Rodrigues et al. (2019), that investigated the Fenton reaction applied for MP extraction from estuarine waters, was adapted to feed samples. Each feed sample (1 g) was placed in a glass flask with 20 mL 0.05 M Fe^{2+} solution and two subsequent aliquots of 20 mL of 30% H_2O_2 (added at t = 0 min and t = 30 min respectively). The reaction was performed on a hot plate at 75 °C under stirring conditions (300 rpm, magnetic stirrer) for 1 h. To obtain the complete removal of organic matter, progressive Fenton reactions were tested (from one to three

subsequent reactions), each one followed by a filtration step through a metallic sieve. In detail, flask content was filtered through a metallic filter (200 mesh, pore dimension <0.03 mm, Humboldt). The flask was rinsed three times with 0.22 µm filtered ultrapure water, to remove residual MPs. Subsequently, the solid retained by the filter was also washed with ultrapure water to remove any residual water-soluble contamination. Afterwards, it was transferred using a metallic spatula in the new flask and, to avoid any possible loss, the filter was rinsed directly into the flask, using the reagent for the subsequent reaction. After the oxidation step, the filtrate was dried at 60 °C overnight and transferred into a glass Petri dish to observe it by a stereomicroscope.

2.3.1.2 Alkaline digestion coupled with Fenton reactions

Due to the high amount of interfering organic matter present in the feed matrices investigated, an alkaline digestion pre-treatment was implemented before the Fenton reactions. Tests were performed with 40 mL of KOH (10%) at 60 °C (Dehaut et al., 2016), studying the effect of different incubation times (namely 6 h, 18 h and 24 h). After the alkaline digestion, one, two or three Fenton reactions (section 2.3.2.1) were performed according to the experimental design of Fig. 1. Each reaction was followed by filtration through a metallic sieve (see paragraph 2.3.1.1). At the end of each trial, the final filtrate was dried at 60 °C and was transferred into a glass Petri dish for the subsequent identification and quantitation procedures.

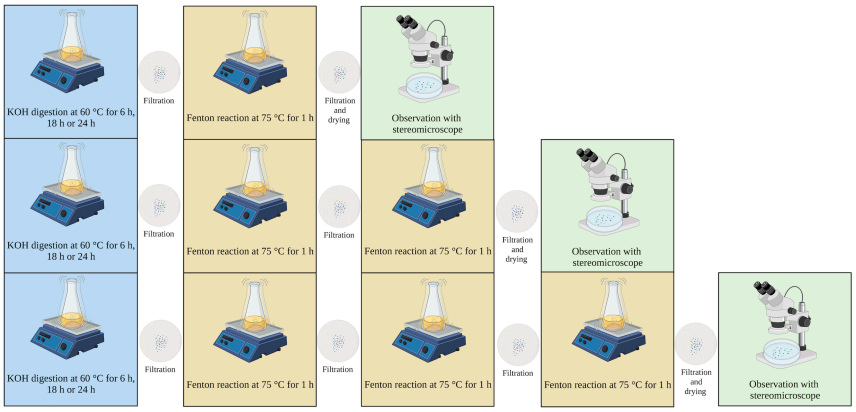


Fig. 1. Degradation of OM. Experimental trials of alkaline digestion coupled with Fenton reactions (created with BioRender.com).

2.3.2 MP Extraction Recoveries and Polymers Integrity Check

MP extraction recoveries (R%) were evaluated fortifying each feed sample (1 g) with 0.01 ± 0.005 g of each of the five laboratory prepared MP standards.

It should be mentioned that the same weight could correspond to different numbers of MP pieces because density, sizes, shapes, and weights of MP pieces can vary from one to another polymer. Hence, the specific MP pieces counted and added to the feed samples are reported in Table S1 of the Supplementary Material.

In addition, fortified MPs were properly prepared to be easily distinguishable from native contamination, potentially present in feeds (see section 2.2.1).

After extraction, MPs were again visually counted and observed using the stereomicroscope in order to both assess that no physical degradation occurred (*e.g.* fragmentation) and to evaluate the extraction recoveries

(R%). The extraction recoveries were calculated according to the following equation:

$$R\% = \frac{n^\circ\ of\ MPs\ after\ the\ extraction}{n^\circ\ of\ MPs\ fortified\ in\ the\ sample} \cdot 100$$

Each protocol was repeated three times for each feed sample. Extraction rates (expressed as percentages) obtained for each MP and each feed after the protocol optimization are reported and commented in the Results and Discussion session (Section 3.1.1.2).

In addition to the physical integrity check, the chemical integrity (potential alteration of polymer chains) of MPs after the sequential application of the oxidation cycles was also performed through micro-Fourier transform infrared (µ-FTIR) spectroscopy analysis on both virgin and post-extracted MPs. The *spectra* were acquired using a Perkin Elmer Spectrum Two µ-FTIR ATR spectrometer (wavenumber range of 4000–400 cm^{-1}, 32 scans per *spectrum*, spectral resolution of 4 cm^{-1}).

2.4 Application of the Optimized Protocol to Real Samples

The optimized protocol was applied in the determination of the native contamination by MPs (HDPE, LDPE, PA fibers/part., PET, PS) in four real feed samples. For each tested matrix (CS, H, HPF and TMR), the analysis was performed in triplicate and two control blanks were run in parallel. MP concentration results were expressed as:

i) total number of MPs detected per gram ($\frac{MPs}{g}$);

ii) total number of MP fibers per gram ($\frac{MP\ fibers}{g}$);

iii) total number of MP particles (all MPs not belonging to fibers) per gram $(\frac{MP\ particles}{g})$

where $\frac{MPs}{g} = \frac{MP\ fibers}{g} + \frac{MP\ particles}{g}$.

2.4.1 Characterization of Polymers by Stereomicroscope and Pyr-GC/MS

After the extraction of MPs from each feed, the residual particles retrieved from the metallic filter were preliminary characterized by visual observation using a Nikon H550S stereomicroscope equipped with Micro Capture Ver6.9.12 software (DIV = 0.01 mm). The flow chart suggested by Lusher et al. (2020) was carefully followed in order to reduce subjectivity and random errors, by classifying suspected fragments into three categories, namely morphology (*e.g.* size, shape and texture), optical properties (*e.g.* the color) and behavior (*e.g.* flexibility and density). Once classified, MPs were further analyzed through pyrolysis-gas chromatography/mass spectrometry (Pyr-GC/MS) to confirm their nature and to identify polymer composition. The use of Pyr-GC/MS as a complementary technique to the visual identification allows to achieve integrated analysis of MPs, for example for source-apportionment and risk assessment studies (Dong et al., 2023; Narloch et al., 2022; Wang and Wang, 2018).

Pyr-GC/MS analysis were performed with a micro-furnace pyrolizer (EGA/Py-3030D, Frontier Laboratories Ltd., Japan) interfaced to a GC/MS system (8890 and 7000D, Agilent Technologies, Inc., USA). The GC was equipped with a fused silica capillary column coated with 0.25 µm film thicknesses of 5% diphenyl 95% dimethylpolysiloxane (HP-5MS, 30 m ×

0.25 mm id, Agilent Technologies, Inc., USA). The GC was operated with a constant helium (He) flow as carrier gas of 1 mL/min. To avoid saturation of the mass *spectrum*, a 1:100 split ratio was chosen. GC injector was set at 300 °C.

For the analysis, the MP was directly placed in a sample cup. Then it was inserted into the pyrolizer furnace. The temperature program curves were set according to the work of Anuar et al. (2022). Polymer identification was achieved using total ion pyrograms and recognizing characteristic pyrolysis products. The recognition of pyrograms and pyrolytic products was performed by comparison with the "*Pyrolysis-GC/MS data book of synthetic polymers: pyrograms, thermograms and MS of pyrolyzates*" which includes pyrograms of 163 polymers and MS data for the main pyrolyzates by means of Pyr-GC/MS (Tsuge et al., 2011).

2.5 Statistical Analysis

Data were analyzed using R statistical software (R 4.2.2) and its extension RStudio (2022.07.2 + 576). The Kruskal-Wallis non-parametric test (significance threshold level 0.05) was used to detect the statistical differences for: MP extraction recoveries, MP concentrations (MPs/g, MP fibers/g and MP particles/g, each variable analyzed separately) and MP range lengths. Kruskal-Wallis non-parametric test was used after the invalidation of the normality variance with Shapiro-Wilk test.

3. Results and Discussion

3.1 Optimization of MP Extraction Protocol

As previously mentioned, the aim of the extraction protocol here optimized is both to remove the interfering matrix, avoiding, at the same time, MP degradation. Feeds are considered complex samples because they have a complex composition. Main feed constituents (such as lignocellulose) are difficult to be removed, similarly to other matrices such as soils, sand, organic-rich sediments, sludge, biota, etc. (Monteiro and Pinto Da Costa, 2022). In this regard, within the protocol, several steps were evaluated.

It should be mentioned that preliminary density separation tests were performed with saturated salt solutions (such as NaCl and $ZnCl_2$), with unsuccessful removal of the organic matter. Hence, density separation step was discarded.

3.1.1 Degradation of organic matter (OM)

Even if it is universally recognized that the organic matter present in samples should be efficiently removed to avoid interferences in MP determination, oxidation techniques can potentially damage MPs themselves. Indeed, depending on the concentration and duration of the oxidation treatment, the oxidizing agents can lead to degradation or alteration of MPs. This can result in changes to their size, shape, and surface characteristics, which may negatively impact the subsequent analysis or characterization (Savino et al., 2022). Based on the above-mentioned premises, the following oxidation treatments were carefully optimized, in order to degrade OM without damaging MPs.

3.3.1.1 Fenton reactions

A single cycle of two Fenton reactions (as described in section 2.3.2.1) was initially tested for all matrices. Visual observation of treated samples showed that for H and TMR, degradation was slightly more efficient than for CS and HPF. However, for all the samples, poor recoveries (below 10%) were obtained, since MPs were fully hindered by interfering matrix clusters (residual organic matter in H and TMR is reported as an example in Fig. S1 in Supported Material). The higher degradation resistance of CS and HPF could be ascribed to some of their components rich in cellulose and lignin (*i.e.* corn stalk for CS and seed shell for HPF) (Jahirul et al., 2012; Passoth and Sandgren, 2019). However, it should be mentioned that for none of the tested matrix, a complete removal of OM was achieved. Hence, two and three consecutive Fenton cycles were tested, but they were still not enough to efficiently remove the OM (Fig. S1 in Supplementary Material). Even if a partial increase in the extraction recoveries was observed, recoveries still remained below 18% for all the feeds.

3.3.1.2 Alkaline digestion coupled with Fenton reactions

Given that the three consecutive Fenton reaction cycles were not efficient enough for the complete OM removal, the effect of a KOH alkaline digestion before Fenton reactions was studied for all feed samples. Three different KOH digestion times were tested, namely, 6 h, 18 h and 24 h. Visual observation of the post-oxidized samples through the stereomicroscope showed that both 6 h and 18 h reaction times provided higher degradation of interfering organic matter than Fenton reactions only, even if a complete degradation could not be achieved. A similar

trend as for Fenton degradation was shown also for KOH digestion, with more efficient OM removal for H and TMR than for CS and HPF.

Finally, 24 h KOH digestion followed by two Fenton reactions (for H and TMR)/ three Fenton reactions (for CS and HPF) allowed the quantitative degradation of OM for H, TMR, CS, and HPF, as shown in Fig. S2 (Supplementary Material).

For CS and HPF, a residual fraction of OM was still observed. Nevertheless, this fraction was considered negligible, since MPs were easily discernible from these residual matrix particles and easily classifiable through the Lusher et al. (2020) identification flow-chart (see section 2.4.1).

Based on these results, the optimized protocol for OM degradation is: 24 h KOH digestion followed by Fenton reactions (two cycles for H and TMR and three for CS and HPF, respectively).

3.2 MP extraction Recoveries and Polymers Integrity Check

In Fig. 2, the MP extraction recoveries obtained through the optimized protocol are reported. Results show that quantitative extractions were obtained for HDPE, LDPE, PA part. and PS for all the tested matrices, while for PA fibers and PET recoveries were above 85%. For each MP type, the extraction recoveries did not differ significantly in the four feeds (N = 3). In addition, repeatability was confirmed with relative standard deviation equal to 0% for HDPE, LDPE, PA part. and PS, and lower than 12% and 8% for PA fibers and PET respectively (N = 3).

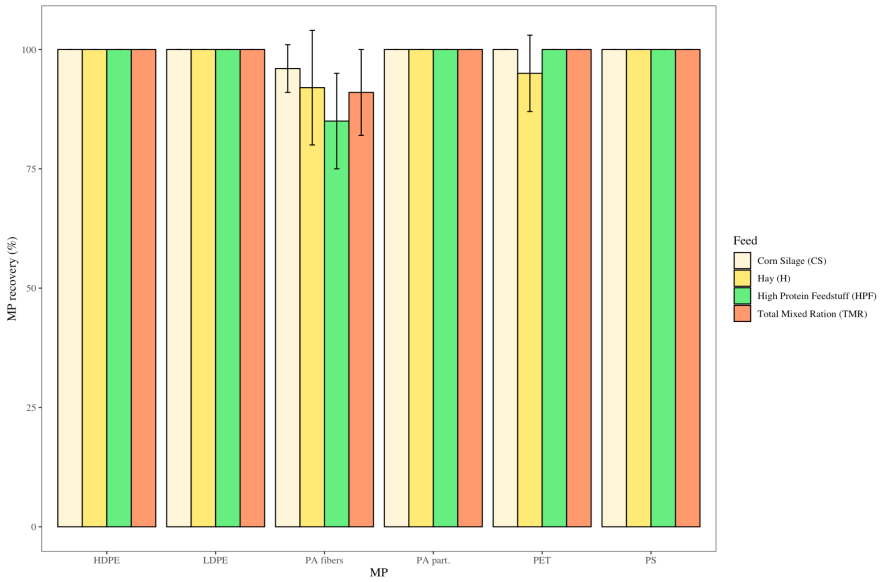


Fig. 2. MP extraction recoveries (%) obtained using the optimized MP extraction protocol on the four fortified feeds. For each MP, the extraction recoveries did not differ significantly in four feeds (Kruskal-Wallis test, significance threshold level 0.05, N = 3).

These results obtained within our study are in good agreement with the work of Way et al. (2022), in which a meta-analysis study was conducted on the extraction yields of 71 protocols available in the literature for the extraction of MPs from different matrices. According to their research, extraction recovery rates were 84.5% for microplastics ranging from 1 µm to 1 mm (n = 60 works) and 84.8% for microplastics sized between 1 mm and 5 mm (N = 30 works). Additionally, according to the above-mentioned work, extraction yields were higher for plant material, whole organisms, and excreta from swine human, chicken, and zebrafish (> 88%), and lower for fishmeal, water, and soil (58-71%); furthermore, high-density MPs (like PA and PET) are characterized by lower extraction recovery yields. However, in the study of Way et al. (2022), no ruminant feed was considered and, to the best of our knowledge, the present work appears

to be the first in which MP extraction yields from ruminant feed are evaluated.

To check the integrity of each target MP after the sequential application of the oxidation cycles, μ-FTIR analysis on both virgin and post extracted MPs was performed. The signals typical of each polymer were still clearly distinguishable, thus demonstrating that no alteration of polymer chains occurred (Fig. S3 of Supplementary Material).

3.3 Optimized MP Extraction Protocol for Ruminant Feeds

The optimized MP extraction protocol for ruminant feeds is summarized, together with all its operational steps, in Table S2 (Supplementary Material).

Considering the studies currently available in the literature regarding the extraction of MPs from complex environmental samples, it can be noted that, due to the differences in density between the MPs and the matrix components, a step based on density separation is generally present. In particular, density separation is exploited for the analysis of MPs in:

i) soils (Isari et al., 2021, Li et al., 2019, Liu et al., 2019;
ii) marine sediments and oysters (Rivoira et al., 2020);
iii) lake sediments (Zobkov et al., 2020);
iv) sand (Kedzierski et al., 2017; Maynard et al., 2021);
v) marine biota (Yu et al., 2019).

In contrast to other complex matrices, the optimized protocol for the extraction of MPs from ruminant feeds has the advantage of bypassing the density separation step, thus reducing the extraction time and increasing the feasibility.

The first step of the protocol here optimized for the extraction of MPs from ruminant feeds consists of a first KOH (10%) digestion at 60 °C for 24 h. This alkaline digestion was shown to be one of the most effective digestive treatments in MP extraction protocols (Phuong et al., 2018; Prata et al., 2019). Alkaline digestion was also applied by Thamizhakaran Stanley et al. (2022) for the pretreatment and degradation of lignocellulosic biomass, which has a similar chemical composition to that of ruminant feeds, thus making it a promising approach to remove organic matrix in the tested feeds. Although alkaline digestion could promote discoloration and degradation of PA fibers (Prata et al., 2019), it was here demonstrated that under the optimized extraction conditions, the chemical structure of target MPs is maintained.

The second step of the optimized protocol was the oxidation of OM by Fenton reaction cycles: two reactions for TMR and H and three for CS and HPF. A similar approach based on sequential Fenton reactions was used to extract MPs from the secondary effluent of a wastewater treatment plant (Dyachenko et al., 2017) and from sludge (Cunsolo et al., 2021), a matrix affected by a high organic matter content.

As discussed in Section 3.1.2, the use of KOH treatment only or multiple Fenton reactions was not sufficient to efficiently remove the interfering OM. Conversely, the proposed synergic combination of both KOH digestion together with Fenton reactions achieved the goal. A similar approach was followed also for other complex matrices, such as marine biota, as shown by Alfonso et al. (2021) and Avila et al. (2023).

3.4 Application of the Optimized Protocol to Real Samples

The optimized protocol previously described was successfully applied in the analysis of target MPs in feed samples (CS, H, HPF and TMR) daily used by local farmers. The results are summarized in Table 3. The highest concentration of MPs/g was detected in H (39.3 ± 7.02 MPs/g); this concentration is more than double that detected in the other matrices. CS, HPF and TMR had MPs/g concentrations not significantly different from each other (12.3 ± 5.76 MPs/g, average concentration). For all four feeds tested, the majority of MPs detected are in fiber form. The MP colors were: colorless, black, blue, green, red, grey, and brown.

Table 3
MP concentrations in the four feeds obtained by applying the optimized MP extraction protocol. Concentrations are expressed in MPs/g, MP fibers/g and MP particles/g, expressed as mean ± SD (Kruskal-Wallis test, significance threshold level 0.05, N = 3).

Feed	MPs/g	MP fibers/g	MP particles*/g
CS	13.3±3.21^{b}	13.0±3.46^{bc}	0.3±0.58^{b}
H	39.3±7.02^{a}	32.0±2.00^{a}	7.3±5.03^{a}
HPF	8.3±3.51^{b}	8.0±3.61^{c}	0.3±0.58^{b}
TMR	15.3±5.77^{b}	15.3±5.77^{b}	0.0±0.00^{b}

Different superscript letters in the same column refer to mean values statistically different according to the Kruskal-Wallis test (significance threshold level 0.05). Conversely, common letters in the same column refer to mean values not statistically different from one to the other. MPs/g = MP fibers/g + MP particles/g; MP fibers = length:diameter > 3; MP particles = length:diameter < 3.
* “MP particles” refers to the overall non-fiber fraction, as detailed in section 2.2.1

The total detected MPs were additionally classified by their dimensions, as reported in Fig. 3 (and further discussed in detail in section S1 of Supplementary Material).

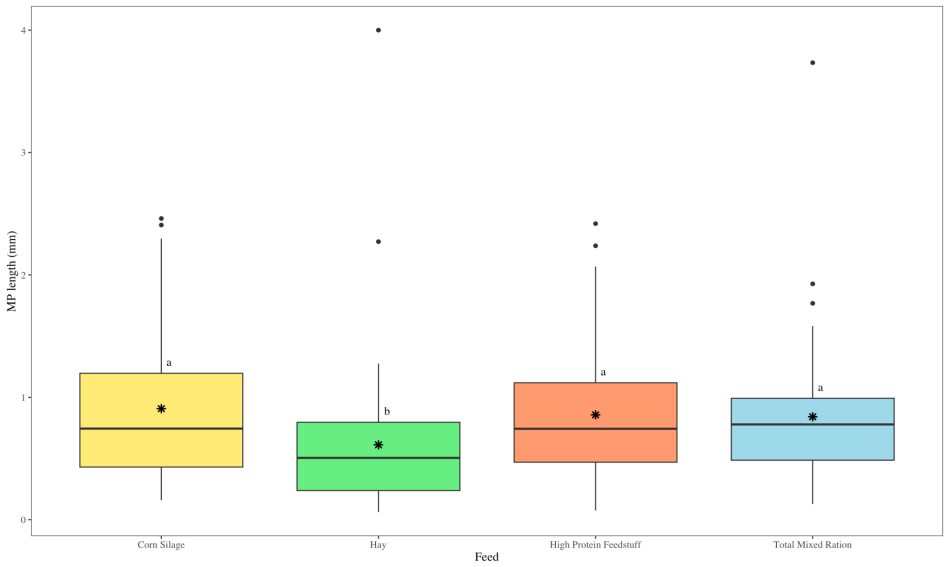


Fig. 3. Range length of MPs found in feed samples expressed in mm. The horizontal line represents the median value of data (Q50), the edges of the boxes are the quartiles (Q25 and Q75), the asterisk is the mean (Kruskal-Wallis test, significance threshold level 0.05, different superscripts (a–b) within feeds indicate significant differences).

For all four feeds, the box plots were positively skewed having the means higher than the median, and the upper quartiles larger than the lower quartiles. All the samples had values outside the quartiles showing wide variability in lengths. H showed to have MPs of significantly shorter lengths than other feeds.

The application of the optimized MP extraction protocol on real ruminant feed samples demonstrated the presence of MPs in all dairy cow feeds: CS, H, HPF and TMR, as also reported for HPF by the pilot study of van der Veen et al. (2022). Regardless of particle size, these results confirm the existence of an "Environmental MP cycle" that involves ruminants, as hereafter phase-by-phase reported and commented. Phase 1: agricultural soils, including those of livestock farms, are contaminated with MPs (from 7.1 to 42.96 MPs/g of agriculture soils; 9.56 MPs/g of pasture soils)

(Álvarez-Lopeztello et al., 2021; Zhang and Liu, 2018). Contamination of soils by MP arises from plastic equipment (*e.g.* mulching), substances already contaminated with plastics (*e.g.* wastewater sludges used as an amendment) or atmospheric deposition (Chang et al., 2022). MPs (especially nano-sized microplastics) could move from soil to plants through root uptake (Yu et al., 2024). Phase 2: from soils and plants they could pass to animal feeds and this contamination could be enhanced by plastic material used for storing ruminant feeds (Borreani and Tabacco, 2017) (19.05 MPs/g of ruminant feeds in our study). Phase 3: ruminants eat contaminated feed and MPs enter their bodies (*i.e.* 38.6 MPs/mL of bovine follicular fluid), interfering with their physiologic functionalities (*e.g.* reproductive system) (Grechi et al., 2023). MPs larger than150 µm can return to the soil via feces (from 0 to 5 MPs/g of sheep feces) (Beriot et al., 2021), becoming a new source of pollution.

3.4.1 Characterization of Polymers by Stereomicroscope and Pyr-GC/MS

In Fig. 4 selected subset of MPs, including three micropolymers for each feed (ten fibers and two particles) are reported. It is possible to observe the variety of colors (green, colorless, red, and blue) shapes (fibers/particles) and lengths.

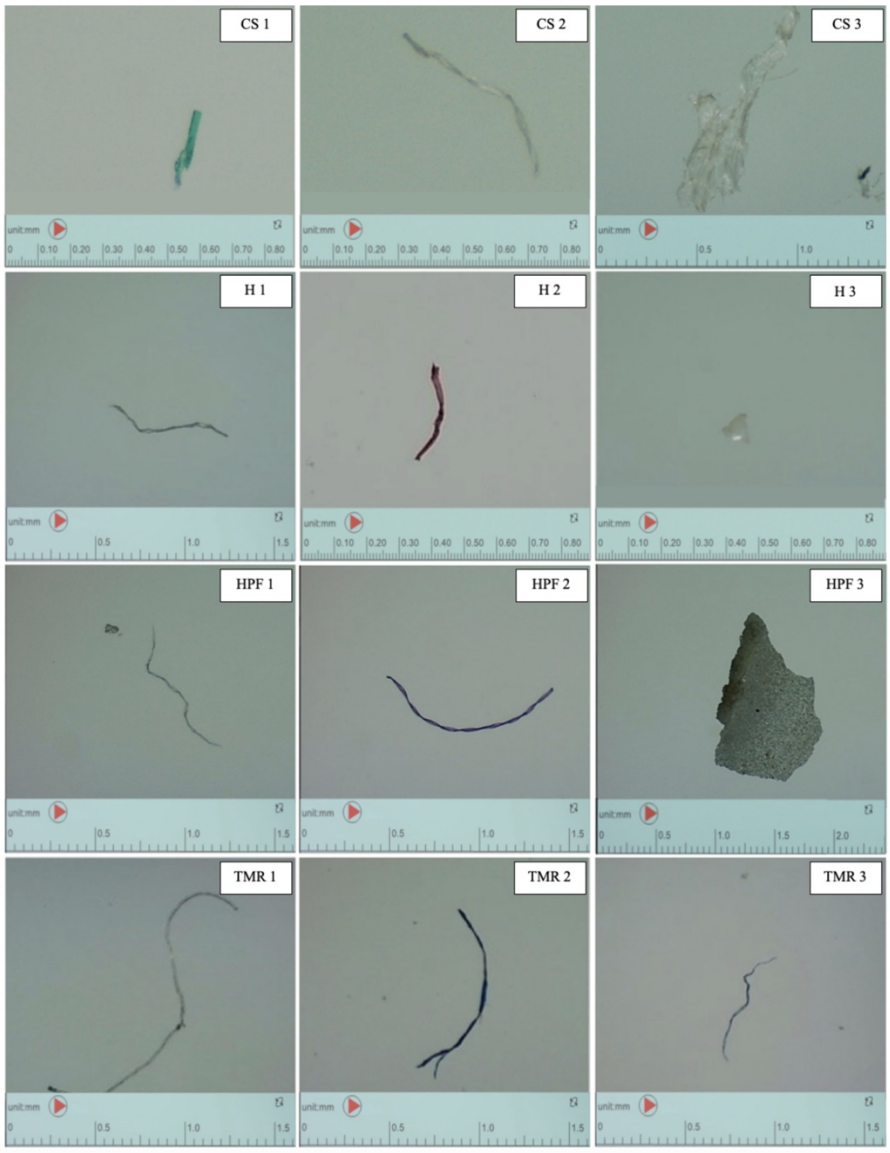


Fig. 4. Pictures captured under stereomicroscope of the three MPs for each feed sample that were selected and identified by Pyr-GC/MS.

After stereomicroscopic inspection, MPs were analyzed by Pyr-GC/MS. The pyrograms are reported in Fig. S4-S7 (Supplementary Material).

The majority of the fragments in CS, H, HPF and TMR were correctly recognized as polyethylene, typically employed for feed wrapping and

storage (Borreani and Tabacco, 2015, Borreani and Tabacco, 2017). Similarly to our study, polyethylene was detected in cow HPF by the only preliminary study by van der Veen et al. (2022). Furthermore, the presence of polyethylene has been detected in blood, milk, meat, and follicular fluid of cows (van der Veen et al., 2022; Grechi et al., 2023). This result confirms the widespread diffusion of polyethylene and the tendency of objects made with polyethylene to degrade and form MPs (Da Costa Filho et al., 2021; Grechi et al., 2023; van der Veen et al., 2022).

It should be mentioned that, among the MPs detected in HPF (see Table 3), one fragment (classified as HPF 3 in Fig. 4) was not ascribable to any of the polymers considered within this study (hence suggesting that the proposed protocol could be also effective for additional polymers than those here studied).

4. Conclusions

For the first time, a non-destructive extraction protocol dedicated to the analysis of five types of MPs from ruminant feed is proposed. High consumption feeds for dairy cows were investigated (corn silage, hay, high protein feedstuff and total mixed ration). The developed protocol, which represents a novelty in the scientific literature, was intentionally developed with the final aim of both removing interfering species and not altering the structure of MPs, to make possible reliable qualitative and quantitative analysis. In this regard, different combinations of procedural steps based on Fenton reactions and KOH digestion were tested and discussed. The final protocol, which leads to recoveries as high as 85% for all the MPs and feeds, requires a KOH digestion at medium temperature (60 °C for 24 h) followed by two/three cycles of Fenton reaction. The

effectiveness of the proposed procedure avoids the use of additional density separation steps, thus reducing the operating times compared to already existing procedures developed for other complex matrices (*e.g.* soil, biota).

The optimized procedure was successfully applied for the extraction, visual quantitation, and mass spectrometric identification of MP contamination in the four tested animal feeds. The detection of polyethylene in all the matrices confirms the exposure of ruminants to MPs.

The effectiveness, ease, and robustness of the proposed protocol suggest its full compatibility with future monitoring campaigns aimed at evaluating MP contamination in ruminant feed and crucial for understanding the real impact of these pollutants in animal farming.

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SUPPLEMENTARY MATERIAL

S1. Dimensional classification of MPs detected in real feed samples.

Detected MPs were classified by their dimensions. In detail, as reported in the box plots of Fig. 3 (in the Chapter 2), CS had a MP range length from 0.159 mm to 2.461 mm, with a mean of 0.907 mm and a median of 0.744 mm (Q25 = 0.430 mm; Q50: 0.744 mm; Q75 = 1.196 mm). H had a MP range length from 0.063 mm to 4.000 mm, with a mean of 0.612 mm and a median of 0.505 mm (Q25 = 0.238 mm; Q50 0.505 mm; Q75 = 0.796 mm). HPF had a MP range length from 0.076 mm to 2.419 mm, with a mean of 0.857 mm and a median of 0.743 mm (Q25 = 0.470 mm; Q50 0.743 mm; Q75 = 1.118 mm). TMR had a range length from 0.128 mm to 3.734 mm, with a mean of 0.841 mm and a median of 0.779 mm (Q25 = 0.486 mm; Q50 = 0.779 mm; Q75 = 0.992 mm).

Figures and Tables

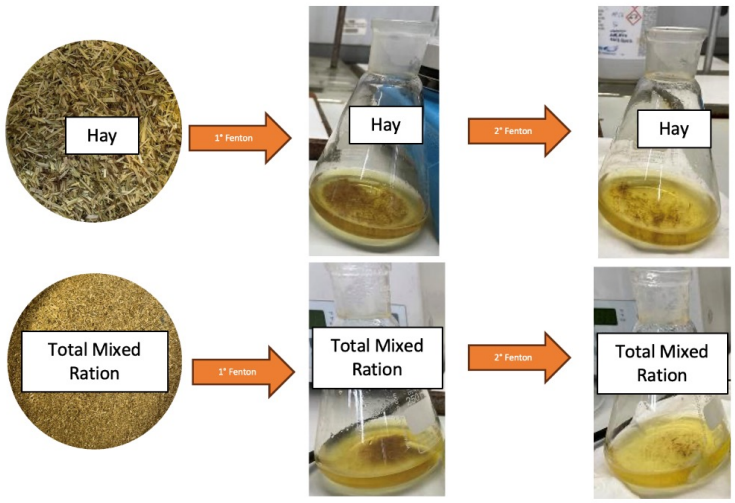


Fig. S1. Residual interfering organic particles in H and TMR after one and two cycles of Fenton reaction.

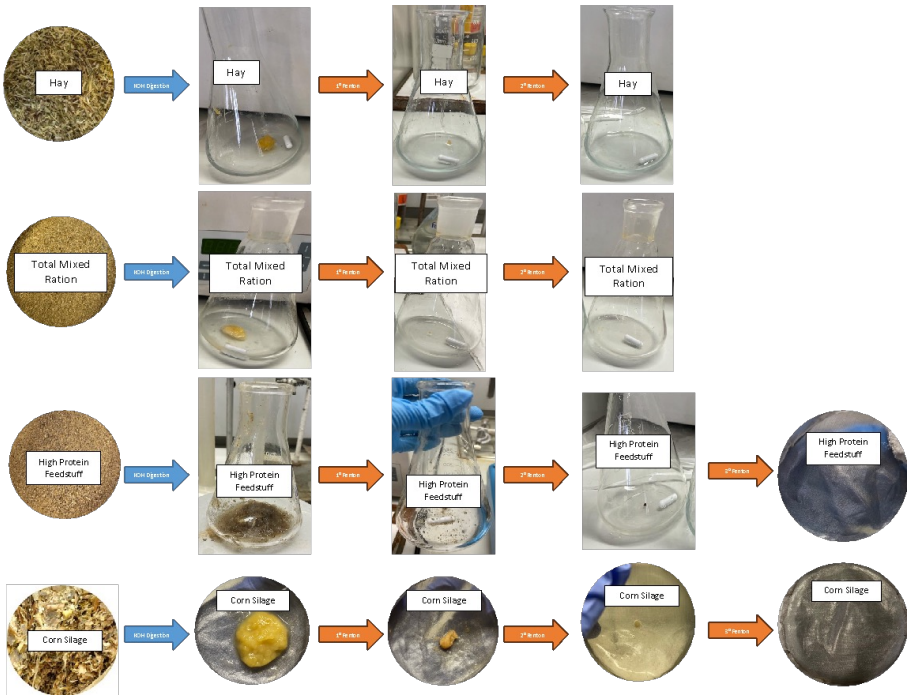


Fig. S2. Pictures of feed samples after each step of the optimized MP extraction protocol.

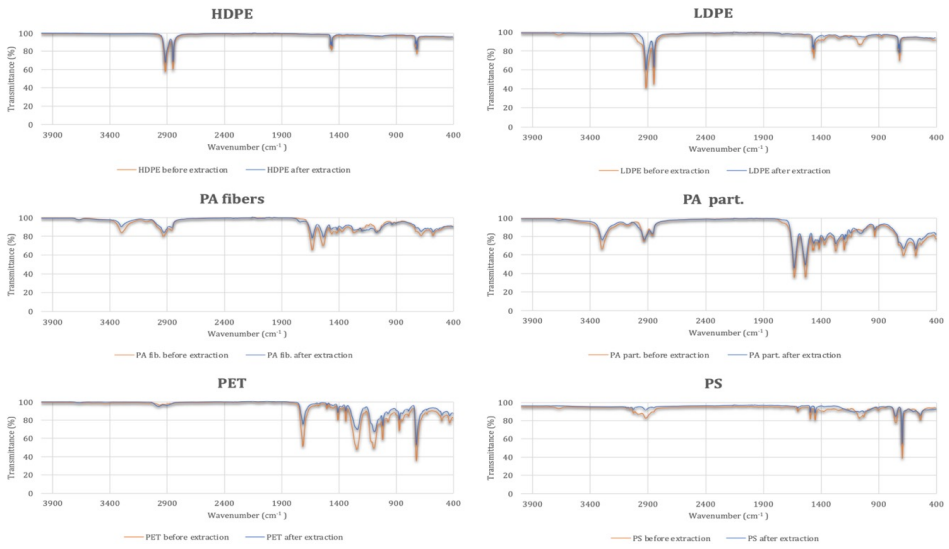


Fig. S3. FT-IR *spectra* of MPs before and after the optimized extraction protocol (24 h KOH digestion coupled with three Fenton reaction cycles).

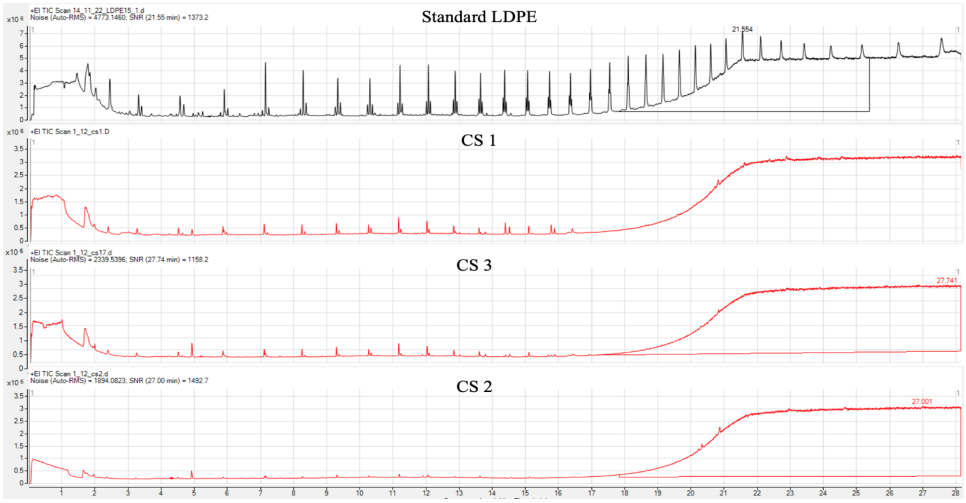


Fig. S4. Pyrograms obtained after Pyr-GC/MS analysis of virgin LDPE standard and LDPE microplastics detected in corn silage (CS) sample. Analytical conditions are reported in Section 2.4.1 of the Chapter 2.

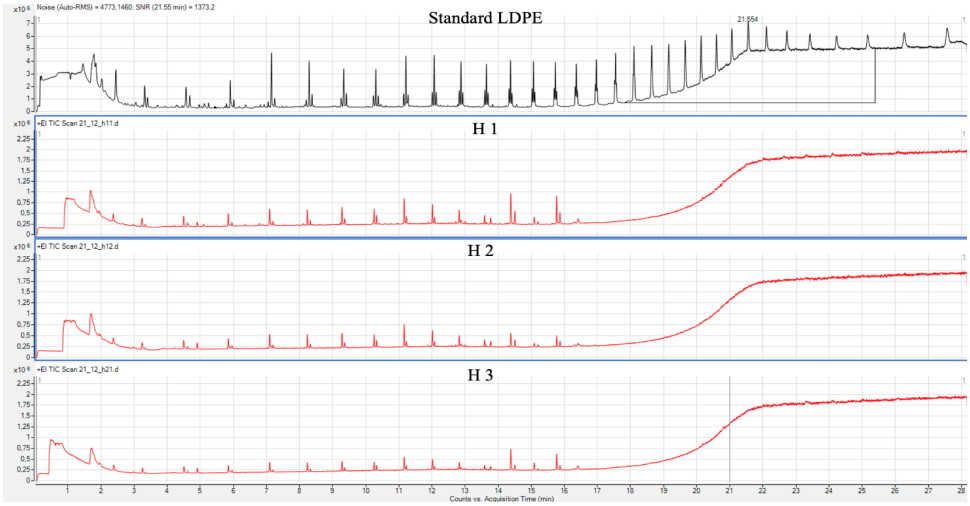


Fig. S5. Pyrograms obtained after Pyr-GC/MS analysis of virgin LDPE standard and LDPE microplastics detected in hay (H) sample. Analytical conditions are reported in Section 2.4.1 of the Chapter 2.

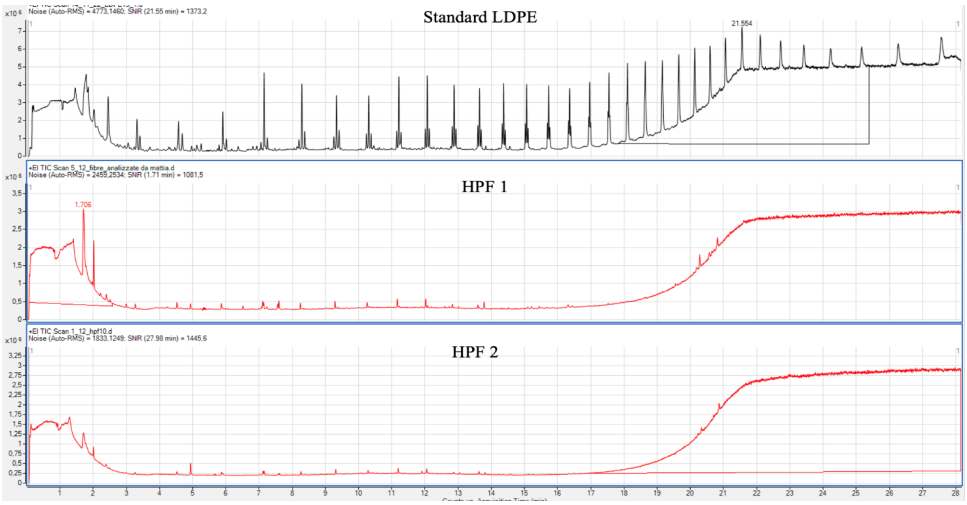


Fig. S6. Pyrograms obtained after Pyr-GC/MS analysis of virgin LDPE standard and LDPE microplastics detected in high protein feedstuff (HPF) sample. Analytical conditions are reported in Section 2.4.1 of the Chapter 2.

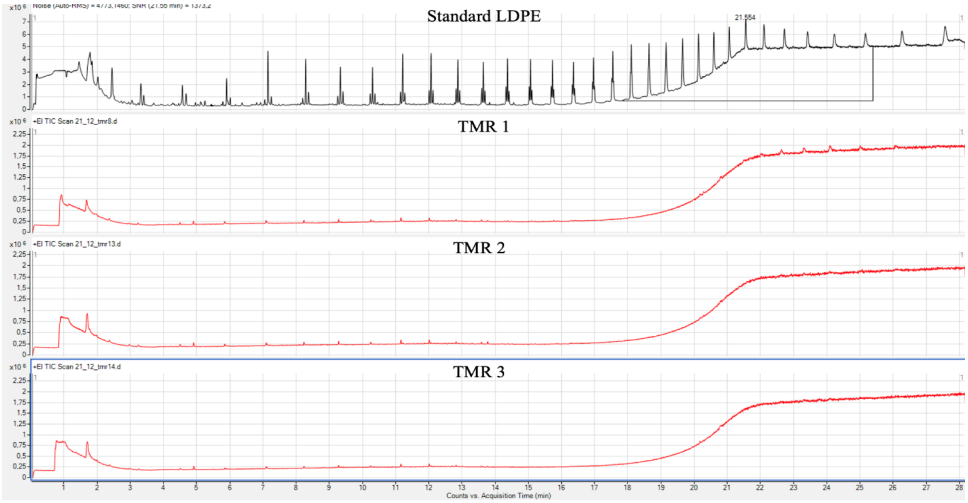


Fig. S7. Pyrograms obtained after Pyr-GC/MS analysis of virgin LDPE standard and LDPE microplastics detected in total mixed ratio (TMR) sample. Analytical conditions are reported in Section 2.4.1 of the Chapter 2.

Table S1
Number of MP pieces counted in 0.01 ± 0.005 g and added to each feed (1 ± 0.05 g). The mean and standard deviation refer to the replicates of all the samples and environmental controls (n = 14).

MP type	MP pieces counted in 0.01 ± 0.005g
HDPE	2.2 ± 0.43
LDPE	33.9 ± 6.76
PA fibers	125.9 ± 30.53
PA part.	3.2 ± 0.44
PET	8.3 ± 1.98
PS	48.1 ± 12.11

Table S2
Steps of the optimized MPs extraction protocol for ruminant feeds.

Step	Explanation	Equipment and reagents preparation
1.Feed sampling (samples collection and preparation)	Collect feed samples (CS, H, HPF and TMR) avoiding the use of plastic materials. Dry (at 60 °C overnight) and grind (< 5 mm sieve) them. After weight each feed directly in a 250 mL flask.	
2.KOH digestion	In each flask add 40 mL of KOH (10%) and a magnetic stir bar.	KOH solution (1.7 M – 10% W/W): weight 25 g of KOH

	Place the flasks on hot plate stirrer (60 °C and 300 rpm) for 24 h.	in a beaker and add 225 mL of milli-Q water. Place the beaker (with a magnetic stir bar) on hot plate stirrer (300 rpm) until the dissolution of KOH.
3.Filtration	Filter with a metallic sieve (mesh < 0.03 mm) the content of each flask and wash it several times with mill-Q water.	Attach the metal filter on to a glass funnel for vacuum filtration with a metal clamp.
4.First Fenton reaction	Place the sample remained on the filter in a flask using a metallic spatula (damages to the MPs were confirmed to be avoided during their transfer by visual observation and counting with stereomicroscope). In each flask add 20 mL of Fenton reagent, 20 mL of H_2O_2 (30%) and a magnetic stir bar. Place the flasks on hot plate stirrer (75 °C and 300 rpm)	Fenton reagent (500 mL): weight 7.5 g of $FeSO_4$ $7H_2O$ in beaker and add 300 mL of milli-Q water. Add 3 mL of H_2SO_4 and raise with milli-Q water to a final volume of 500 mL. Place the beaker (with a magnetic stir bar) on hot plate stirrer (300 rpm) until the complete dissolution.

	for 1 h. After 30 min add again 20 mL of H_2O_2 (30%).	
5.Filtration	As point 3	As point 3
Repeat points 4 and 5 (one time for H and TMR and two times for CS and HPF).		
6.MPs counting	After the last filtration, place the metallic filter with the remaining sample into a Petri dish. Dry it at 60° C. Transfer the dried sample from the metallic filter to another Petri dish using a metallic spatula to allow the stereomicroscope observation and counting (damages to the MPs were confirmed to be avoided during their transfer by visual observation and counting with stereomicroscope). For each MP take a picture, measure the length (< 5 mm), describe the shape and color.	
7.MPs identification	Choose a MP from the sample. Transfer it from Petri dish to a	

	pyrolizer Eco-cup. Do the Pyr-GC/MS. Analyze and compare the pyrograms and related mass *spectra* with a database of plastic pyrograms to identity the polymer.	

Chapter 3: Assessment of MP Contamination in Ruminant Feeds

Assessment of Microplastic Contamination in Ruminant Feeds of Northwestern Italy

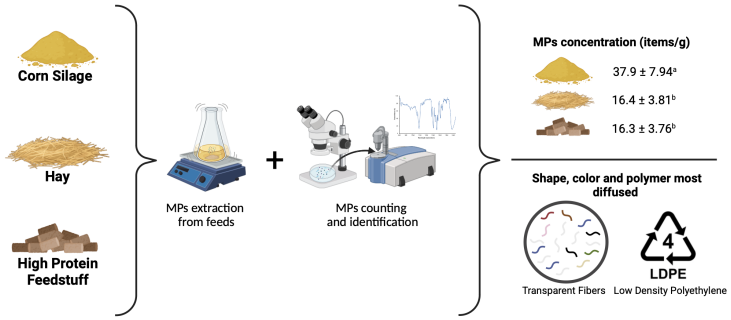
Prepared paper

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Graphical Abstract



Abstract

Ruminant feeds are a crucial link in microplastic (MP) contamination through the trophic chain, representing one of the potential primary sources of MPs exposure for ruminants. Plastic is widely used for storing feeds and in cultivating crops intended for animal consumption. Additionally, atmospheric agents can transport plastics and MPs onto crops and feeds. Despite this, most research to date has focused on the presence of MPs in ruminant bodies, their feces, and animal products, leaving a significant gap in understanding one of the possible sources of MPs in ruminants: their feeds. This study aimed to assess MP contamination in three ruminant feeds: corn silage (CSG), hay (HAY), and high protein feedstuff (HPF) of northwestern Italian farms. MPs were extracted through a specific protocol previously optimized for ruminant feeds. After the extraction, MPs were quantified and identified using a stereomicroscope and micro-Fourier transform infrared spectroscopy. CSG resulted to be the most significant polluted feed (37.9 ± 7.94 items/g), followed by HAY (16.4 ± 3.81 items/g) and HPF (16.3 ± 3.76 items/g). Transparent fiber-shaped MPs were the most abundant items in all feeds. MP lengths ranged from 0.030 to 5 mm and low-density polyethylene (LDPE) was the most common polymer in feeds, reflecting its extensive use in agricultural practices.

Highlights

- Application of a new MPs extraction protocol to ruminant feeds.
- Corn silage resulted to be the most polluted feed (37.9 ± 7.94 items/g).
- Hay: 16.4 ± 3.81 MPs items/g; high protein feedstuff: 16.3 ± 3.76 MPs items/g.
- Transparent fiber-shaped MPs were the most abundant items in all feeds.
- LDPE was the most spread polymer, reflecting its extensive use in agricultural.

1. Introduction

Microplastics (MPs), defined as plastic between 1 µm and 5 mm in size (Dong et al., 2023), are an alarming, but underestimated, concern in livestock sector (Khan et al., 2024; Ramachandraiah et al., 2022; Rodríguez-Seijo and Pereira, 2017). MPs have been detected in various body parts of ruminants, including blood (van der Veen et al., 2022), follicular fluid (Grechi et al., 2023), sperm (Grechi et al., 2024), feces (Beriot et al., 2021; Wu et al., 2024), as well as in animal products intended for human consumption, such as milk, meat, liver and tripe (Bahrani et al., 2024; Da Costa Filho et al., 2021; van der Veen et al., 2022; Visentin et al., 2024). This contamination poses a serious threat to both animal and human health (Corte Pause et al., 2024). The effects of MPs on living organisms are pronounced among various systems, including the digestive (Eichinger et al., 2024; Tassone et al., 2024b, 2025; Chang et al., 2024), reproductive (Grechi et al., 2024, 2023; Yang et al., 2023), and circulatory (Liang et al., 2024) ones. Moreover, MPs toxicity is amplified

by molecules that are adhered to them, such as persistent organic pollutants, heavy metals, pesticides, and additives used in the production of plastic as bisphenol A (Lackner and Branka, 2024; Urli et al., 2023).

Ruminants are exposed to MPs through three main intake sources: inhalation, drinking, and eating (Khan et al., 2024). Focusing on eating, MPs can be ingested by ruminants both indirectly, by eating plastic that is broken into MPs during digestion (Dong et al., 2023; Khan et al., 2024), and directly, by consuming MPs present in feeds (Glorio Patrucco et al., 2024b). In the former case, ruminants can ingest plastic debris that is not correctly disposed of. Plastics have been found in the rumen and reticulum of various ruminants (cattle, buffalos, sheep, goats, dromedary camels and dairy cows) (Corte Pause et al., 2024; Khan et al., 2024; Yemelyanenko et al., 2021). Approximately 72% of foreign bodies in the rumen of domestic cattle are plastic-based materials (65.5% plastic bags + 6.3% plastic ropes) (Mushonga et al., 2015). Plastic in the rumen can cause health issues such as ruminal impaction (Khan et al., 2024), a decreased ruminal motility (Akraiem and Abd Al-Galil, 2016), erosion and ulcerations of ruminal papillae (Otsyina et al., 2017). Plastic ingested, is fragmented into MPs during digestion and not all of them are excreted through feces (Dong et al., 2023; Khan et al., 2024). MPs with a diameter of less than 150 µm can cross the intestinal mucosal barrier, enter the bloodstream, and reach various organs and tissues (Khan et al., 2024). The second way of MPs ingestion by ruminants is through the consumption of feeds contaminated with these pollutants (Glorio Patrucco et al., 2024b; Khan et al., 2024). MPs pollution in feeds can originate from plastic used

to store them and to cultivate crops dedicated to animal nutrition (Urli et al., 2023). Plastic is intensively used to cover silages and to wrap hay bales and forages (Borreani and Tabacco, 2017; Urli et al., 2023). The plastic films that are utilized to cover bunker silages represent from 33% to 45% of the entire amount of plastic purchased by dairy farmers for feed conservation, depending on herd size and are mainly transparent and black (Borreani and Tabacco, 2017). In a survey conducted in Northern Italy, about 10.3 kg of plastic per cow was shown to be used each year, of which 3.9 kg per cow were specifically used for covering bunker silages (Borreani and Tabacco, 2015). MPs in feeds can derive from plastic applied for cultivation of feed crops as mulch, irrigation, and plastic waste not properly disposed of (Urli et al., 2023). Plastics from feed storage and crop cultivation undergo fragmentation due to physical and chemical processes, including ultraviolet photodegradation, mechanical abrasion, oxidative degradation, and abiotic weathering processes (Klimasz and Grobelak, 2024). These actions generate MPs, which can be accumulated in feeds (Khan et al., 2024). Furthermore, atmospheric agents like wind, rainfall, and snowfall carry MPs from urban and industrial areas to agricultural and remote rural regions. This movement spreads MPs widely, increasing their presence in livestock farms and their feeds (Ramachandraiah et al., 2022; Roy et al., 2023).

Despite MPs in feeds, especially, in ruminant feeds, being a crucial link in MP contamination through the trophic chain, to date the only studies that investigated MPs in ruminant feeds were the ones of van der Veen et al. (2022), Maganti and Akkina (2023) and Wu et. (2021). Moreover, to the

best of our knowledge, our team was the first one that optimized a protocol to specifically extract MPs from ruminant feeds (Glorio Patrucco et al., 2024b).

Therefore, we hypothesized that applying our optimized MP extraction protocol for ruminant feeds (Glorio Patrucco et al., 2024b) would allow for the detection of MPs in ruminants feeds and reveal variations in contamination levels across different feed types.

The aim of this work was to apply this protocol to study MP contamination in different ruminant feeds, providing a preliminary monitoring of the level and types of contamination posed by this emerging pollutant in livestock systems.

2. Materials and Methods

2.1 Feed Samples

Plastic materials were avoided during sampling procedures with all clothing including lab coats and gloves made of cotton to minimize contamination. The analyzed feeds represented the three most used feeds in dairy cow diets in northwestern Italy: corn silage (CSG), mixed hay (HAY), and high protein feedstuff (HPF). Samples were collected in the autumn season from three dairy farms in Piedmont, Italy. CSG and HPF were sampled using a stainless-steel trowel which was thoroughly cleaned with disposable paper sheets between samples to prevent cross-contamination. HAY samples were collected by hand, using cotton gloves that were carefully cleaned in the same way as stainless-steel trowel. Approximately 500 g of each feed in each farm was collected from random

points within the storage area. Since three types of feed were collected from each of three farms, a total of nine feed samples were obtained. CSG, HAY and HPF samples were stored in natural fiber bags. Each bag was labelled with the sample name and farm, tied with a cotton string, and immediately transported to the laboratory for analysis.

Feed samples were dried at 60 °C for 24 h, ground to a particle size of 5 mm using a granulator (MLI 204; Bühler AG, Uzwil, Switzerland), and stored in glass bottles until further use. Chemical compositions of CSG, HAY and HPF were analyzed by measuring: dry matter (DM, #930.15), ash (#942.05), ether extract (EE, #942.05) and crude protein (CP, #984.13) according to AOAC methods (2000). Fiber fractions, including neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL), were determined using an Ankom 200 Fiber Analyzer (Ankom Technology, Macedon, NY, USA) according to the procedures of Van Soest et al. (1991). The chemical composition of CSG, HAY and HPF is reported in Table 1.

Table 1
Chemical composition of corn silage (CSG), mixed hay (HAY) and high protein feedstuff (HPF) collected in the three farms expressed in mg/g of dry matter (DM) (Mean ± SD, N = 3).

Chemical composition	CSG	HAY	HPF
DM[1]	254 ± 12.3	896 ± 23.1	887 ± 4.7
Ash	53 ± 5.8	97 ± 3.7	94 ± 26.0
EE	28 ± 4.1	14 ± 4.3	25 ± 13.2

CP	98 ± 22.6	115 ± 15.2	387 ± 170.2
NDF	500 ± 69.6	700 ± 17.8	223 ± 116.3
ADF	252 ± 45.4	386 ± 10.4	94 ± 33.1
ADL	38 ± 8.4	51 ± 12.9	20 ± 14.4

EE = Ether Extract; CP = Crude Protein; NDF = Neutral Detergent Fiber; ADF = Acid Detergent Fiber; ADL = Acid Detergent Lignin
[1](mg/g of fresh matter)

2.2 MP Detection and Identification

2.2.1 MP Extraction Reagents

Throughout this work, ultrapure water (18.2 MΩcm at 25 °C) obtained with a Milli-Q Academic system (Millipore, Billerica, MA, USA) was used. In addition, the following reagents were applied: 30% hydrogen peroxide (H_2O_2) (VWR Chemicals, Milan, Italy); iron sulphate heptahydrate ($FeSO_4$ $7H_2O$) (VWR Chemicals, Milan, Italy); 95-97% sulfuric acid (H_2SO_4) (Honeywell, Charlotte, North Carolina, USA) and potassium hydroxide (KOH) (Honeywell, Charlotte, North Carolina, USA).

2.2.2 MP Background Contamination: Quality Assurance

Contamination from laboratory tools or airborne particles during sample handling is a key source of background interference in MP research. Presently, there are no universally accepted protocols to mitigate background contamination in MP studies. In this study, the following preventive measures were taken:

i) when possible, avoiding the use of plastic materials;

ii) the operators using cotton clothes, coats and nitril gloves;
iii) preventing equipment contamination by thoroughly rinsing all glassware and containers with 0.45 µm filtered ultrapure water prior to use and covering them with aluminum foil;
iv) conducting extraction processes within a chemical hood to limit exposure to unfiltered air;
v) running two blanks (no feed sample, only reagents) in parallel with each extraction to monitor contamination and subtracting the MPs found in blanks from the sample results.

2.2.3 MP Extraction from Ruminant Feeds

To extract MPs from CSG, HAY and HPF, the extraction protocol described by Glorio Patrucco et al. (2024b), specifically optimized for ruminant feeds, was followed. In brief, 1 g for each dried feed was weighted directly in 250 mL glass flasks. To ensure representativeness, the feed was collected by thoroughly mixing the contents of the storage bottle and sampling from multiple points. Each sample underwent multiple steps of organic degradation: KOH (10% w/w) digestion (40 mL at 60 °C for 24 h at 300 rpm) and Fenton reactions (20 mL of 0.05 M Fe^{2+} solution at 75 °C for 1 h at 300 rpm and two subsequent aliquots of 20 mL of H_2O_2 (30%) added at time 0 and 30 min) repeated twice for HAY and three times for CSG and HPF. Each feed from each farm was extracted in triplicate, resulting in a total of nine replicates per feed (N = 9).

2.2.4 MP Quantification and Identification

After the extraction, MPs from each feed were quantified by visual observation using a Nikon H550S (Tokyo, Japan) stereomicroscope

equipped with Micro Capture Ver6.9.12 software (DIV = 0.01 mm), expressing the results as MP items/g DM of feed sample (N = 9). MPs were categorized according to morphology, color and size (longest axis) (Lusher et al., 2020; Markley et al., 2024). Once classified, one replicate for each farm was further analyzed through micro-Fourier transform infrared (μ-FTIR) spectroscopy (Perkin Elmer Spectrum Two μ-FTIR ATR spectrometer - wavenumber range of 4000–400 cm^{-1}, 32 scans per *spectrum*, spectral resolution of 4 cm^{-1}) to confirm their nature and to identify polymer composition (N = 3). Perkin Elmer Spectrum software and database were used to identify polymer composition.

2.3 Statistical Analysis

Data analysis was performed using R statistical software (version 4.2.2) along with RStudio (version: 2022.07.2 + 576, Boston, USA). Statistical tests were performed to evaluate the significance of MP concentrations (across CSG, HAY, HPF), shape (fibers *vs* particles), lengths (across CSG, HAY, HPF) and polymer types (across polymers found in each feed) applying a linear model using the “lm” function from the “stats” package (version 4.2.2). Analysis of variance (ANOVA), also from the “stats” package, was used to further examine the significance (threshold of p-value < 0.05). Post-hoc analyses were conducted with the “emmeans” package (version 1.8.3), and pairwise comparisons were performed using Bonferroni adjustments to account for multiple comparisons. Results were expressed as means ± standard deviation (SD). All data visualizations were created using the “ggplot2” package (version 3.5.1).

3. Results

Fig. 1 shows the concentration of MP items in the analyzed feed samples, along with their color distribution. In 100% of feeds analyzed MPs were detected with CSG exhibited the significantly highest concentration of MPs (37.9 ± 7.94 MP items/g DM). In contrast, HAY and HPF contained approximately less than half of MPs found in CSG. No significant differences were observed between these two feeds (HAY 16.4 ± 3.81 MP items/g DM; HPF 16.3 ± 3.76 MP items/g DM).

In terms of color distribution, transparent MPs were the most abundant in all samples, followed by blue, black, red and brown. Notably, green MPs were found exclusively in HAY and HPF, while yellow and pink MPs were the rarest, appearing only in HAY.

In Table 2 the concentration of MPs in feeds classified according to their shape are reported. Fibers were the predominant shape in all feed samples. Particles were present at very low levels.

The detected MPs were further categorized based on their length, as shown in Fig. 2. No statistical differences were detected among CSG, HAY and HPF. Despite that, as shown by the shape of the violin plots, the distribution of MP lengths varied. The widest range was detected in CSG (from 0.039 to 5 mm), followed by HPF (from 0.030 to 4 mm) and HAY (from 0.059 to 3 mm).

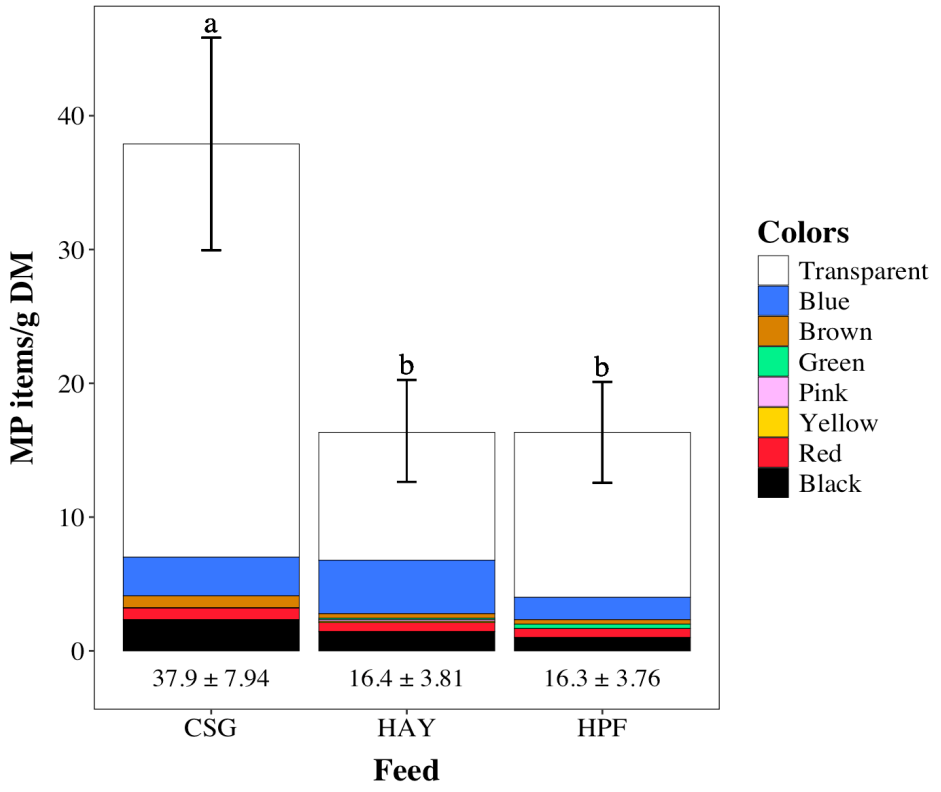


Fig. 1. Concentration of microplastics (MPs) in the feed samples expressed as items/g DM and distribution of MP colors in each feed. Results are expressed as mean ± SD (one-way analysis of variance followed by Bonferroni's multiple comparison test, different superscripts (a–b) indicate significant differences, N = 9, p-value <0.001). CSG = corn silage; HAY = mixed hay; HPF = high protein feedstuff.

Table 2
Concentration of microplastics in feeds divided according to their shape (Fiber items/g DM and Particle items/g DM). Results are expressed as mean ± SD (One-way analysis of variance followed by Bonferroni's multiple comparison test, different superscripts (a–b) indicate significant differences inside each row, N = 9).

Feed	Fiber items/g DM	Particle items/g DM	p-value
CSG	37.6 ± 8.00[a]	0.3 ± 0.70[b]	<0.0001
HAY	16.2 ± 3.63[a]	0.2 ± 0.67[b]	<0.0001
HPF	16.0 ± 3.46[a]	0.3 ± 0.50[b]	<0.0001

CSG = corn silage; HAY = mixed hay; HPF = high protein feedstuff

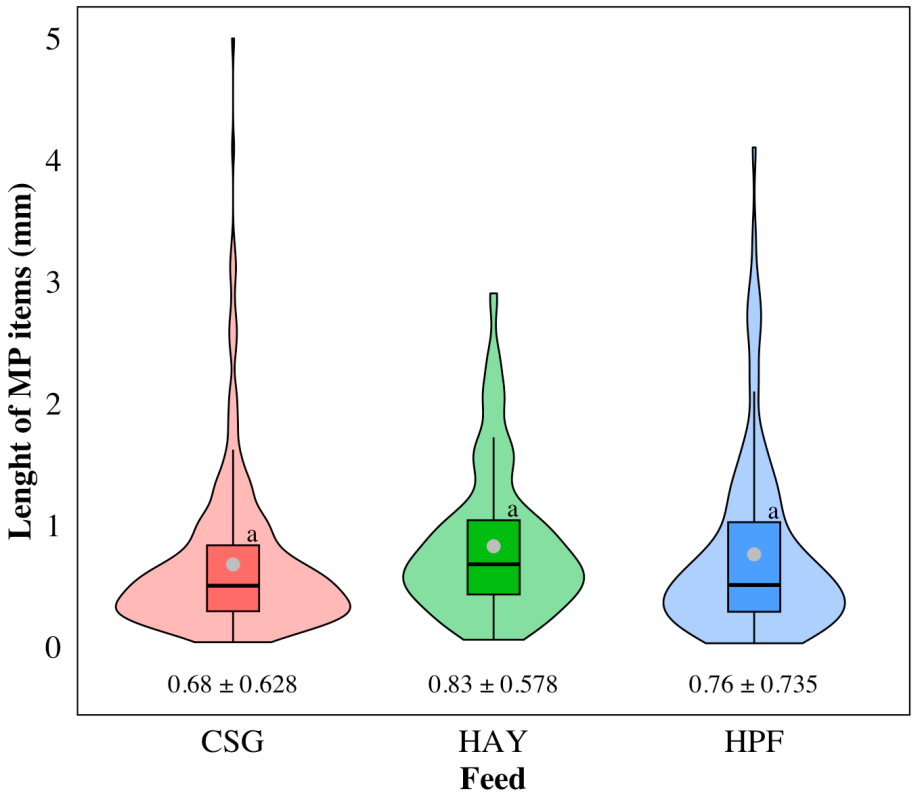


Fig. 2. Box plot combined with violine plot of length of MP items found in feed samples expressed in mm. The horizontal line represents the median value of data (Q50), the edges of the boxes are the quartiles (Q25 and Q75), the white circle is the mean, the lower and upper whiskers are the minimum and maximum data values (excluding outliers) and the violine plot indicates the distribution of the data (One-way analysis of variance followed by Bonferroni's multiple comparison test, N = 9, p-value = 0.052). The values above the x axis are the mean ± SD. CSG = corn silage; HAY = mixed hay; HPF = high protein feedstuff.

Table 3 illustrates the polymer percentages found in the three feeds. Three polymers were found in all the investigated feeds: LDPE, high-density polyethylene (HDPE) and PA.

Table 3
Microplastic polymers found in the three feeds expressed in percentages (mean ± SD) (One-way analysis of variance followed by Bonferroni's multiple comparison test, different superscripts (a–c) indicate significant differences inside each row, N = 3). The last column reports the polymer distribution inside the same feed as pie charts.

Feed	LDPE	HDPE	PA	p-value	HDPE LDPE PA
CSG	88 ± 5.2^{a}	10 ± 6.6^{b}	2 ± 1.6^{b}	<0.0001	
HAY	73 ± 10.3^{a}	25 ± 7.1^{b}	2 ± 3.2^{c}	<0.0001	
HPF	74 ± 30.8^{a}	18 ± 17.6^{ab}	8 ± 13.3^{b}	0.0209	

CSG = corn silage; HAY = mixed hay; HPF = high protein feedstuff; LDPE = low-density polyethylene; HDPE = high-density polyethylene; PA = polyamide

LDPE was the predominant polymer in all the feeds, accounting for 88% in CSG, 73% in HAY, and 74% in HPF. In CSG, both HDPE and PA occurred in lower proportions compared to LDPE, with no significant difference between them. In HAY, HDPE levels were lower than LDPE but higher than PA. For HPF, although the mean percentage of LDPE exceeded that of HDPE, a high standard deviation rendered the difference statistically insignificant, while PA appeared in smaller amounts than LDPE and was comparable to HDPE. Three examples of micro-FT-IR *spectra* for each polymer in each feed are reported in Figures S1-S3 of the Supplementary Material.

Fig. 3 reported three pictures of MPs for each feed analyzed. Their micro-FT-IR *spectra* are reported in Figures S1-S3 of the Supplementary Material.

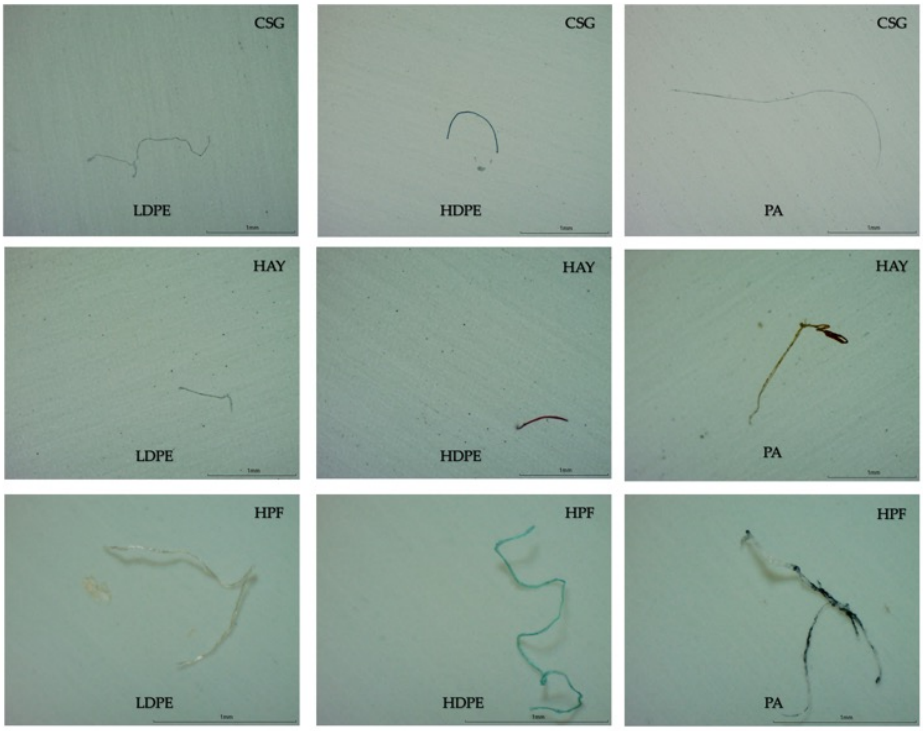


Fig. 3. Examples of microplastics found in the analyzed feeds captured by a stereomicroscope. CSG = corn silage; HAY = mixed hay; HPF = high protein feedstuff; LDPE = low-density polyethylene; HDPE = high-density polyethylene; PA = polyamide.

4. Discussion

Ruminant feeds are a crucial link in the contamination of MPs through the food chain. Despite this growing concern, research on MP pollution in livestock feeds remains limited (Maganti and Akkina, 2023; van der Veen et al., 2022; Wu et al., 2021). Previous studies on livestock focused on MPs presence in their bodies, excreta and food products (*e.g.* milk and meat), leaving a significant gap in understanding how MPs enter in their bodies (Bahrani et al., 2024; Beriot et al., 2021; Da Costa Filho et al., 2021; van

der Veen et al., 2022; Visentin et al., 2024; Wu et al., 2024). Our study is among the first to monitor MP contamination in three dairy cow feeds.

4.1 MP Concentration in Ruminant Feeds

MPs were detected in all three analyzed feeds, from a minimum of 16 items/g DM (HAY and HPF) to a maximum of 38 items/g DM (CSG). This aligns with our previous work, in which the concentrations of MPs ranging from 8 items/g DM (HPF) to 39 items/g DM (HAY) were reported (Glorio Patrucco et al., 2024b). Accordingly, in the work of Maganti and Akina (2023), all cow feeds proved to be polluted by MPs (from 336 µg/g to 1057 µg/g). Similarly, in the pilot study of van der Veen et al. (2022), the majority of cow feeds (except for fresh feed roughage) were contaminated by MPs (<14 to ≥ 2600 µg/g). In contrast, Wu et al. (2021) found that only the feed from one farm out of three tested positive for MPs (1.10 x 10^2 items/kg, corresponding to 0.11 items/g). In both the studies by Maganti and Akina (2023) and van der Veen et al. (2022), results are presented in µg/g, while in our study as items/g DM. This difference arises from the distinct quantification methods used. Their approach employed pyrolysis-gas chromatography-mass spectrometry (Pyr-GC-MS), yielding results in w/w, but being destructive, thus precluding visual observation and classification. In our study, we used stereomicroscope and micro-FT-IR spectroscopy giving additional important qualitative information: shape, length, color of MPs. The lower concentration of MPs found by Wu et al. (2021) compared to our study could be due to differences in extraction methods (density separation *vs*

organic matter degradation) and the type of feed analyzed (not declared *vs* CSG, HAY and HPF).

Expanding the discussion to other animal feeds, Jeyasanta et al. (2024) investigated MPs in feeds of chickens, fishes and shrimps, concluding that the potential exposure for chicken was the highest one ranging from 3 to 434 items/g. Wu et al. (2021) studied also MPs presence in pig feed, detecting 3.33×10^2 items/kg (corresponding to 0.33 items/g). MPs were found also in pet food (up to 12 µg/g and 4.6 µg/g in cat and dog foods, respectively) (Zhang et al., 2019).

Comparing MP contamination in ruminant feed with other ruminant matrices, MPs had a wide range of concentrations due to the different nature of the matrices, extraction and identification methods. In ruminant products for human consumption, Bahrani et al. (2024) found 0.19, 0.14, and 0.15 items/g in cow meat, liver, and tripe respectively while Visentin et al. (2024) measured up to 30 items/g in beef hamburgers. Moreover, Da Costa Filho et al. (2021) detected from 204 to 1004 items/100 mL in cow milk. In ruminant feces, Beriot et al. (2021) found a mean of 1 item/g, while Wu et al. (2024) a mean of 21 items/g. Inside ruminant body, various MP concentrations were demonstrated, ranging from 0.8 to 6.1 µg/g of cow blood (van der Veen et al., 2022), 38.6 items/mL of bovine follicular fluid and 72.5 items/mL of bovine epididymal sperm (Grechi et al., 2024, 2023).

In our study, CSG proved to be the most contaminated feed compared to HAY and HPF. The higher pollution in CSG could be ascribed to the way of conservation of this feed since its fermentation and storage are taken

place under multiple layers of plastic that are subjected to atmospheric fragmentation (Astner et al., 2019; Briassoulis, 2023; Ramachandran et al., 2021). Also, Maganti and Akina (2023) found that the diet in which one of the main ingredients was corn silage (mix of corn silage, crushed maize, wheat bran, minerals) had the highest presence of MPs (up to 1057 µg/g).

4.2 MP Color Distribution in Ruminant Feeds

The color of MPs is a useful parameter for tracing their origin, helping to identify the type of plastic products they may have derived from, as well as detecting potential contamination during sample preparation and extraction (Zhang et al., 2020). Transparent MPs were the most prevalent in all the feed samples analyzed, followed by blue, black, red, brown, green, yellow and pink. It was also reported that the dominant color was transparent (up to 45%) in fish and farmed feeds analyzed by Jeyasanta et al. (2024). In general, transparent MPs are the most common colors found across different matrices and environments (Ibrahim et al., 2021; Manbohi et al., 2023; Radhakrishnan et al., 2024). In contrast, Wu et al. (2021) found only green MPs in cow feed, while red was the predominant color in pig feed followed by white, blue and green. Considering other ruminant matrices, in the work of Bahari et al. (2024) the most abundant colors were transparent and black in ruminant liver, meat and tripe, followed by orange, blue and red while in the work of Visentin et al. (2024) on beef hamburgers grey > brown > transparent > green > black > blue > yellow.

4.3 MP Shape and Length in Ruminant Feeds

Shape and length are two important parameters to consider when assessing MPs. The shape of MPs can help identify their sources, and studies are ongoing to better understand shape-dependent effects on toxicity (Bostan et al., 2023; Osman et al., 2023). Length also plays a critical role in determining their biological fate: MPs longer than 150 µm are more likely to be excreted, while those shorter than 150 µm can cross the intestinal mucosal barrier, enter the bloodstream, and reach organs (Khan et al., 2024). Regarding the shape, fibers were the most commonly detected in all the feeds studied, as reported also by our previous study Glorio Patrucco et al. (2024b), by Jeyasanta et al. (2024) in all feeds analyzed (up to 93%) and by Wu et al. (2021) in cow and pig feeds (100%). In addition, in other ruminant matrices as edible tissues, fibers were the predominate MPs (Bahrani et al., 2024). As for transparent color, fiber is the most common shape for MPs across different matrices and environments (Ibrahim et al., 2021; Manbohi et al., 2023; Radhakrishnan et al., 2024).

MP length ranged from a minimum of 0.03 mm (HPF) to a maximum of 5 mm (CSG), demonstrating a wide length range of distribution. The mean values were 0.68 mm for CSG, 0.83 mm for HAY and 0.76 mm for HPF, with no statistical differences across feeds. In our previous work, the length ranged from 0.06 mm to 4 mm and the mean length ranged from 0.61 mm to 0.91 mm (Glorio Patrucco et al., 2024b). In the work of Jeyasanta et al. (2024), the mean size of fish feeds was 0.72 mm, while the dominant particle size of MPs was 0.10-0.50 mm for farmed ones. Moving to other

ruminant matrices the highest numbers of MPs were observed in the size ranges of 0.50-1 mm, in the liver, meat, and tripe of cows, while, the most abundant MPs were found within the size ranges of 0.10-0.50 mm in the same edible tissues of sheep (Bahrani et al., 2024). The size range of MPs observed was between 0.03 and 3 mm in beef hamburgers, with an overall average of 0.12 mm (Visentin et al., 2024).

4.4 MP Polymers in Ruminant Feeds

As previously mentioned, in each feed the most abundant polymer was LDPE. LDPE is the main polymer applied in farms, in particular to store feeds as silage bags, bale wraps, plastic bunker silo covers and the net that is used to tie bales (Borreani and Tabacco, 2017; Ramachandran et al., 2021). Moreover, LDPE is the most common plastic for mulching in agriculture systems (Sarkar et al., 2018). LDPE together with polypropylene (PP) are the most produced and wasted plastics globally (Geyer, 2020). LDPE MPs proved to significantly alter rumen fermentation dynamics, rumen protozoal populations and ammonia-nitrogen levels (Tassone et al., 2025), while feed degradability was not affected (Glorio Patrucco et al., 2024a).

The other polymers found in our study were HDPE and PA. HDPE is primarily used for containers that hold liquid fertilizers, agrochemicals, and veterinary medicines, as well as for netting used to tie bales. PA is occasionally constructed with LDPE for the application previously described for this polymer (Borreani and Tabacco, 2017). Also, PA demonstrated to affect rumen fermentation reducing feed degradability, microbial efficiency, rumen protozoal population and increasing total gas

production (Tassone et al., 2024a), while for HDPE, there are currently no existing studies on its effects on ruminants, but proved to affect nutritional status, nutrient metabolism, fish health, and gut microbial community of juvenile yellow perch (*Perca flavescens*) (Lu et al., 2022).

Other works targeting feeds also stated LDPE as a main polymer (Glorio Patrucco et al., 2024b; Jeyasanta et al., 2024; Wu et al., 2021). In contrast, the most abundant polymer in cow feeds was polyvinyl chloride (PVC) in the works of Maganti and Akkina (2023) and van der Veen (2022). PVC is used as an alternative of LDPE in mulching and is the material of water pipes, including those used to irrigate forage crops (Ramachandran et al., 2021; Sheriff et al., 2023; Wu et al., 2021). In other ruminant matrices the main polymer found was PA in ruminant edible tissues (Bahrani et al., 2024); polycarbonate (PC) in beef hamburgers (Visentin et al., 2024); PE in milk (Da Costa Filho et al., 2021); PVC in cow blood (van der Veen et al., 2022) and PP in bovine epididymal sperm (Grechi et al., 2024).

5. Conclusions

This monitoring study demonstrated that 100% of the ruminant feeds analyzed were contaminated with MPs, confirming that feeds are a significant pathway for animal contamination, which poses a risk of transferring MPs throughout the food chain.

Corn silage showed the highest pollution of MPs. Transparent fiber-shaped MPs were the most common, with lengths ranging from 0.03 to 5 mm. LDPE was the most abundant polymer, reflecting its extensive use in agricultural practices.

Future research should focus on conducting large-scale, geographically diverse surveys across a wide variety of farm types worldwide to develop a more comprehensive understanding of MP contamination in ruminant feeds. It should also aim to identify the sources of MP pollution, helping to determine key contributors and pathways of contamination. Expanding the scope of these investigations to include additional types of ruminant feeds, as well as feeds for monogastric animals, would further enhance insights into the extent and impact of MP pollution across the livestock sector.

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SUPPLEMENTARY MATERIAL

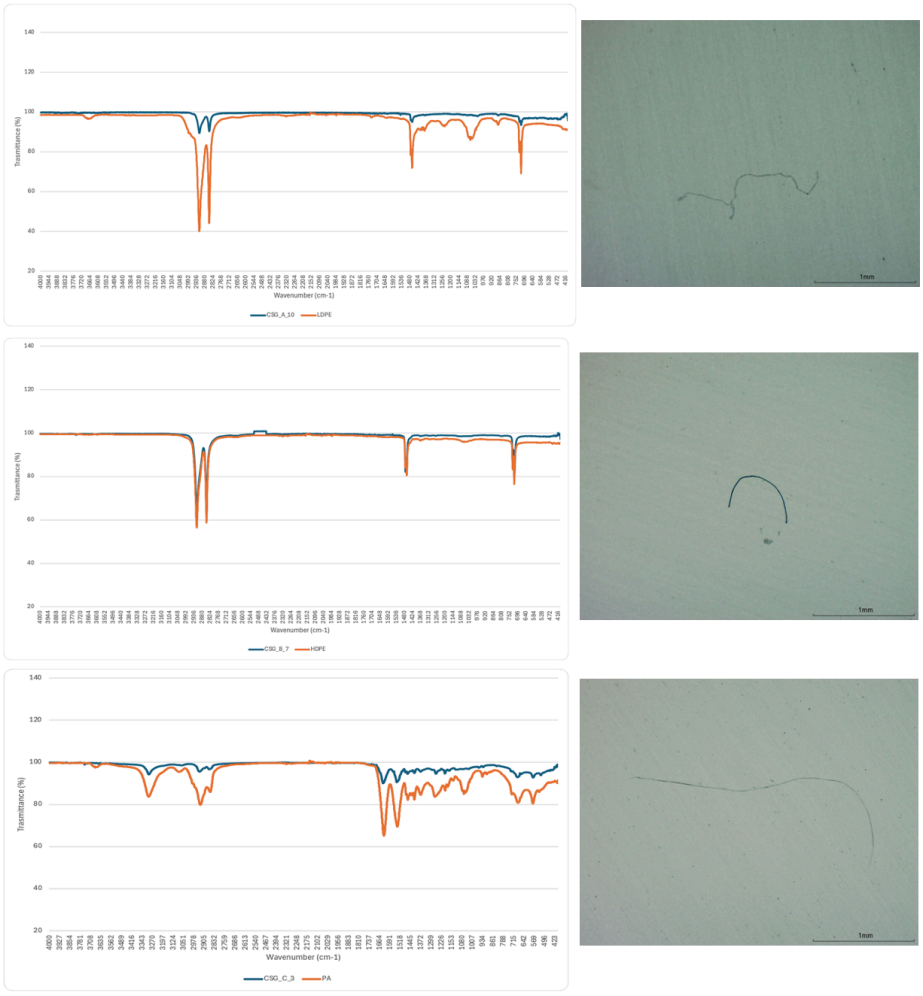


Fig. S1. One example of micro-FT-IR *spectra* for each polymer found in corn silage. Blue line MP detected; orange line virgin MP. LDPE = low-density polyethylene; HDPE = high-density polyethylene; PA = polyamide.

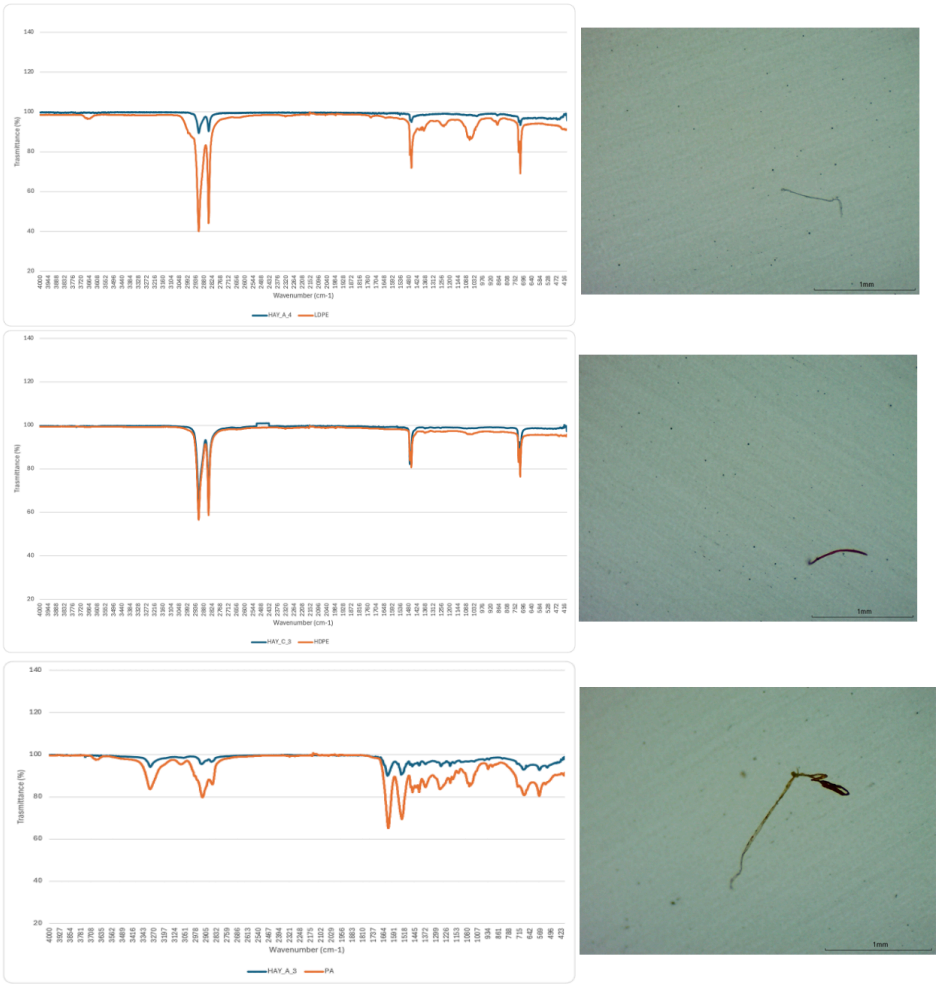


Fig. S2. One example of micro-FT-IR *spectra* for each polymer found in hay. Blue line MP detected; orange line virgin MP. LDPE = low-density polyethylene; HDPE = high-density polyethylene; PA = polyamide.

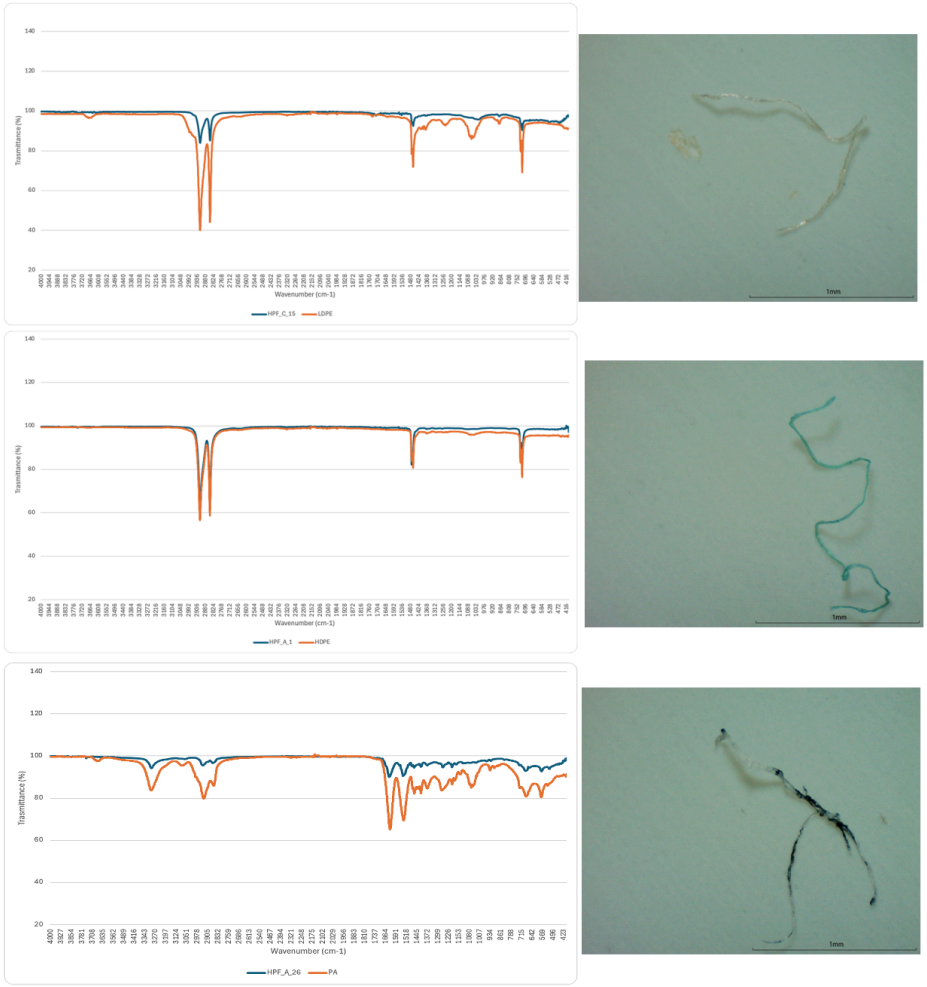


Fig. S3. One example of micro-FT-IR *spectra* for each polymer found in high protein feedstuff. Blue line MP detected; orange line virgin MP. LDPE = low-density polyethylene; HDPE = high-density polyethylene; PA = polyamide.

Chapter 4: Investigation of MP Effects on Ruminant Digestion

Published paper on Animals (MDPI)

https://doi.org/10.3390/ani14152139

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First Evidence of the Effects of Polyethylene Terephthalate Microplastics on Ruminal Degradability and Gastro-Intestinal Digestibility of Mixed Hay

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Simple Summary: Simple Summary: Microplastics have emerged as a pressing global environmental issue and have been alarmingly detected in both the feed and feces of ruminants. However, their effects on the ruminal and intestinal digestive tract have not yet been studied. Our study represents the first investigation of the effects of polyethylene terephthalate microplastics on the ability of the ruminal-gastro-intestinal system to degrade and digest mixed hay. Our findings reveal that polyethylene terephthalate significantly reduce the digestibility of crude protein in mixed hay. Notably, low levels of PET MPs negatively impacted the intestinal phase, while medium and high levels disrupted the ruminal phase. Moreover, medium and high concentrations of polyethylene terephthalate hindered the degradation of fiber fractions, specifically of neutral detergent fiber. These insights underscore the potential risks posed by polyethylene terephthalate microplastics to ruminal–gastro-intestinal functionality and highlight the urgent need for innovative strategies to combat this emerging environmental challenge, ensuring the sustainability and productivity of ruminant farming.

Abstract: Microplastics (MPs) raise environmental concerns. However, their effects on the ruminal–gastro-intestinal system have not yet been studied. This study aims to investigate the effects of polyethylene terephthalate (PET) MPs on the ability of the ruminal–gastro-intestinal system to degrade and digest mixed hay. Using a three-step in vitro ruminal–gastro-intestinal incubation system, PET MPs were introduced at concentrations of 0, 5, 10, and 15 g/L in ruminal and gastro-intestinal solutions. Ruminal fluid was collected from three 16-month-old Piedmontese bulls. The experiment was conducted on three mixed hays and was repeated three times, with triplicate incubations in each run. The results reveal that PET MPs reduced the degradability and digestibility of crude protein. Specifically, crude protein degradation was reduced by 9% at medium and 16% at high PET MP concentrations in the ruminal phase, while the crude protein digestibility of undegraded crude protein was reduced by 8% at the lowest PET MPs concentration in the gastro-intestinal tract. Additionally, PET MPs reduced the degradation of neutral detergent fiber at medium and high PET MP concentrations in the ruminal phase by 9% and 13%, respectively. These results highlight the risks of PET MPs contamination on ruminal–gastro-intestinal functions and underscore the urgent need to mitigate MPs contamination in the livestock sector.

Keywords: microplastics; polyethylene terephthalate; ruminal degradability; gastro-intestinal digestibility; mixed hay

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Citation: Tassone, S.; Barbera, S.; Kaihara, H.; Glorio Patrucco, S.; Abid, K. First Evidence of the Effects of Polyethylene Terephthalate Microplastics on Ruminal Degradability and Gastro-Intestinal Digestibility of Mixed Hay. *Animals* **2024**, *14*, 2139. https://doi.org/10.3390/ani14152139

Academic Editor: Alejandro Plascencia

Received: 28 June 2024
Revised: 18 July 2024
Accepted: 20 July 2024
Published: 23 July 2024

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1. Introduction

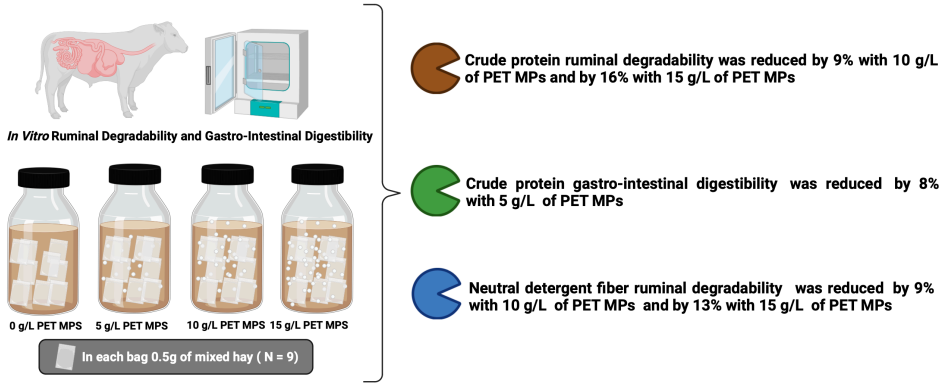
Microplastics (MPs), defined as plastic debris between 1 µm and 5 mm in size, have emerged as a global environmental problem affecting both marine and terrestrial ecosystems [1]. In terrestrial environments, the presence of MPs in soil contributes to the destruction of soil structure and microbial communities, which in turn leads to nutrient depletion

First Evidence of the Effects of Polyethylene Terephthalate Microplastics on Ruminal Degradability and Gastro-Intestinal Digestibility of Mixed Hay

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Graphical Abstract



Abstract

Microplastics (MPs) raise environmental concerns. However, their effects on the ruminal-gastro-intestinal system have not yet been studied. This study aims to investigate the effects of polyethylene terephthalate (PET) MPs on the ability of the ruminal-gastro-intestinal system to degrade and digest mixed hay. Using a three-step *in vitro* ruminal-gastro-intestinal incubation system, PET MPs were introduced at concentrations of 0, 5, 10, and 15 g/L in ruminal and gastro-intestinal solutions. Ruminal fluid was

collected from three 16-month-old Piedmontese bulls. The experiment was conducted on three mixed hays and was repeated three times, with triplicate incubations in each run. The results reveal that PET MPs reduced the degradability and digestibility of crude protein. Specifically, crude protein degradation was reduced by 9% at medium and 16% at high PET MP concentrations in the ruminal phase, while the crude protein digestibility of undegraded crude protein was reduced by 8% at the lowest PET MPs concentration in the gastro-intestinal tract. Additionally, PET MPs reduced the degradation of neutral detergent fiber at medium and high PET MP concentrations in the ruminal phase by 9% and 13%, respectively. These results highlight the risks of PET MP contamination on ruminal-gastro-intestinal functions and underscore the urgent need to mitigate MP contamination in the livestock sector.

Highlights

- First study on PET MPs effects on hay ruminal-gastro-intestinal degradability and digestibility.
- PET MPs reduced crude protein degradation by up to 16% in the ruminal phase.
- PET MPs decreased crude protein digestibility by 8% in the gastro-intestinal phase.
- Neutral detergent fiber degradation declines by up to 13% with PET MPs exposure.

1. Introduction

Microplastics (MPs), defined as plastic debris between 1 µm and 5 mm in size, have emerged as a global environmental problem affecting both marine and terrestrial ecosystems (Ajithkumar et al., 2025). In terrestrial environments, the presence of MPs in soil contributes to the destruction of soil structure and microbial communities, which in turn leads to nutrient depletion and reduces plant growth and crop production (Ajithkumar et al., 2025). When these MPs enter the bodies of animals and humans, they can cause physical harm, oxidative stress, cell death, cytotoxicity, neurotoxicity, reproductive toxicity, and immune system disruption (Ajithkumar et al., 2025; Bostan et al., 2023). Consequently, MPs have the potential to disrupt ecological processes, contributing to the population declines of certain species and altering community dynamics (Ajithkumar et al., 2025).

Recent studies have revealed alarming amounts of MPs in the digestive systems of wild prey birds, migratory birds, and ducks in intensive farming, with polyethylene terephthalate (PET) MPs and polyethylene MPs being the predominant MP polymers (Carlin et al., 2020; Hoang and Mitten, 2022; Susanti et al., 2021). A recent pilot study conducted on an Italian dairy farm revealed that 100% of ryegrass hay samples were contaminated with MPs, averaging 39.3 ± 7.02 MPs per gram of dry ryegrass hay (Glorio Patrucco et al., 2024). Another study in India found that 100% of cow diet samples were contaminated with PET MPs, with alarming levels ranging from 89 to 326 g per kilogram of feed (Maganti and Akkina, 2023). Additionally, a study in Spain reported the presence of

MPs in 92% of sheep feces samples, with an average of 997 ± 971 particles per kilogram of dry feces (Beriot et al., 2021). These results confirm that PET MPs can be ingested by ruminants via feed and pass through the ruminal-gastro-intestinal tract.

Recent *in vitro* studies have simulated the ruminal digestive system of cattle to investigate the degradation of MPs. In the study by Glorio Patrucco et al. (2023), it was found that approximately 1% of PET MPs can be degraded by the rumen microbiota. Moreover, Quartinello et al. (2021) identified specific by-products formed during this degradation process, including 0.15 mM terephthalic acid, mono(2-hydroxyethyl) terephthalate, and bis(2-hydroxyethyl) terephthalate. An *in vivo* study conducted on mouse models has shown that administering PET MPs at 4 mg/day over a short period of time (7 days) altered the intestinal microenvironment, changed the microbiota community, and reduced its diversity (Xie et al., 2022). Additionally, mouse oral administration of PET MPs at a rate of 3×10^4 particles every 3 days over 8 weeks caused a slight chronological change in microbial composition (Harusato et al., 2023). For human, an *in vitro* study using a digestion model that simulated human colonic digestion, including fermentation of the gut microbiota, showed that ingestion of PET MPs at 166 mg/intake modified both the composition and diversity of the microbial communities in the human colon and decreased the amount of total viable bacteria (Tamargo et al., 2022). Another *in vitro* study simulating human saliva and stomach and intestinal solutions demonstrated that adding PET MPs at 80 mg/L of small intestine solution reduced lipid digestion (Tan et al., 2020).

However, the effects of MPs evaluated on *in vivo* mouse models and *in vitro* models simulating the human digestive system cannot be fully extrapolated to ruminants, due to significant differences in their digestive systems (Chang et al., 2024). Only non-toxic doses of MPs confirmed in mice can be applied to *in vivo* studies for ruminants, due to ethical restrictions (Chang et al., 2024). Furthermore, the Three Rs (Replacement, Reduction, and Refinement) apply to animal studies, encouraging the development of alternative *in vitro* approaches to reduce the use of animals in research and replace them with simulated systems (Fournier et al., 2023a).

Given these considerations, our hypothesis is that PET MP contamination in the ruminal-gastro-intestinal system of bulls will adversely impact their digestive processes. Specifically, this study aims to investigate, for the first time, the effects of PET MP contamination in the ruminal-gastro-intestinal system of bulls on their ability to degrade and digest hay, using an *in vitro* simulated system.

2. Materials and Methods

2.1. Feedstuff Sample Preparation

In October 2023, first-cut mixed hay samples, one of the most crucial feed resources for ruminants globally (Dynes et al., 2003), were systematically collected from three farms across the north region of Italy in the Piedmont region. The mixed hays included grasses (ryegrass, fescue, and timothy) and legumes (clover). Approximately 2 kg of mixed hay was collected from each farm, gathered from various round bales stored in different positions, to ensure a representative sampling of the hay used in

these studies related to ruminant nutrition. These representative samples of mixed hay were then transported to the laboratory.

The samples were dried at 60 °C for 24 h, ground to a particle size of 1 mm using a granulator (MLI 204; Bühler AG, Uzwil, Switzerland), and stored in glass bottles until further use. The chemical composition of the ground mixed hay was analyzed for dry matter (DM, #930.15), ash (#942.05), crude protein (CP, #984.13), and ether extract (EE, #942.05), according to AOAC methods (2000). Fiber fractions, including neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL), were determined using an Ankom 200 Fiber Analyzer (Ankom Technology, Macedon, NY, USA) according to the procedures of Van Soest et al. (1991). The non-fiber carbohydrates (NFC) were calculated following the equation of NRC (2001):

$$\mathrm{NFC} = 1000 - \mathrm{CP} - \mathrm{EE} - \mathrm{NDF} - \mathrm{ash} \quad (1)$$

Where:

- NFC are non-fiber carbohydrates in mg/g dry matter;
- CP is crude protein in mg/g dry matter;
- EE is ether extract in mg/g dry matter;
- NDF is neutral detergent fiber in mg/g dry matter;
- Ash refers to ash in in mg/g dry matter.

The chemical composition of the mixed hays used in this study is presented in Table 1.

Table 1
Chemical composition of mixed hays collected in three farms (mg/g dry matter).

Chemical composition	Farm 1	Farm 2	Farm 3
Dry matter[1]	882.8	881.6	903.8
Ash	97.4	99.8	112.7
Ether extract	16.5	17.3	17.5
Crude protein	107.1	105.5	110.5
Neutral detergent fiber	718.8	697.1	684.4
Acid detergent fiber	386.1	375.5	370.3
Acid detergent lignin	60.6	55.1	54.1
Non fiber carbohydrates	60.2	80.3	74.9

[1]Dry matter in mg/g fresh matter

The dried and ground mixed hay samples were weighed into Ankom filter bags (F57 filter bags with 25 µm porosity, Ankom Technology, Macedon, NY, USA) at 0.50 ± 0.01 g. In total, 108 bags containing mixed hays were prepared (36 bags/mixed hay), to be used in three different *in vitro* incubations.

2.2 Microplastic Characteristics

Pure PET MPs in granular form were provided from an Italian company and utilized without any additional processing that could alter their crystallinity or chemical reactivity. The PET MPs used were opaque and had a heterogeneous shape, as depicted in Fig. 1, captured with a stereomicroscope (Nikon H550S, Nikon Corporation, Tokyo, Japan). The company provided the average of the particles size: 522 µm for PET (ISO 13320) and the MP size distribution as can be observed in Fig. 2, along with the density value of 1.38 g/cm^3.

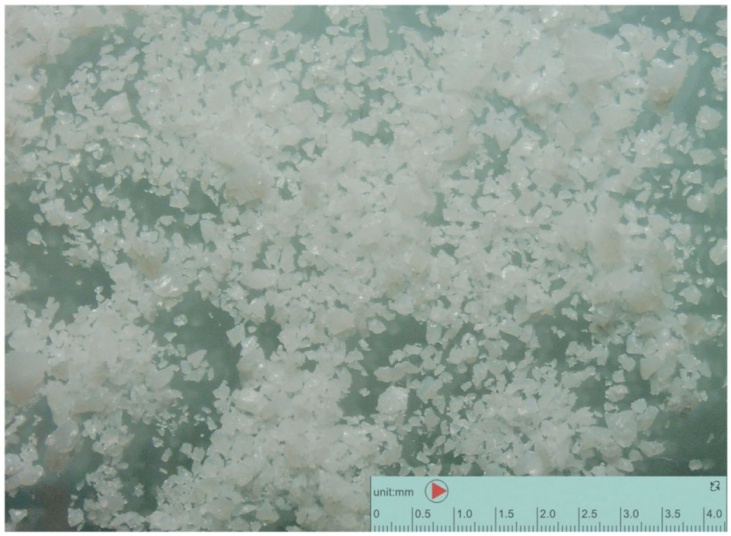


Fig. 1. Stereomicroscope pictures of polyethylene terephthalate microplastics used in the experiment.

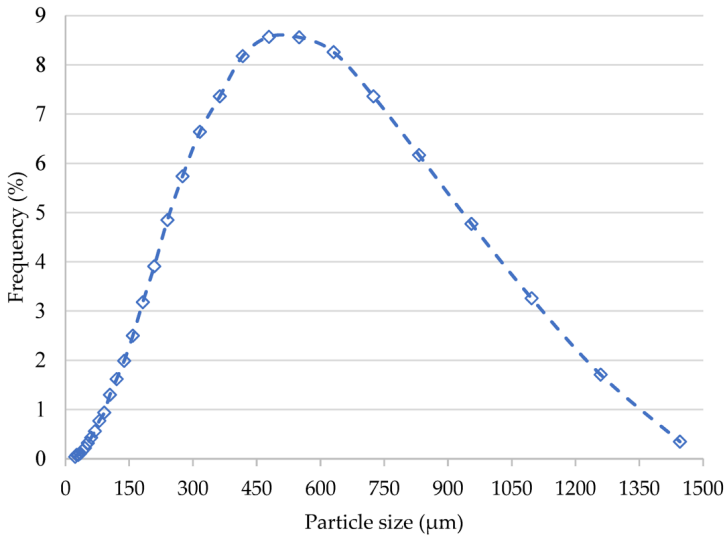


Fig. 2. Distribution of the polyethylene terephthalate microplastic particle sizes used in the experiment, based on particle counts.

2.3 *In Vitro* Ruminal Degradability and Gastro-Intestinal Digestibility Analysis

The experiment was conducted using simulated ruminal-gastro-intestinal digestion systems to investigate the effects of PET MPs on mixed hay ruminal degradability and gastro-intestinal digestibility, using a three-steps method (De Evan et al., 2019; Hippenstiel et al., 2015). The study included four treatment groups with varying concentrations of PET MPs: 0 g/L (control), 5 g/L, 10 g/L, and 15 g/L in the ruminal/gastric/intestinal buffer. To our knowledge, no previous studies have quantified the actual concentrations of MPs in ruminal or gastrointestinal environments, nor examined their effects on microbial activity, fermentative capacity, or nutrient digestion in ruminants. This approach allowed us to establish baseline data and assess the potential dose-dependent impact of PET MPs on both ruminal degradation and gastrointestinal digestion.

This study serves as the pioneering effort to explore the direct effects of MP contamination in this specific environment.

Step 1. Ruminal Degradation

Ruminal degradation was conducted using the Ankom Daisy^{II} Incubator (Ankom Technology Corp., Fairport, NY, USA), following the protocols of Hippenstiel et al. (2015) and de Evan et al. (2019). The procedure was repeated across three consecutive weeks (runs) in December 2023. Each run included three repetitions of mixed hay samples per jar. For each experimental run, 2 L of ruminal fluid was collected at a slaughterhouse, immediately after slaughter, from different positions within the rumen of three healthy Piedmontese bulls, following the standardized protocol of

Fortina et al. (2022). The bulls were each approximately 16 months old and received a daily diet composed of 2 kg of barley straw, 4 kg of ryegrass hay, and 10 kg of concentrate. The concentrate was composed of 68.9% corn meal, 11.7% soybean meal, 5.9% beet pulp, 5% bran, 1% soybean oil, 1% extruded flaxseeds, 0.5% hydrogenated fat, 3.5% calcium carbonate, 0.5% tampon, 0.9% multi acid, and 1% urea. The ruminal fluid was then transported to the laboratory within 20 min from slaughter in a pre-warmed flask at 39 °C to maintain its integrity. Upon arrival at the laboratory, it was filtered through layers of cheesecloth and then mixed with the buffer solution at a ratio of 1:4 (v/v) (Goering and Van Soest, 1970) under continuous flushing with carbon dioxide (CO_2) at 39 °C to mimic the ruminal environment (Goering and Van Soest, 1970).

The samples, prepared as described in Section 2.1, were divided into 4 Ankom Daisy jars. Each jar contained 9 bags of mixed hays (3 hays weighed in triplicate) and 2 L of prewarmed mixed buffer-ruminal solution. PET MPs were added to each jar at different concentrations: 0, 5, 10, and 15 g PET MPs/L buffer-ruminal solution, respectively, in jar 1, 2, 3, and 4. The jars were flushed with CO_2 and placed in the Ankom Daisy^{II} Incubator for 48 h at 39 °C, with a constant rotation at 1 rpm to simulate ruminal motility (Tassone et al., 2023). After the incubation period, the bags were washed in a turbine washing machine with cold water to remove any residue. They were then dried in a forced-air oven at 60 °C for 48 h to ensure that all moisture was removed. At the end of the drying process, the bags were weighed to determine the DM of mixed hay residue after ruminal incubation, according to the procedures of AOAC (2000).

After determining the DM of mixed hay residues post-ruminal incubation, bags containing undegraded mixed hay from each jar (from step 1, ruminal phase) were used as follows: 3 bags to determine the CP of mixed hay and another 3 bags for NDF and ADF analysis. The CP of the mixed hay residues was measured using the Kjeldahl method, according to AOAC (2000), while the NDF and ADF were determined using the Ankom 200 Fiber Analyzer (Ankom Technology, Macedon, NY, USA), following the procedures of Van Soest et al. (1991).

Step 2. Gastric Digestion of Mixed Hay Residue After Ruminal Incubation

Gastric digestion was conducted according to the protocols described by Hippenstiel et al. (2015) and de Evan et al. (2019). Briefly, the remaining 3 bags from each jar that underwent step 1 were incubated in 4 jars with 2 L of of pre-warmed 0.1 N HCl buffer (pH 1.9) with 1 g/L of pepsin (P-7000, Sigma, St. Louis, MO, USA) and the same doses of PET MPs (0, 5, 10, and 15 g PET MPs/L gastric solution). After 1 h of incubation in an Ankom Daisy^{II} Incubator, all the bags were washed under running tap water to remove any residual gastric contents.

Step 3. Intestinal Digestion of Mixed Hay Residue After Ruminal and Gastric Incubation

Intestinal digestion was successively conducted after step 2 following the protocols described by Hippenstiel et al. (2015) and de Evan et al. (2019). The bags used in step 2 were placed into 4 jars with 2 L of pre-warmed 0.5 M KH_2PO_4 buffer (pH 7.75) with 3 g/L of pancreatin (P-7545, Sigma, St. Louis, MO, USA), 50 ppm of thymol (antimicrobial agent), and the same

concentrations of PET MPs and incubated in an Ankom Daisy^{II} Incubator for 24 h.

After intestinal digestion, all bags were washed in a turbine washer to remove any remaining residue and dried in a forced-air oven at 60 °C for 48 h, to determine the DM of the mixed hay residue after ruminal-gastro-intestinal incubation according to the AOAC (2000). The CP of the mixed hay residue after ruminal-gastro-intestinal incubation was determined using the Kjeldahl method according to the AOAC (2000).

2.4. *In Vitro* Ruminal Degradability and Gastro-Intestinal Digestibility Calculations

After all the analysis, the values of ruminal degradability, gastro-intestinal digestibility, and total tract digestibility were calculated as follows:

$$DM_{deg} = \frac{DM_{hay\ before\ ruminal\ incubation} - DM_{hay\ residue\ after\ ruminal\ incubation}}{DM_{hay\ before\ ruminal\ incubation}} \times 1000 \quad (2)$$

$$CP_{deg} = \frac{CP_{hay\ before\ ruminal\ incubation} - CP_{hay\ residue\ after\ ruminal\ incubation}}{CP_{\ hay\ before\ ruminal\ incubation}} \times 1000 \quad (3)$$

$$NDF_{deg} = \frac{NDF_{hay\ before\ ruminal\ incubation} - NDF_{hay\ residue\ after\ ruminal\ incubation}}{NDF_{\ hay\ before\ ruminal\ incubation}} \times 1000 \quad (4)$$

$$ADF_{deg} = \frac{ADF_{hay\ before\ ruminal\ incubation} - ADF_{hay\ residue\ after\ ruminal\ incubation}}{ADF_{\ hay\ before\ ruminal\ incubation}} \times 1000 \quad (5)$$

$$DM_{dig} = \frac{DM_{hay\ residue\ after\ ruminal\ incubation} - DM_{hay\ residue\ after\ ruminal-gastro-intestinal\ incubation}}{DM_{hay\ residues\ after\ ruminal\ incubation}} \times 1000 \quad (6)$$

$$CP_{dig} = \frac{CP_{hay\ residue\ after\ ruminal\ incubation} - CP_{hay\ residue\ after\ ruminal\ -\ gastro\ -\ intestinal\ incubation}}{CP_{hay\ residue\ after\ ruminal\ incubation}} \times 1000 \quad (7)$$

$$DM_{deg+dig} = \frac{DM_{hay\ before\ ruminal\ incubation} - DM_{hay\ residue\ after\ ruminal\ -\ gastro\ -\ intestinal\ incubation}}{DM_{hay\ before\ ruminal\ incubation}} \times 1000 \quad (8)$$

$$CP_{deg+dig} = \frac{CP_{hay\ before\ ruminal\ incubation} - CP_{hay\ residue\ after\ ruminal\ -\ gastro\ -\ intestinal\ incubation}}{CP_{hay\ before\ ruminal\ incubation}} \times 1000 \quad (9)$$

Where:

- DM_{deg} is dry matter degradability of mixed hay in mg/g dry matter of mixed hay;

- CP_{deg} is crude protein degradability of mixed hay in mg/g dry matter of mixed hay;
- NDF_{deg} is neutral detergent fiber degradability of mixed hay in mg/g neutral detergent fiber of mixed hay;
- ADF_{deg} is acid detergent fiber degradability of mixed hay in mg/g acid detergent fiber of mixed hay;
- DM_{dig} is dry matter gastro-intestinal digestibility of ruminal non-degraded residue of dry matter of mixed hay in mg/g;
- CP_{dig} is crude protein gastro-intestinal digestibility of ruminal non-degraded residue of crude protein in mg/g;
- $DM_{deg+dig}$ is dry matter total tract degradability and digestibility of mixed hay in mg/g dry matter of mixed hay;
- $CP_{deg+dig}$ is the crude protein total tract degradability and digestibility of mixed hay in mg/g crude protein of mixed hay.

2.5. Statistical Analysis

All the data were statistically analyzed using a one-way analysis of variance (ANOVA) with PROC GLM of the SAS software (version 9.1; SAS Institute, Cary, NC, USA). The statistical significance of the differences between the mean values was assessed using the Tukey test. Orthogonal polynomial contrast was used to detect linear and quadratic effects in increasing PET MP levels. A significant difference was declared at a p-value < 0.05.

3. Results

3.1. Ruminal Degradability

The effects of PET MP contamination on the DM_{deg}, CP_{deg}, NDF_{deg}, and ADF_{deg} are summarized in Table 2. Overall, the increment in PET MPs level in the buffer-ruminal solution (at 0, 5, 10, and 15 g/L) linearly decreased (p-value < 0.05) the CP_{deg} and NDF_{deg} of the mixed hay. In particular, at the medium and high doses of PET MPs, the CP_{deg} declined by 9 and 16% respectively and the NDF_{deg} decreased by 9 and 13% respectively. However, the PET MPs did not influence the DM_{deg} and ADF_{deg} (p-value > 0.05) at any level.

Table 2.
Effects of polyethylene terephthalate microplastics in buffer–ruminal solutions on ruminal degradability of nutrient compounds of mixed hay (mg/g).

	PET MPs Added in Buffer-Ruminal Solutions (g/L)				SEM	*p*-Value	Orthogonal Polynomial Contrast	
	0	5	10	15			Linear	Quadratic
DM_{deg}	504	486	488	492	38.9	0.749	0.353	0.212
CP_{deg}	446^{a}	417^{ab}	408^{bc}	375^{c}	34.4	0.003	0.0003	0.888
NDF_{deg}	469^{a}	446^{ab}	426^{b}	409^{b}	31.9	0.003	0.0002	0.790
ADF_{deg}	375	360	348	347	66.7	0.760	0.309	0.711

$^{a-c}$ Values within row with different superscripts differ significantly at *p*-value < 0.05; ADF_{deg}: acid detergent fiber degradability of mixed hay; CP_{deg}: crude protein degradability of mixed hay; DM_{deg}: dry matter degradability of mixed hay; PET MPs: polyethylene terephthalate microplastics; NDF_{deg}: neutral detergent fiber degradability of mixed hay; SEM: standard errors of means.

3.2. Gastro-Intestinal Digestibility

The effects of PET MPs on the DM_{dig} and CP_{dig} are shown in Table 3. The contamination of the gastro-intestinal system with different doses of PET MPs at 0, 5, 10, and 15 g/L did not affect the DM_{dig} of the mixed hay (p-value > 0.05). However, it had a quadratic effect on the CP_{dig}. In particulary it diminished the CP_{dig} at a low addition of PET MPs.

Table 3.
Effects of addition of polyethylene terephthalate microplastics to gastro-intestinal solutions on gastro-intestinal digestibility of ruminal undegraded dry matter and crude protein content of mixed hay (mg/g).

	PET MPs Added in Gastro-Intestinal Solutions (g/L)				SEM	*p*-Value	Orthogonal Polynomial Contrast	
	0	5	10	15			Linear	Quadratic
DM_{dig}	189	204	229	226	42.1	0.193	0.054	0.550
CP_{dig}	553[a]	506[b]	520[ab]	538[ab]	34.1	0.04	0.482	0.011

[a-c] Values within row with different superscripts differ significantly at *p*-value < 0.05; CP_{dig}: crude protein gastro-intestinal digestibility of ruminal non-degraded residue of crude protein of mixed hay; DM_{dig}: dry matter gastro-intestinal digestibility of ruminal non-degraded residue of dry matter of mixed hay; PET MPs: polyethylene terephthalate microplastics; SEM: standard errors of means.

3.3. Total Tract Digestibility

The *in vitro* effects of PET MPs at 0, 5, 10, and 15 g/L of the buffer-ruminal and gastro-intestinal solutions on the $DM_{deg+dig}$ and $CP_{deg+dig}$ of the mixed hay is shown in Table 4. The $DM_{deg+dig}$ of the mixed hay remained the same (p-value > 0.05) for all PET MP additions compared to the control. However, increasing the level of MPs from 0 to 5, 10, and 15 g/L of the buffer-ruminal and gastro-intestinal solutions led to a linear decrease in the $CP_{deg+dig}$ of the mixed hay, with no significant differences between low, medium, and high levels of PET MPs.

Table 4.
Effects of addition of polyethylene terephthalate microplastics to buffer–ruminal gastro-intestinal solutions on total tract digestibility of dry matter and crud protein content of mixed hay (mg/g).

	PET MPs Added in Buffer-Ruminal Gastro-Intestinal Solutions (g/L)				SEM	*p*-Value	Orthogonal Polynomial Contrast	
	0	5	10	15			Linear	Quadratic
$DM_{deg+dig}$	600	590	606	607	33.8	0.61	0.239	0.885
$CP_{deg+dig}$	752[a]	712[b]	716[b]	712[b]	22.0	0.001	0.001	0.021

[a-b] Values within row with different superscripts differ significantly at *p*-value < 0.05; CP: crude protein tract degradability and digestibility of mixed hay; DM: dry matter total tract degradability and digestibility of mixed hay; MPs: polyethylene terephthalate microplastics; SEM: standard errors of means.

4. Discussion

The effects of the presence of MPs in the ruminal-gastro-intestinal system on the digestive efficiency of ruminants have not yet received sufficient attention. Our *in vitro* study found that the addition of PET MPs to the ruminal-gastro-intestinal digestive system of bulls reduced their ability to degrade and digest certain mixed hay nutrient components, such as NDF and CP. Despite NDF representing a substantial portion of mixed hay DM, its reduced degradability did not translate into lower overall DM degradation. We hypothesize that this could be due to compensatory improvements in the degradability of NFC, EE, and/or ash, although these components were not directly measured in our study. An increased degradability of these fractions may have offset the observed reductions in NDF and CP digestion, thereby maintaining total DM degradability. These results align with a previous *in vivo* study in sheep, which demonstrated that the daily intake of 100 mg of polystyrene MPs at a particle size of 50 µm reduced the apparent digestion of some dietary components, such as NDF and EE fraction, while the apparent digestion of the DM of diets remained unchanged (Chang et al., 2024).

The results from our study are further supported by *in vivo* research on aquatic animals that proved the exposure of mussels to polyethylene MPs and polystyrene MPs caused significant changes in digestive enzyme activities (Trestrail et al., 2021). These modifications in enzyme activities may explain the negative effects detected on specific compounds (CP and NDF) and maybe the increased degradation and digestion of other nutrient compounds not analyzed in our study. This selective effect could

be due to changes in the microbial population in the digestive system, such as increased *Bacteroidetes* and *Actinobacteria* and decreased *Oriobacteriales incertae Sedis* and *Prevotellaceae YAB2003*, as proved in *in vivo* studies on the ruminal microbiota of sheep contaminated by polyester MPs (Chang et al., 2024), the intestinal microbiota of mice contaminated by PET MPs (Xie et al., 2022), the intestinal microbiota of zebrafish contaminated by PET MPs (Duan et al., 2022), and an *in vitro* study on a human simulated digestive system contaminated by polyethylene MPs (Fournier et al., 2023b).

In the ruminal phase, PET MPs reduced the NDF_{deg} with significant differences between levels of 0 and 10, and 15 g/L, with reductions at medium and high doses by 9% and 13%, respectively, while it did not alter the ADF_{deg}. This selective inhibition of fiber compounds is consistent with findings in aquatic animals, although they are phylogenetically distant from ruminants. Trestrail et al. (2021) indicated that mussels exposed to polyethylene MPs and polystyrene MPs showed a reduction in xylanase activity, crucial for degrading the hemicellulose part of NDF, but not in cellulase activity, crucial for degrading the cellulose part of ADF and NDF. Therefore, ADF degradation remains unaffected. This reduction in the NDF_{deg} can lead to lower feed intake and diminish ruminal performance, resulting in significant economic losses for producers (Oba and Allen, 1999). In addition, in this phase, PET MPs also reduced the CP_{deg} with significant differences between levels of 0 and 10, and 15 g/L, with reductions at medium and high doses by 9% and 16% respectively. This reduction can be attributed to the potential inhibition of protease enzyme

activity produced by the ruminal microbiota, as proved in a previous study in mussels exposed to polystyrene MPs (Wang et al., 2020). Wang et al. (2020) showed that protease activity in the digestive system of these aquatic animals decreased with increasing polystyrene MP concentrations. These findings indicate a broader impact of MPs on protein degradation in both aquatic animals and ruminants. The reduction in the CP_{deg} and NDF_{deg} of the mixed hay, detected only at a medium and high addition of PET MPs, can be attributed to the ability of the ruminal microbiota to release terephthalic acid from PET MPs (Quartinello et al., 2021), which can be toxic at high concentrations (Maurya et al., 2023). Additionally, PET MPs can interact with the gut microbiota and impair its normal function (Duan et al., 2022; Tamargo et al., 2022; Xie et al., 2022). However, this interaction may be more pronounced at higher levels of PET MPs, where a greater quantity of particles can more significantly disrupt the balance and activity of microbial populations. While differential enzymatic activity may explain the selective nutrient degradation observed, this does not preclude the role of TPA toxicity. Both mechanisms may coexist: MPs could alter microbial community structure-reducing fibrolytic and proteolytic activity-while concurrently shifting enzymatic activity toward more readily fermentable substrates. Future studies should assess changes in microbial composition and enzyme profiles across varying MP concentrations to clarify these effects.

In the gastro-intestinal phase, the CP_{dig}, was significantly reduced by the presence of PET MPs at a low dose, decreasing by 8%. This negative effect,

observed only in the gastro-intestinal phase at this dose and not in the ruminal phase, could be due to gastric acid altering the characteristics of PET MP particles or changing their shape, potentially making them more bioactive (Hippenstiel et al., 2015). Alternatively, the MPs might absorb enzymes, like pepsin, thereby reducing their efficacy (Tan et al., 2020; Wang et al., 2020) or they could reduce the activity of pancreatic enzymes (Frank et al., 2023). However, at medium and high levels of PET MP contamination, the CP_{dig} of mixed hay did not show significant differences in comparison to the control. These results underscore the need for further investigation and highlight the complex nature of MP-enzyme interactions within the gastrointestinal environment. Future studies should aim to elucidate these mechanisms, to better understand the complex interactions between PET MPs and the CP_{dig} in the gastro-intestinal tract.

In addition, PET MPs significantly decreased the $CP_{deg+dig}$ of the mixed hay at all doses. Similar to our finding, previous *in vivo* studies on lambs showed that daily ingestion of 100 mg of polystyrene MPs at 100 µm reduced the apparent CP digestion (Chang et al., 2024). This reduction in the $CP_{deg+dig}$ can decrease feed efficiency and ruminant productivity, increase feed costs, and ultimately reduce overall farm profitability and sustainability (Abbasi et al., 2018). Additionally, the decreased $CP_{deg+dig}$ contributes to increased nitrogen residue in manure, which is converted into ammonia and nitrous oxide that volatilize into the atmosphere, contributing to air pollution and greenhouse gas emissions (Abbasi et al., 2018; Kebreab et al., 2002).

5. Conclusions

This study has demonstrated, for the first time, the adverse effects of PET MPs pollution on the digestive activity of the ruminal-gastro-intestinal system of ruminants. Significant adverse effects were observed at PET MP concentrations of 10 and 15 g/L in the buffer-ruminal solution. Increasing concentrations of PET MPs in the ruminant digestive system led to a linear decline in the ability to degrade the NDF content of mixed hay. Furthermore, the addition of PET MPs impaired the performance of the ruminal-gastro-intestinal digestive system on the $CP_{deg+dig}$ of mixed hay, across all levels of addition, albeit not uniformly throughout the different digestive phases. Specifically, PET MPs negatively affected the CP_{deg} during the ruminal phase at medium and high concentrations, whereas their detrimental effects on the CP_{dig} during the gastro-intestinal phase were observed only at the lowest concentrations. Further research is needed to explore these mechanisms and clarify the specific factors contributing to the detrimental effects of PET MPs on ruminant digestion. Moreover, it should be assessed whether the observed effects are attributable to the chemical properties of the MP material itself, additives in the MPs, degradation products of the MP material, residual starting materials from the polymerization process, or a combination of these factors. Future studies can build upon these initial findings by evaluating the effects of additional doses of PET MPs.

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Chapter 5: Study Rumen Microbiota Ability to Biodegrade MPs

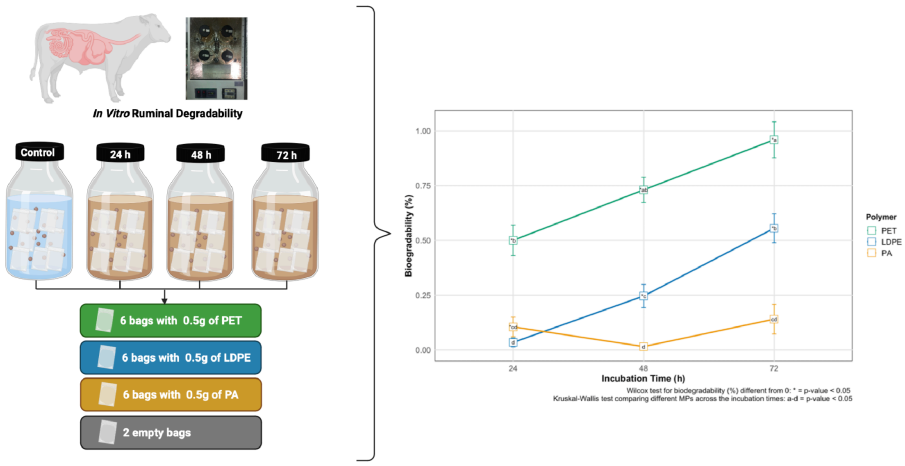
From Pollution to Solution: Preliminary Study on the Ability of Rumen Microbiota to Biodegrade Microplastics

Prepared paper

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Graphical Abstract



Abstract

Microplastics (MPs) pollution is an emerging concern in ruminant livestock, as animals are exposed to MPs through air, water, and feeds. Ruminants play a key role in MPs transmission to humans via animal products (*e.g.* milk and meat) and contribute to MPs return to the environment through excreta. Identifying effective strategies to mitigate MP pollution in the ruminant sector is crucial. A promising yet understudied approach involves the potential ability of rumen microbiota to biodegrade MPs. Rumen microorganisms produce fibrolytic enzymes capable of degrading not only complex plant molecules but potentially also plastics and MPs. This study preliminary investigated the *in vitro* biodegradation of three widely distributed MPs -low-density polyethylene (LDPE), polyethylene terephthalate (PET), and polyamide (PA)- by rumen microbiota over 24, 48, and 72 h. PET exhibited the highest biodegradation rates (24 h: 0.50 ± 0.070%; 48 h: 0.73 ± 0.057%; 72 h: 0.96 ± 0.082%), followed by LDPE (24 h: 0.03 ± 0.020%; 48 h: 0.25 ± 0.053%; 72 h: 0.56 ± 0.066%) and PA (24 h: 0.10 ± 0.045%; 48 h: 0.02 ± 0.015%; 72 h: 0.14 ± 0.067%). No colorimetric changes were observed in LDPE and PET, whereas PA exhibited progressive browning over time indicating possible structural alterations. These findings suggest that rumen microbiota could serve as a promising tool for MP biodegradation. Further research is needed to elucidate the mechanisms involved, potentially enhancing ruminants' natural capacity to degrade MPs.

Highlights

- Prelimanary investigation of rumen microbiota ability to biodegrade MPs.
- PET exhibited the highest biodegradation rates *in vitro* (0.96 ± 0.082% at 72 h).
- LDPE was the second most degraded polymer (0.56 ± 0.066% at 72 h).
- PA exhibited the lowest degradation (0.14 ± 0.067% at 72 h) and progressive browning.

1. Introduction

Plastic pollution has emerged as a critical environmental challenge, with global plastic waste projected to reach 11,000 million tons by 2025. Despite efforts to manage this crisis, only 9% of plastic waste is recycled, leaving the majority - 91% - to persist in the environment (Aardema et al., 2024). In the environment, plastic undergoes fragmentation due to physical and chemical processes, including ultraviolet photodegradation, mechanical abrasion, oxidative degradation, and abiotic weathering, generating microplastics (MPs, plastic with dimensions between 1 µm to 5 mm) (Dong et al., 2023; Klimasz and Grobelak, 2024). MPs are nowadays ubiquitous representing a serious concern for both aquatic and terrestrial ecosystems (Kumari et al., 2022). This contamination poses a dangerous threat to both animal and human health (Corte Pause et al., 2024). Focusing on livestock, ruminants are one of the key players in MP cycle in the environment. They are mainly exposed to MPs through three main polluted sources: inhalation of air, drinking of water, and ingestion of feeds (Glorio Patrucco et al., 2024; Khan et al., 2024). These

contaminations could originate from the fragmentation of plastics commonly used in farms, primarily composed by low-density polyethylene (LDPE) alone or constructed with polyamide (PA) (Borreani and Tabacco, 2017; Klimasz and Grobelak, 2024). This includes materials used for feed storage, such as hay bale wraps and silage covers, as well as plastics employed in crop cultivation for animal consumption, such as mulching films, irrigation pipes, and plastic-coated fertilizers (Borreani and Tabacco, 2017; Urli et al., 2023). Additionally, atmospheric agents like wind, rain, and snow play a significant role in the transport and dissemination of MPs (Ramachandraiah et al., 2022; Roy et al., 2023). Moreover, ruminants can ingest large plastic debris that could be broken down in MPs during the digestion process (Dong et al., 2023). MPs were found inside ruminant bodies: blood, follicular fluid and sperm (Grechi et al., 2024, 2023; van der Veen et al., 2022). From ruminants they can pass to humans through the consumption of contaminated animal products such as meat and milk (Bahrani et al., 2024; Rbaibi Zipak et al., 2024; van der Veen et al., 2022). MPs can also return to the environment from ruminants through their feces. (Beriot et al., 2021; Wu et al., 2024). As the demand for animal-based food products continues to rise to meet the needs of a growing global population, projected to reach 9.7 billion by 2050, it is crucial to develop effective strategies to mitigate MP pollution in the ruminant sector (Henchion et al., 2021; Lackner and Branka, 2024). A promising but understudied solution could come from ruminants themselves. Ruminants naturally ingest feed that is contaminated with MPs (Glorio Patrucco et al., 2024b; Chapter 3), leading to the unintentional accumulation of MPs in the rumen. Rumen microorganisms

(bacteria, archea, fungi, protozoa) allow ruminants to digest plant fiber materials. In fact, they secrete fibrolytic enzymes that hydrolyze complex plant cell molecules as cutin, cellulose, hemicellulose into smaller molecules that can be metabolized by ruminants (Sanjorjo et al., 2023). Microorganisms that produce these enzymes are currently being studied for their potential to degrade plastics and MPs through biodegradation. In this process, microorganisms decompose large organic compounds into smaller ones. This process is influenced by various factors, including substrate availability, surface characteristics, polymer morphology, and molecular weight (Mohanan et al., 2020).

Up to now the majority of the work, on MP and plastic biodegradation, are focused on:

i) pure culture of bacteria, fungi and microalgae (Gao et al., 2024). Examples of bacteria are *Ideonella sakainensis* for polyethylene terephthalate (PET) (Yoshida et al., 2016); *Acinetobacter* for low-density polyethylene (LDPE) (Zhang et al., 2022); *Bacillus* for LDPE (Yao et al., 2022). Examples of fungi are *Aspergillus*, *Penicillium*, *Fusarium* for LDPE, PET and polyamide (PA) (Sánchez, 2020). Examples of microalgae: *Phormidium lucidum* and *Oscillatoria subbrevis* for polyethylene (PE) (Sarmah and Rout, 2018);
ii) natural consortia of microorganisms, as demonstrated for gut microbiota of insects, such as *Galleria mellonella* for polystyrene (PS), polyvinyl chloride (PVC) and polypropylene (PP); *Tenebrio molitor* for PVC and PP (Siddiqui et al., 2024).

The natural consortium of rumen microbiota holds significant potential for the biodegradation of plastics and microplastics (MPs). However, this area remains relatively underexplored in scientific research, with most studies focusing on the biodegradability of biodegradable and bio-based polymers. To date, key studies include Quartinello et al. (2021), who investigated the ability of rumen microbiota to degrade the synthetic polyester PET, the biodegradable polyester polybutylene adipate-co-terephthalate (PBAT), and the bio-based polyester poly(ethylene furanoate) (PEF). Additionally, Galyon and colleagues conducted *in vitro*, *in situ*, and *in vivo* trials to assess the ability of rumen microbiota to biodegrade the biodegradable polymers poly(butylene succinate-co-adipate) (PBSA) and polyhydroxyalkanoates (PHA), both individually and in blends. These *in situ* and *in vivo* studies also included the synthetic polymer LDPE (Galyon et al., 2022, 2023a, 2023b).

Based on the urgent need to find solution to MP pollution in livestock and the potential, but understudied ability of rumen microbiota to degrade MPs, the aim of this work was to study the *in vitro* ability of rumen microbiota to biodegrade three widely diffused MPs: LDPE, PET and PA.

2. Materials and Methods

2.1 Microplastic Samples

MPs chosen for the experiment were: LDPE, PET and PA. LDPE and PA were selected due to their extensive use in farms, and previously demonstrated presence as pollutants in ruminant feeds (Chapter 3 *Assessment of MP Contamination in Ruminant Feeds*). PET, on the other hand, was included because of its widespread environmental

contamination, as plastic and MPs, and its preliminary biodegradation by rumen microbiota, as previously demonstrated by Quartinello et al. (2021).

LDPE, PET and PA were provided by an industrial company specialized in the production and sale of thermoplastic polymers. MPs were in particles' shape with a length-to-diameter ratio of less than 3, in accordance with the classification outlined by the European Chemicals Agency (ECHA, 2019). All three polymers were white, without the inclusion of any additives. The company provided the average of the particles size: 643 µm, 522 µm and 993 µm respectively for LDPE, PET and PA (ISO 13320) and the MP size distribution as can be observed in Fig. 1 along with stereomicroscope picture for each polymer.

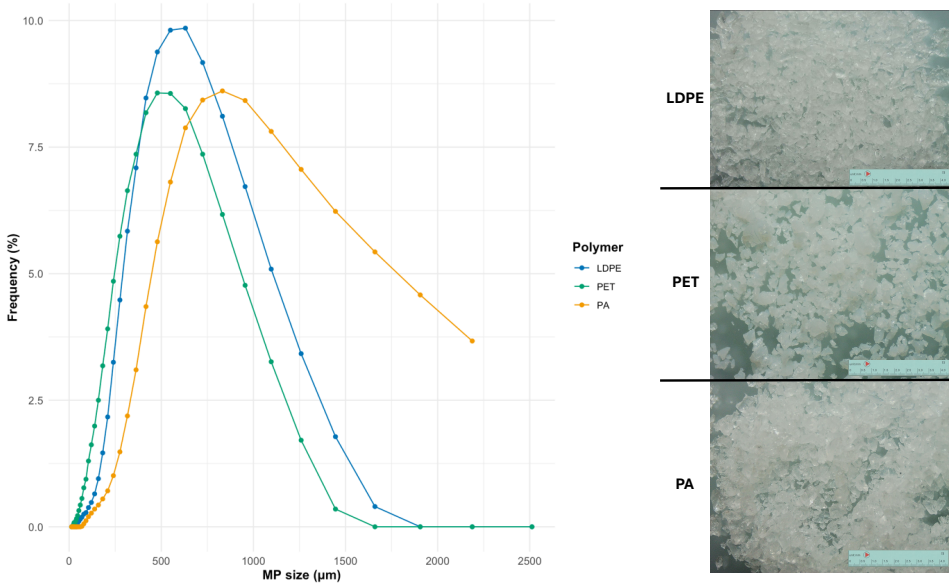


Fig. 1. On the left microplastic (MP) size distribution (in µm) of LDPE (low-density polyethylene), PET (polyethylene terephthalate) and polyamide (PA) provided by the industrial company. On the right stereomicroscope pictures of LDPE, PET and PA used in the experiment.

2.2 Rumen Fluid Collection

For each experimental run, 2 L of rumen fluid were collected at a slaughterhouse immediately after the slaughter from three healthy Piedmontese bulls, following the standardized protocol of Fortina et al. (2022). The bulls, approximately 16 months old, were fed a daily diet consisting of 2 kg of barley straw, 4 kg of ryegrass hay, and 10 kg of concentrate. The concentrate contained 68.9% corn meal, 11.7% soybean meal, 5.9% beet pulp, 5% bran, 1% soybean oil, 1% extruded flaxseeds, 0.5% hydrogenated fat, 3.5% calcium carbonate, 0.5% buffer, 0.9% multi-acid, and 1% urea. The rumen fluid was transported to the laboratory in pre-warmed thermic bottles at 39 °C within 20 min to preserve its integrity. Upon arrival, the rumen fluid was filtered through multiple layers of cheesecloth and mixed with the buffers' solution described in the "*In Vitro True Digestibility using the ANKOM DAISY^{II} Incubator (Method 3)*" (ANKOM Technology, 2023) under continuous CO_2 flushing at 39 °C to simulate the ruminal environment.

2.3 Microplastic Biodegradability in *In Vitro* Ruminal Fermentation

In vitro ruminal fermentation to assess LDPE, PET, PA MPs biodegradability by rumen microbiota was performed with the Ankom Daisy^{II} Incubator (AD^{II}, Ankom Technology Corporation Fairport, Macedon, NY, USA), a thermostatically controlled chamber (at 39 °C) containing 4 rotating digestion jars that allow to simulate rumen environment. The protocol followed was an adaptation of the method for feed digestibility provided by ANKOM Technology (*In Vitro* True Digestibility using the ANKOM DAISY^{II} Incubator (Method 3)" (ANKOM Technology, 2023). In brief, each jar (1, 2,

3, and 4) was loaded with LDPE, PET, and PA samples, each previously weighed (0.5 g) and thermo-sealed in F57 ANKOM bags (25 µm pore size; Ankom Technology Corporation Fairport, Macedon, NY, USA) using a thermo-sealer. Each jar contained six replicate bags for each polymer (6 LDPE, 6 PET, and 6 PA) and 2 empty bags to account for rumen fluid residuals, totaling 20 bags per jar. Jars 1, 2, and 3 were filled with buffered rumen fluid (1600 mL of buffers A:B (1:5) + 400 mL of rumen fluid) and 20 g of dried and ground TMR (the diet provided to the rumen fluid donor bulls, as described in Section 2.2 *Rumen Fluid Collection*). Jar 4, intended to assess potential MP loss, contained only the buffer solution (1600 mL of buffers A:B (1:5)). Before the incubation, each jar was insufflated with CO_2 to maintain anaerobic conditions. The incubation times were 24 h for jar 1, 48 h for jar 2, and 72 h for jar 3. In jar 4, two MP bags from each polymer were removed at 24, 48, and 72 h to check potential MP loss. At the end of each incubation MP sample bags were removed and washed in cold tap water to clean them from buffered rumen fluid residues. After the washing, samples were dried at 60 °C forced-air oven overnight. The experiment was repeated for 3 runs (N = 18). The summary of the work is illustrated in Fig. 2.

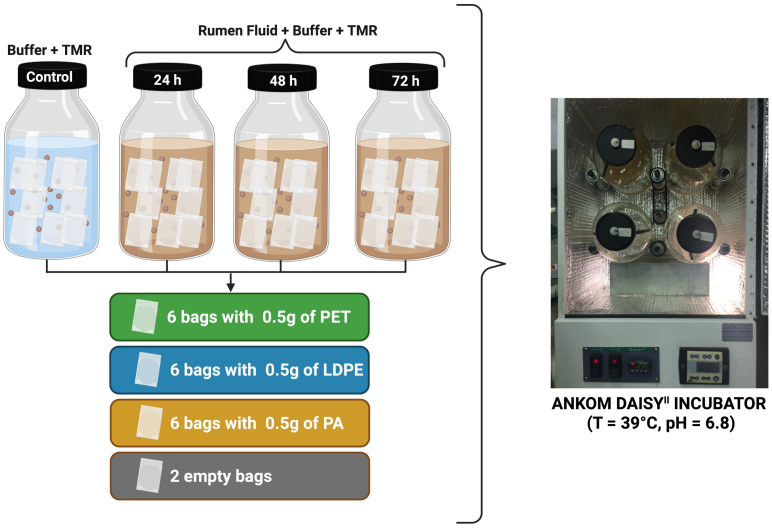


Fig. 2. Summary of microplastics biodegradability in *in vitro* ruminal fermentation. LDPE = low-density polyethylene; PET = polyethylene terephthalate; PA = polyamide; TMR = Total Mixed Ration.

2.4 Microplastic Biodegradability Quantification and Optical Observation

MP biodegradability was measured by weight loss, that is one of the main parameters most used to preliminary detect MP biodegradability (Mohanan et al., 2020; Venkatesh et al., 2021).

MPs were weighted before and after each incubation with 5 decimals analytical balance (Sartorius - CP225D). MP biodegradability by rumen microbiota was calculated as follow:

$$MP\ biodegradability\ (\%):\ \left(\frac{(Pre\ inc.\ \ MPs\ weight\ -Post\ inc.\ \ MPs\ weight\ corrected)}{Pre\ inc.\ \ MPs\ weight}\right)\cdot 100$$

where:

$$Pre\ inc.MPs\ weight:\ Pre\ inc.MPs\ weight - Tare\ of\ the\ bag$$

$$Post\ inc.MPs\ weight:\ Post\ inc.\ \ MPs\ weight - Tare\ of\ the\ bag$$

$$Post\ inc.MPs\ weight\ corrected:\ (Post.inc.MPs\ weight - Rumen\ fluid\ residues\ of\ the\ bags + MPs\ loss)$$

Moreover, MP samples pre and post incubation were visually observed using a stereomicroscope. Further investigation of changes in the polymers' chemical structure is currently underway at the University of Salerno.

2.5 Statistical Analysis

Data from the 3 runs (N = 18) were analyzed using RStudio (version 2022.07.2). Statistical analyses were conducted to assess the biodegradation of LDPE, PET, and PA MPs by rumen microbiota across different incubation times (24 h, 48 h, and 72 h). Since the data were not normally distributed, non-parametric tests were applied. For each MP and incubation time, Wilcoxon test was performed using the 'wilcox.test' function from the 'stats' package (version 4.2.2) to determine if biodegradability significantly differed from zero. To evaluate differences in biodegradability among LDPE, PET, and PA MPs across incubation times, the Kruskal-Wallis test was conducted using the 'kruskal' function from the 'agricolae' package. Pairwise comparisons were carried out with Bonferroni adjustments to correct for multiple comparisons. Results are presented as Means ± SE. Data visualization was performed using the ggplot2 package (version 3.5.1).

3. Results

As illustrated in Fig. 3, PET was the MP most biodegraded by rumen microbiota, followed by LDPE and PA. For PET, the biodegradability at all three incubation times was significantly different from zero ($p < 0.0001$). Furthermore, the biodegradability of PET at 72 h was significantly higher than at 24 h (PET 24 h = 0.50 ± 0.070%; 48 h = 0.73 ± 0.057%; 72 h = 0.96

± 0.082%). Regarding LDPE, biodegradability was significantly different from zero at 48 h ($p = 0.001$) and 72 h ($p < 0.001$), with a clear increase over incubation times (LDPE 24 h = 0.03 ± 0.020%; 48 h = 0.25 ± 0.053%; 72 h = 0.56 ± 0.066%). However, the biodegradability of LDPE at 72 h was only comparable to that of PET at 24 h. For PA, biodegradability was significantly different from zero only at 24 h ($p = 0.02$) but remained close to zero (PA 24 h = 0.10 ± 0.045%; 48 h = 0.02 ± 0.015%; 72 h = 0.14 ± 0.067%).

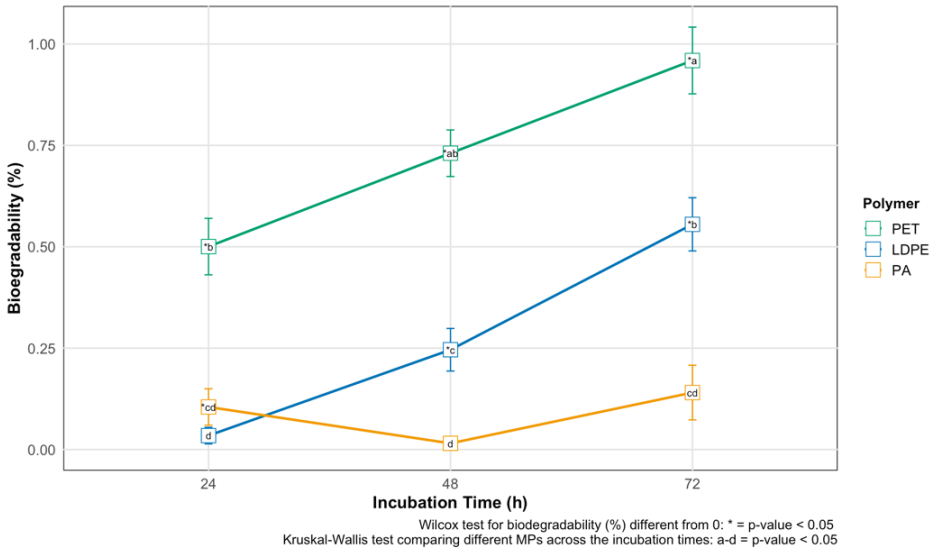


Fig. 3. Biodegradability (%) of polyethylene terephthalate (PET), low density polyethylene (LDPE) and polyamide (PA) in the *in vitro* incubation with rumen fluid (incubation times: 24 h, 48 h and 72 h). Results are expressed as Mean ± SE.

Fig. 4 presents stereomicroscope pictures of LDPE, PET, and PA at incubation times of 0, 24, 48, and 72 h. No observable colorimetric changes were detected in LDPE and PET throughout the incubation periods. In contrast, PA underwent the most pronounced colorimetric change, displaying a progressive browning with increasing incubation

times. These colorimetric changes can be due to rumen fluid residuals in MPs or structural alteration in MPs. Further investigations are currently underway at the University of Salerno to clarify these phenomena and examine chemical structure changes through detailed structural analysis.

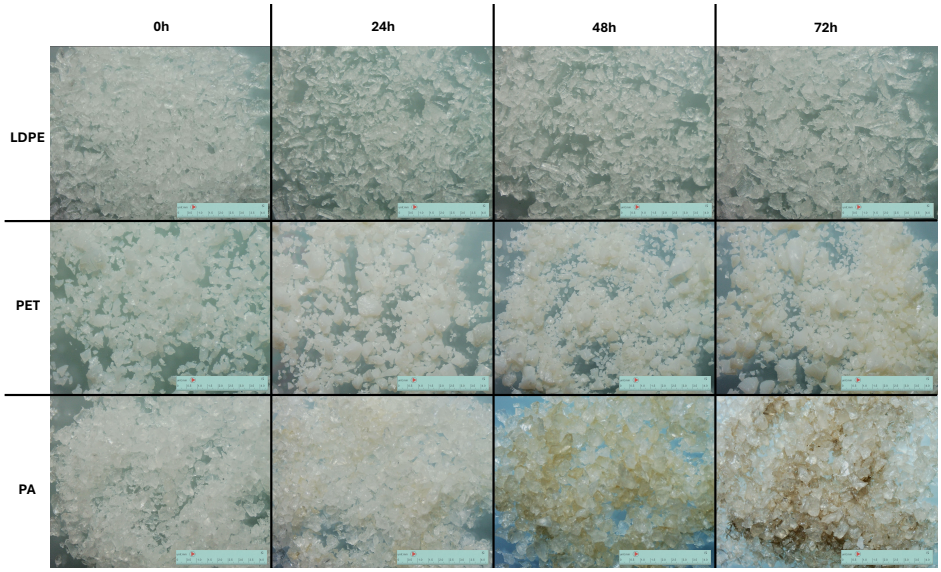


Fig. 4. Stereomicroscope pictures of low-density polyethylene (LDPE), polyethylene terephthalate (PET), and polyamide (PA) in the *in vitro* incubation with rumen fluid (incubation times: 24 h, 48 h and 72 h).

4. Discussion

This study provides one of the first indications of the potential ability of rumen microbiota to biodegrade synthetic polymers, specifically LDPE, PET, and PA MPs.

The findings revealed that PET exhibited the highest biodegradability, reaching 0.96 ± 0.082% after 72 h of incubation with rumen fluid. LDPE and PA showed lower biodegradability, with maximum values of 0.56 ± 0.066% (72 h) and 0.10 ± 0.045% (24 h), respectively. The higher

biodegradability of PET compared to LDPE and PA in the rumen environment can be attributed to the similarities in structure between PET and cutin (Din et al., 2023; Quartinello et al., 2021). Rumen microbiota naturally produce cutinases (EC 3.1.1.74), enzymes that hydrolyze cutin, a primary component of plant cuticles situated between the primary cell wall and the surface wax (Quartinello et al., 2021; Xin et al., 2021). Both cutin and PET are polyesters containing ester bonds, making them susceptible to hydrolysis by cutinases (Din et al., 2023). Consequently, cutinases from rumen microbiota may also have the potential to cleave the ester bonds in PET. Cutinases of various organisms have been investigated for the ability to biodegrade PET, in the database "Plastics-Active Enzymes Database (PAZy)" the cutinases active against PET are up to now 26 and are produced by various bacteria, archea and eukarya. In addition to cutinases, lipases (EC 3.1.1.3) and carboxylases (EC 3.1.1.1) have also been studied for their potential role in PET biodegradation (Buchholz et al., 2022). Considering all three enzymes the total amount registered in PAZy database able to degrade PET are 127. Up to now the best-studied model for the hydrolysis of PET is the one of *Ideonella sakaiensis* in which PET (composts of repeating units of terephthalic acid - TPA and ethylene glycol - EG) is hydrolased in the intermediate mono(2-hydroxyethyl) terephthalic acid (MHET). MHET is internalized by the cell and hydrolyzed into the two monomers: TPA and EG. The resulting monomers are then used for bacterial metabolism (Danso et al., 2019; Yoshida et al., 2016).

Compared to PET, the biodegradation of LDPE and PA has been less extensively studied, and not all biodegradation steps have been fully identified yet. LDPE is made of long chain polymers of ethylene; it undergoes cleavage into oligomers containing 10-50 carbon atoms, which can then be transported into microbial cells for further metabolism. Enzymes known for degrading lignin, have also been implicated in PE degradation. These include manganese peroxidase (EC 1.11.1.13), lignin peroxidase (EC 1.11.1.14), and laccases (EC 1.10.3.2), which facilitate the breakdown of the polymer structure and promote microbial assimilation (Ghatge et al., 2020). Other enzymes studied for LDPE degradation are proteases, lipases, cutinases, and alkane hydroxylases (Dey et al., 2020). In the PAZy database only to enzymes are registered for the biodegradation of PE (Buchholz et al., 2022). PA 6 is made by the ring-opening polymerization of caprolactam and has 6 carbon atoms in its repeat unit liked by amide bonds (Buchholz et al., 2022; Cooper, 2013). The biodegradation process involves the breakdown of amide bonds by various enzymes, such as cutinases, proteases, and amidases, leading to the formation of shorter polymer fragments. Additionally, reactive oxygen species and other oxidative agents further degrade the polymer chain, causing fragmentation and oxidation of functional groups. Ultimately, microorganisms metabolize the resulting compounds, completing the degradation process (Nierstrasz, 2009; Oulidi et al., 2024). In the PAZy database are listed 26 enzymes involved in PA biodegradation (Buchholz et al., 2022).

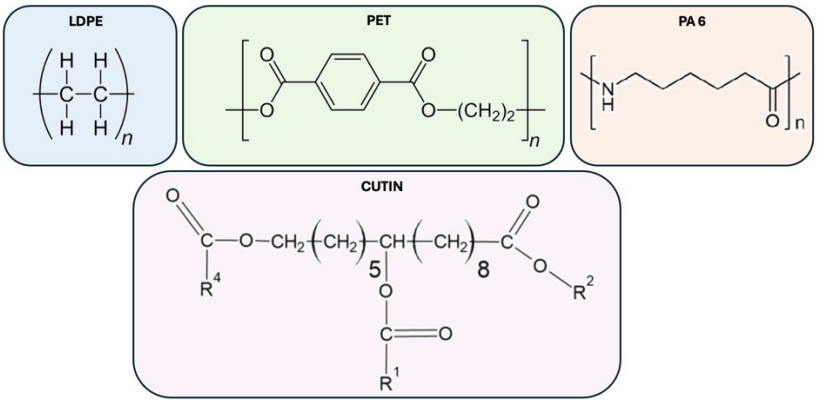


Fig. 5. Chemical structure of LDPE (low-density polyethylene), PET (polyethylene terephthalate), polyamide (PA) and cutin. The sources of the pictures are Plastics-Active Enzymes Database (Buchholz et al., 2022) for LDPE, PET; Castelvetro et al. (2021) for PA and Sales et al. (2021) for cutin.

Comparing our results with the limited literature available on MPs/plastic biodegradation by rumen microbiota, the observed biodegradation of PET is consistent with the findings of Quartinello et al. (2021), who demonstrated that rumen microbiota could partially degrade PET and other two natural polyesters (PBAT and PEF). In their study, the biodegradability of PET increased with prolonged incubation times (24 h, 48 h, and 72 h), consistent with our results; the biodegradability was assessed using a different approach, focusing on the analysis of hydrolysis by-products of PET, PBAT and PEF and employing scanning electron microscopy (SEM) to examine polymer surfaces before and after incubation. Similar to our approach, Galyon et al. (2022) used the Daisy^{II} Incubator to test polymers biodegradability by measuring sample weights before and after incubation. However, their research was limited to biodegradable polymers (PBSA (powder), PHA (powder), PBSA:PHA in powder and filament) and the incubation times were: 24 h, 48 h, 96 h and 240 h. The biodegradation percentages reported at 240 h were: PHA 0.53

± 0.13%, PBSA 0.49 ± 0.16%, PHA:PBSA (powder) 0.82 ± 0.20%, and PHA:PBSA (filament) 1.68 ± 0.33%.

In contrast to our findings, the *in situ* trial conducted by Galyon et al. (2023a) showed that LDPE did not exhibit partial degradation. In their study, LDPE, PHA, PBSA, and PHA:PBSA disks were tested, and degradation was monitored by measuring the weight of the samples before and after incubation at specific time points (days 0, 1, 14, 30, 60, 90, 120, and 150). This discrepancy could be attributed to differences in the dimensions of the LDPE samples (MPs in our study *vs* plastic disks in theirs), which affect the surface:volume ratio and the bioavailability of the material. Additionally, the incubation conditions (*in situ* in their study *vs* *in vitro* in ours) could further account for the observed differences in biodegradation.

Observing the MP stereomicroscope images, a progressive browning of PA is clearly visible with increasing incubation times with buffered rumen fluid (Fig. 4). The browning could be ascribed to two possible explanation:

i) absorption of rumen fluid residues;
ii) Maillard reaction between PA and reducing sugars (present in TMR, the substrate given to rumen *inoculum* during incubations), in which the amine ($-NH_2$) groups present in PA react with the carbonyl group of the reducing sugar in presence of water forming a Schiff base, which further undergoes complex rearrangements and condensation reactions, leading to the formation of advanced Maillard reaction products (Fig. 6). These products contribute to the progressive browning observed in PA over increasing incubation times (Ohe and

Yoshimura, 2014). The structural analyses conducing at University of Salerno will clarify if the browning of PA is caused by rumen fluid residues, Maillard reaction or another reason.

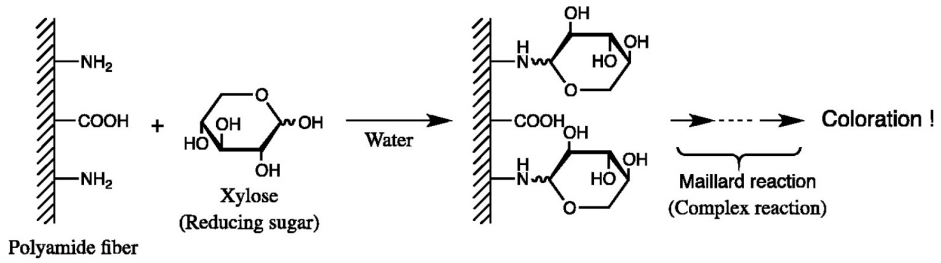


Fig. 6. Scheme of Maillard reaction between amine (-NH_2) group of polyamide and the carbonyl group of the reducing sugar (Ohe and Yoshimura, 2014).

5. Conclusions

This preliminary study is the first to demonstrate the ability of rumen microbiota to biodegrade different MP polymers in the order of PET > LDPE > PA during *in vitro* incubation. These findings highlight the potential of the rumen microbiota consortium as a promising tool in fighting MP pollution. Future research should focus on elucidating the hydrolysis by-products generated during MP biodegradation by rumen microbiota, identifying the microorganisms and enzymes involved, and expanding the investigation to other MP polymers. Additionally, studies should assess whether the process leads to complete mineralization and evaluate the potential health risks associated with MP by-products in ruminants. A deeper understanding of these mechanisms will provide insights into enhancing the natural ability of ruminants to biodegrade MPs, which they inadvertently assume through their diet, water, and air.

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Chapter 6: Conclusions

Ruminants are a fundamental component of global food systems, providing essential animal products (Gerber et al., 2013; Place, 2024; Pulina et al., 2017). With the continuous growth of the global population, the demand for ruminant-derived products is projected to increase accordingly, driving the expansion of ruminant farming and necessitating sustainable strategies to mitigate their environmental footprint (Du et al., 2022; United Nations, 2019; Worldometer, 2025).

Among the major challenges facing the ruminant livestock sector, MP contamination in ruminants remains an emerging and largely unexplored issue (Corte Pause et al., 2024; Lackner and Branka, 2024). MPs are insidious and often invisible contaminants that can pose toxicological risks to both animal and human health through the consumption of contaminated animal products (Su et al., 2025). Due to their small size and mobility, MPs can easily disperse throughout the environment, infiltrate the food chain, adsorb toxic chemical pollutants, cross trophic levels, and accumulate in various animal tissues (Peng et al., 2020; Su et al., 2025).

One of the primary sources of MP contamination in ruminant farming are feeds (Su et al., 2025). However, no standardized protocol had previously been developed to extract MPs from these complex organic matrices. In Chapter 2 (*Development of MP Extraction Protocol for Ruminant Feeds*), a novel extraction protocol was optimized for the first time, enabling the efficient degradation of organic matter in feeds without damaging MPs.

This protocol provides a valuable tool for assessing MP contamination levels in ruminant feeds (Glorio Patrucco et al., 2024).

Applying this protocol, Chapter 3 (*Assessment of MP Contamination in Ruminant Feeds*) investigated MP contamination in feed samples collected from farms in northwestern Italy. The results revealed that 100% of the tested feeds contained MPs, confirming that feeds represent a significant entry point for MPs into the livestock production system and a potential risk for contamination across the food chain. Among different feed types - corn silage, hay, and high-protein feedstuffs - corn silage exhibited the highest levels of contamination. LDPE was the most abundant polymer, likely reflecting its widespread use in farms (Borreani and Tabacco, 2017; Ramachandran et al., 2021).

Beyond their presence in ruminant feeds, MPs have been detected in various tissues and biological fluids, including feces (Beriot et al., 2021; Wu et al., 2024), blood, meat (van der Veen et al., 2022), follicular fluid (Grechi et al., 2023), and epididymal sperm (Grechi et al., 2024). Once ingested, MPs have the potential to interfere with physiological processes, raising concerns about their impact on ruminant health. Despite these concerns, research on the effects of MPs on ruminants remains limited (Corte Pause et al., 2024; Su et al., 2025). The few works conduceted investigated production performace, immune fuction, reproductive system and rumen microbiota (Chang et al., 2024; Grechi et al., 2023,2024; Wang et al., 2024; Su et al., 2025). In Chapter 4 (*Investigation of MP Effects on Ruminant Digestion*), it was demonstrated that PET MPs negatively affect ruminal degradability and gastro-intestinal digestibility,

suggesting potential consequences for nutrient utilization and overall animal performance (Tassone et al., 2024).

The presence of MPs in ruminants and their potential health consequences underscore the urgent need for solutions to mitigate this emerging problem. A promising solution for addressing MP contamination could come from ruminants themselves (Quartinello et al., 2021; Galyon et al., 2022, 2023a, 2023b). In Chapter 5 (*Study Rumen Microbiota Ability to Biodegrade MPs*), it was preliminary demonstrated the ability of rumen microbiota to biodegrade MPs. Among the tested polymers, PET exhibited the highest degree of biodegradation, followed by LDPE and PA. These findings highlight the potential of the rumen microbial community as a biological tool for MP biodegradation, offering a novel and environmentally sustainable approach to fight MP pollution.

To conclude, this PhD thesis has addressed critical challenges and potential solutions for improving livestock sustainability, transitioning from pollution to solution in the field of MPs. It highlighted the issue of MP contamination in ruminant feeds, demonstrating its negative impact on nutrient degradability and digestibility, while also identifying the rumen microbiota's partial ability to biodegrade MPs as a promising solution.

Future research should focus on:

i) applying the MP extraction protocol for feeds in large-scale, geographically diverse surveys, including both ruminant and non-ruminant feeds, and identifying the sources of contamination;

ii) investigation of the mechanisms underlying the harmful effects of PET MPs on ruminant digestion, including the evaluation of different contamination doses. Furthermore, extend the study to other polymers and feed types;

iii) studying the hydrolysis by-products generated during MP biodegradation by rumen microbiota, identifying the microorganisms and enzymes involved, and expanding the study to other MP polymers. Additionally, assessing whether the process leads to complete mineralization and the enhancement of this natural promising ability.

Appendix: From Pollution to Solution: Greenhouse Gas Emissions in Ruminant Livestock

1.1 Ruminant Greenhouse Gas Emissions

GHG emissions are a major concern for the planet. While GHGs naturally occur in the atmosphere and help regulate Earth's temperature, human activities have dramatically increased their levels, intensifying the greenhouse effect. This accelerated warming is driving climate change (Chen and Kong, 2023; European Parliament, 2024). In 2023 GHG emissions reached 53.0 Gt CO_2eq (without Land Use, land Use Change and Forestry = LULUCF), corresponding to an increase of 1.9% compared to the levels in 2022 (European Commission, 2024).

As can be observed in Fig. 1 the main anthropogenic sectors contributing to GHGs, ranked by emissions, are energy (primary subsector: electricity and heat), agriculture (primary end use: livestock and manure), industrial processes, waste, and LULUCF (Friedrich, 2024; United Nations, 2024). While the main GHGs, consistently ranked by emissions, are carbon dioxide (CO_2), CH_4, nitrous oxide (N_2O), and fluorinated gases (F-gases) (European Parliament, 2024; Friedrich, 2024; United Nations, 2024).

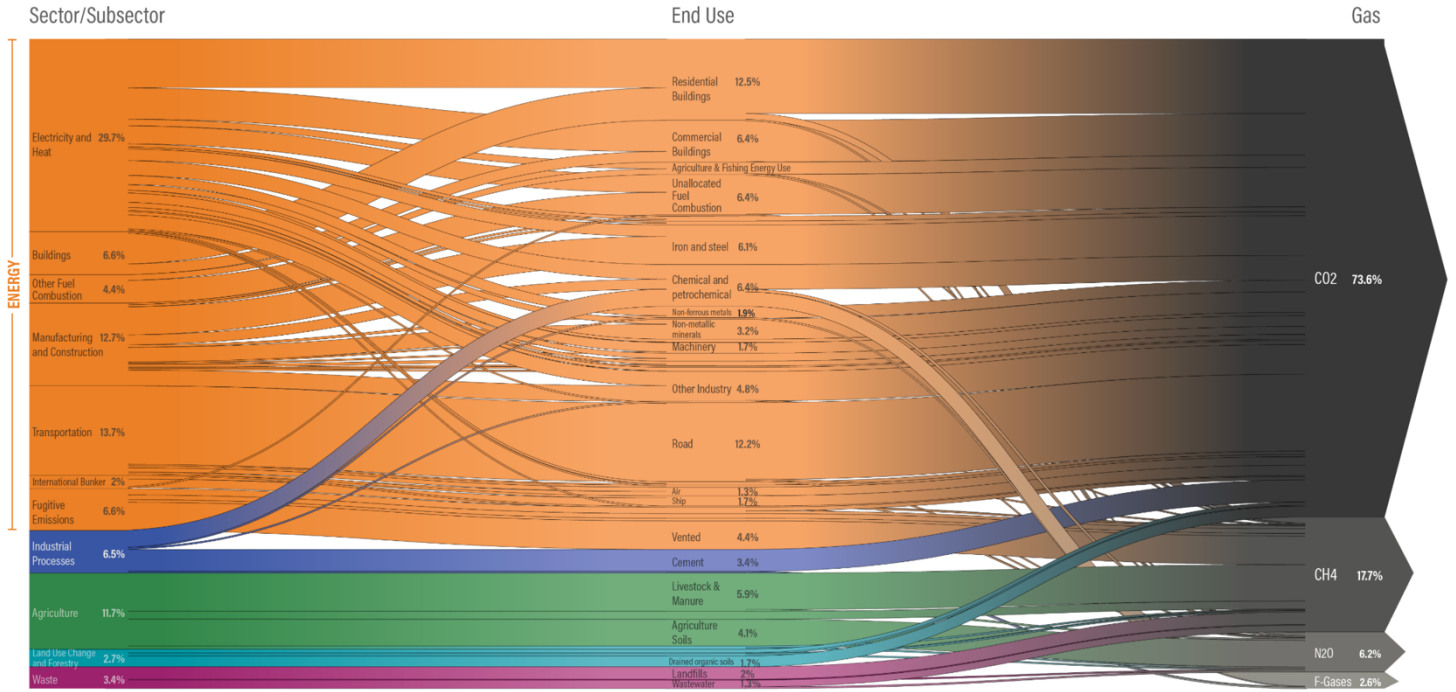


Fig. 1. Sankey flow chart illustrating the flow of global greenhouse gas (GHG) emissions by sector/subsector, end use, and gas type in 2021. The width of each flow represents the relative share of emissions (Friedrich, 2024).

Focusing on livestock, according to FAO, their GHG emissions account for about 12% of all anthropogenic GHGs - 6.2 Gt CO_2eq out of a total 50-52 Gt CO_2eq (FAO, 2022). In Fig. 2 is illustrated the distribution of GHG emissions in livestock done by FAO (2023b). Ruminants are the largest emitters, reaching 77% (cattle 62%, buffalo 8%, goat 4% and sheep 3%) of livestock GHG emissions. Only 23% of GHGs origin from monogastric animals (pig 14% and chichen 9%) (2023b). The primary GHG emitted by ruminants is CH_4, accounting for approximately 54% of total emissions from livestock, with the majority originating from enteric fermentation. A smaller portion of CH_4 emissions is attributed to anaerobic decomposition of manure. The second most significant GHG is CO_2, which predominantly arises from land use change (LUC), feed production, and post-farm activities. N_2O, the third most abundant GHG, primarily results from manure and feed production processes.

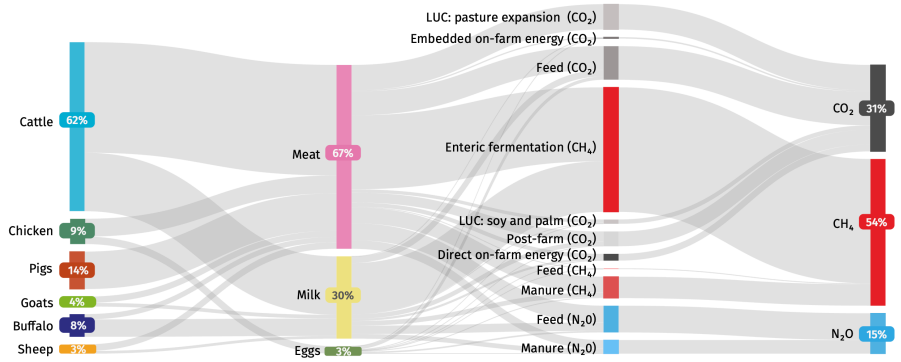


Fig. 2. Sankey flow chart of greenhouse gas emission sources by species, products, sources of emissions and gases (FAO, 2023b).

It is important to note that FAO accounted for both direct and indirect GHG emissions from ruminants. When considering only direct sources - namely enteric fermentation and manure (Sejian et al., 2016) - CH_4 remain

the dominant GHG, but N_2O become the second GHG followed by CO_2, that is also partially emitted by enteric fermentation and manure (Alonso-Moreno et al., 2018). About 3.7 Gt CO_2eq are emitted from direct sources of livestock, corresponding to 60% of total livestock emissions (FAo, 2023b). Focusing on the main sources of GHGs from ruminants, enteric fermentation allows ruminants to digest plant fiber materials through the symbiotic action of rumen microbiota (Waters et al., 2025). Methanogenic archaea play a crucial role in the ruminal ecosystem by utilizing CO_2 and H_2, to produce CH_4 through the process of methanogenesis (Mackie et al., 2024). This process prevents H_2 accumulation maintaining fermentation stability (Choudhury et al., 2022). However, as previously discussed CH_4 from enteric fermentation represent a significant contribution to global GHG emissions destinated to increase by 30% by 2050 due to a growing human population (Mackie et al., 2024).

1.2 Nutritional Strategies to Reduce Ruminant Greenhouse Gas Emissions

Decrease ruminant GHG emissions is a critical goal for limiting the global temperature rise over the next 30 years (Króliczewska et al., 2023). Regarding animal nutrition the main mitigation strategies can be divided in feed manipulation, probiotic, and additives as shown in Fig. 3 (Tseten et al., 2022).

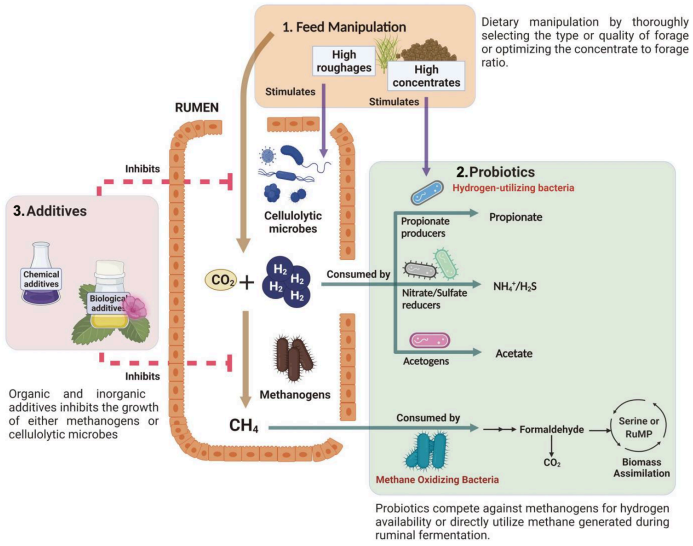


Fig. 3. Nutritional strategies to mitigate CH_4 from ruminants. Light brown line: flow of rumen fermentation, pink line: inhibition, violet line: stimulation, and green line: consumption (Tseten et al., 2022).

Concerning feed manipulation, the main strategies studied and applied are: change the type or quality of forage (as increase forage digestibility and chose forage legume species) and decrease dietary forage-to-concentrate ratio (Arndt et al., 2022; Eugène et al., 2021). These actions stimulate microorganisms that ferment starches and sugars into propionate. Propionate formation competes for H_2, reducing the amount

available for methanogens, leading to lower CH_4 production (Tseten et al., 2022). Must be noted that high-concentrate diets, rich in fermentable carbohydrates, can lead to increased production of lactic acid and VFAs in the rumen. This accumulation can lower ruminal pH, potentially causing subacute ruminal acidosis (SARA) (Lorenz, 2024).

Another strategy is the incorporation of probiotics. They are live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host (FAO, 2016). These microorganisms play a vital role in shaping the gastro-intestinal microflora, thereby influencing rumen fermentation (Króliczewska et al., 2023). Various rumen bacteria are thought to compete with methanogens for the hydrogen supply as propionate producers, nitrate/sulfate reducers and acetogens. Another category of probiotics is CH_4 oxidizing bacteria/methanotrophs that can use CH_4 as energy source (Pandey et al., 2014; Ncho et al., 2024).

Feed additives are considered the most promising strategy for mitigating CH_4 emissions from ruminants (Belanche et al., 2025). According to their mode of action they can be divided in direct or indirect inhibitors of methanogenesis. The direct ones act agaist methanogens, while the indirect ones alter the metabolic pathways leading to a reduction of the substrate for methanogenesis (Belanche et al., 2025; Durmic et al., 2025; Tseten et al., 2022). While considering their origin, they can be categorized in chemical and biological additives. The chemical ones are compounds produced synthetically, as 3-nitrooxypropanol (3-NOP), while biological molecules are naturally produced by plants, macroalgae, fungi, and microorganisms (Durmic et al., 2025). The main natural feed additives

studied are saponins, tannins, flavonoids, lipids and essensial oils (EOs) (Króliczewska et al., 2023).

Focusing on EOs, they are bioactive compounds, originating from the secondary metabolism of plants (Caroprese et al., 2023). EOs are characterized by two or three major compounds at relatively high concentrations (20-70%) that are responsible for their biological effects. They are produced in special cells or groups of cells including roots, bark, seeds, flowers, petals, leaves, fruit, and stems (Cobellis et al., 2016). Among EOs, the most studied for mitigating ruminant GHGs are those from thyme, oregano, lemongrass, clove, cinnamon, rosemary, dill, eucalyptus, garlic, and anise (Cobellis et al., 2016; Wells, 2024).

EOs antimicrobial properties enable them to influence methanogenesis both directly or indirectly (Michalak et al., 2021). Additionally, in some studies, their antimicrobial properties also contribute to lower CO_2 production in the rumen (Alabi et al., 2023; Bokharaeian et al., 2023; Ike et al., 2024). Beyond their effects on rumen fermentation, research highlights their benefits in improving milk yield, digestion, immune function, oxidative stress management, and feed palatability (Tudor, 2023). Recognized as safe (GRAS) by the Food and Drug Administration, EOs present a promising alternative for enhancing animal health, performance, and sustainability in livestock production (Food and Drug Administration, 2024). Ongoing research is actively investigating their effects, optimal dosages, and synergistic EO combinations.

Effects of Essential Oils on Rumen Fermentantion and GHGs

Published paper on Italian Journal of Animal Science (TAYLOR & FRANCIS)

https://doi.org/10.1080/1828051X.2025.2450509

ITALIAN JOURNAL OF ANIMAL SCIENCE
2025, VOL. 24, NO. 1, 266–280
https://doi.org/10.1080/1828051X.2025.2450509

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RUMINANTS NUTRITION AND FEEDING

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Influence of lemongrass and oregano essential oils and their combination on *in vitro* ruminal fermentation and greenhouse gas emissions in total mixed ration for dairy cows

Sara Glorio Patrucco[a], Alessandro Lotto[b], Martina Dorigo[b], Rita Fornaciari[b], Antonio Sagliano[b], Nicola Martinelli[b], Alessandra Cosani[b], Khalil Abid[a], Salvatore Barbera[a], Hatsumi Kaihara[a] and Sonia Tassone[a]

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ABSTRACT

Ruminants play a crucial role in the food chain, but are also considered contributors of greenhouse gas (GHG) emissions . Essential oils (EOs) are emerging as natural feed additives in ruminants' nutrition to enhance animal health, performance and reduce environmental footprint. Among EOs, lemongrass (*Cymbopogon winterianus*) and oregano (*Origanum vulgare*) EOs (LEO and OEO) have attracted attention as modulators of ruminal fermentations, but their role needs to be clarified. The experiment was designed using a randomised setup to assess the effects of LEO and OEO on *in vitro* ruminal fermentation and GHGs, using total mixed ration (TMR) as substrate (incubation time 24h). Experimental treatments included (doses as % of TMR on DM basis): 1) control only TMR (0% EOs) 2) 0.07% LEO 3) 0.07% OEO 4) 0.035% LEO + 0.035% OEO 5) 0.07% LEO + 0.07% OEO. Each treatment was repeated three times in two experimental runs. Only EO combinations reduced total gas (−9%, p=0.001). All EOs decreased CO_2 emissions by −5 to −12% with no significant differences between treatments (p<0.001), although anti-methanogenic effects were not observed (p=0.192). Volatile fatty acids were slightly affected only by EOs blend at the highest dose, resulting in a reduction of propionate (−1.3%, p=0.02), an increase in acetate:propionate (+0.16%, p=0.04) and isovalerate (+0.7%, p=0.03). LEO reduced pH (−0.6%, p=0.004), while OEO increased oxidation capacity (+4.2%, p=0.004), but both parameters remained within physiological ranges. Canonical discriminant analysis confirmed distinct EOs effects, highlighting their potential as natural additives for improving ruminal fermentation and mitigating ruminant environmental footprint.

HIGHLIGHTS

1. Lemongrass and oregano essential oils (LEO and OEO) alone and in blends demonstrated to reduce greenhouse gases, in particular carbon dioxide.
2. Only the blend of LEO and OEO at the highest dosage slightly influenced volatile fatty acids.
3. LEO and OEO did not modify, out of ruminant physiological ranges, pH, oxidation reduction potential and conductivity of rumen fluid.

ARTICLE HISTORY
Received 12 September 2024
Revised 23 December 2024
Accepted 2 January 2025

KEYWORDS
Lemongrass and oregano essential oils; *in vitro* ruminal fermentation; greenhouse gas emissions; methane; carbon dioxide

Introduction

Ruminant production sector plays a crucial role in the world food chain, significantly contributing to the provision of meat and dairy products (Godde et al. 2021; Michalak et al. 2021). As the global population is projected to reach 9.7 billion by 2050, the demand for these animal products is expected to rise accordingly (Henchion et al. 2021). However, livestock are a climate change driver, generating 14.5% of total anthropogenic greenhouse gas (GHG) emissions in the world (Giamouri et al. 2023). According to the Italian Institute for Environmental Protection and Research (ISPRA), agriculture in Italy accounted for 7.4% of GHGs in 2022, with 79% of these emissions originating from livestock (Vitullo et al. 2024). The main GHGs in

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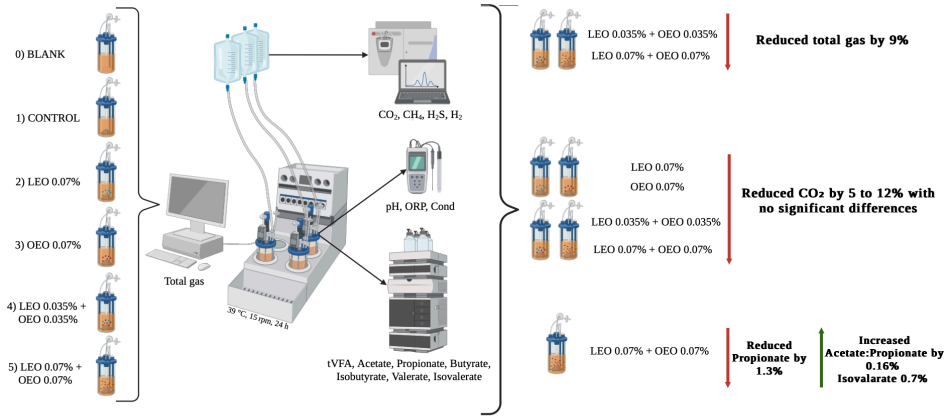
Influence of Lemongrass and Oregano Essential Oils and Their Combination on *In Vitro* Ruminal Fermentation and Greenhouse Gas Emissions in Total Mixed Ration for Dairy Cows

Sara Glorio Patrucco[a], Alessandro Lotto[b], Martina Dorigo[b], Rita Fornaciari[b], Antonio Sagliano[b], Nicola Martinelli[b], Alessandra Cosani[b], Khalil Abid[a], Salvatore Barbera[a], Hatsumi Kaihara[a], Sonia Tassone[a]

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Graphical Abstract



Abstract

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1. Introduction

Ruminant production sector plays a crucial role in the world food chain, significantly contributing to the provision of meat and dairy products (Godde et al., 2021; Michalak et al., 2021). As the global population is projected to reach 9.7 billion by 2050, the demand for these animal products is expected to rise accordingly (Henchion et al., 2021). However, livestock are a climate change driver, generating 12% of total anthropogenic greenhouse gas (GHG) emissions in the world (FAO, 2022). According to the Italian Institute for Environmental Protection and Research (ISPRA), agriculture in Italy accounted for 7.4% of GHGs in 2022, with 79% of these emissions originating from livestock (Vitullo et al., 2024). The main GHGs in livestock are methane (CH_4) and nitrous oxide (N_2O), followed by carbon dioxide (CO_2). CH_4 and CO_2 are primarily emitted through enteric fermentation and anaerobic manure decomposition, while N_2O is released through denitrification during manure management (Alonso-Moreno et al., 2018; Moran, 2005; Sejian et al., 2016). The challenge lies in balancing the need to increase livestock

production with the necessity to reduce climate impacts and GHGs (Michalak et al., 2021). One promising approach in livestock sector is the inclusion of feed additives in ruminant diets (Kataria, 2016).

Essential oils (EOs) have gained increasing interest as natural feed additives in livestock farming. They are volatile aromatic bioactive compounds originating from the secondary metabolism of plants (Caroprese et al., 2023; Cobellis et al., 2016b). EOs are studied and applied in ruminant diets especially for their potential to modulate ruminal fermentation and consequently GHGs by enhancing or inhibiting specific rumen microbiota populations (Brice et al., 2022; Caroprese et al., 2023; Kholif and Olafadehan, 2021). Some EOs can directly inhibit methanogenic archaea and/or indirectly decrease CH_4 production by depressing some microbial metabolic processes contributing to methanogenesis (Michalak et al., 2021). EOs can also reduce CO_2 production in the rumen due to their antimicrobial proprieties (Alabi et al., 2023; Bokharaeian et al., 2023; Ike et al., 2024). EOs' studies and applications in animal nutrition have significantly increased over the years, largely as a result of the ban imposed by European Union on the use of antibiotics as feed additives, due to the transmission and proliferation of resistant bacteria via the food chain (European Union, 2003; Zeng et al., 2015). The Food and Drug Administration categorised EOs as generally recognised as safe (GRAS) for human and animal consumption, making them an attractive alternative for enhancing animal health, performance and reducing their environmental footprint (Benchaar et al., 2008; Food and Drug Administration, 2024). In fact, EOs in ruminant nutrition are studied not only for their direct impacts on

ruminal fermentations, but also for their beneficial effects on milk yield, digestion, oxidative stress, immune system and feed palatability (Tudor, 2023).

Lemongrass (*Cymbopogon* spp.) and oregano (*Origanum vulgare*) essential oils (LEO and OEO) have attracted considerable attention in animal nutrition research due to their potential modulation of ruminal fermentation. LEO has been the focus of studies exploring this property (Nanon et al., 2014; Singh et al., 2018; Soares et al., 2023), and OEO has similarly been investigated for the same purpose (Cui et al., 2024; Zhang et al., 2021; Zhou et al., 2020). They have promising abilities to decrease total gas production, reduce CH_4 emissions and influence the quantity of total volatile fatty acids (tVFA) (Cobellis et al., 2016a; Singh et al., 2018; Zhou et al., 2020), but their roles and dosages are not completely defined (Cobellis et al., 2016b; Luo et al., 2024; Soares et al., 2023). Moreover, ruminal fermentation modulation of EOs can be enhanced combining them (Cobellis et al., 2016a), but to the best of our knowledge no research investigated the interaction effects of LEO and OEO blend. Furthermore, measuring often-overlooked parameters such CO_2 (Hernandez et al., 2017) hydrogen sulphide (H_2S) (Shah et al., 2020), hydrogen (H_2) (McCauley et al., 2020), oxidation reduction potential (ORP) (Huang et al., 2018) and conductivity (Fang, 2023) could offer crucial insights into LEO's and OEO's effects on ruminal fermentation and GHG emissions.

Based on the previous assumptions the aim of this study was to investigate the *in vitro* influence of LEO and OEO, both individually and in combination, on ruminal fermentation and GHGs, hypothesising that LEO

and OEO would individually reduce GHG emissions in ruminal fermentation, and that their combination would produce a synergistic effect, significantly enhancing fermentation efficiency and offering a sustainable strategy to mitigate the environmental impact of ruminant production.

2. Materials and Methods

2.1 Experimental Design

This study was conducted to investigate the influence of LEO and OEO additives on *in vitro* ruminal fermentation of the total mixed ration (TMR) of dairy cows for 24 h. A completely randomised design was applied, incorporating five experimental treatments and a blank (no EOs and no TMR) needed to correct for the effect of the *inoculum*. The experimental treatments included (doses as % of dry matter TMR): (1) control only TMR (0 % EOs), (2) LEO at 0.07% (L), (3) OEO at 0.07% (O), (4) a combination of LEO (0.035%) and OEO (0.035%) (LO1) and (5) a combination of LEO (0.07%) and OEO (0.07%) (LO2). The 0.07% dosage used in this study was determined through Nutristar S.p.A. extensive R&D experience. Initial dosages selection was based on studies investigating EOs as ruminal fermentation modulators (*e.g.* Hart et al., 2008; Kolling et al., 2018; Zhou et al., 2020). Building on this foundation, the company further refined the dosage through years of trial-and-error, ultimately identifying 0.07% EOs in the dry matter of TMR as the optimal concentration, which provided the best performance and proved practical for large-scale farm applications.

The parameters measured were total gas, ruminal gases (CO_2, CH_4, H_2S, H_2), pH, ORP, Cond and VFA. The obtained data (except pH, ORP and Cond) were corrected by subtracting the blank. The experiment was conducted in triplicate across two runs, resulting in a total of six replicates for each treatment (N = 6), as was done by Sahebi Ala et al. (2021). The overall experimental design is summarised in Fig. 1.

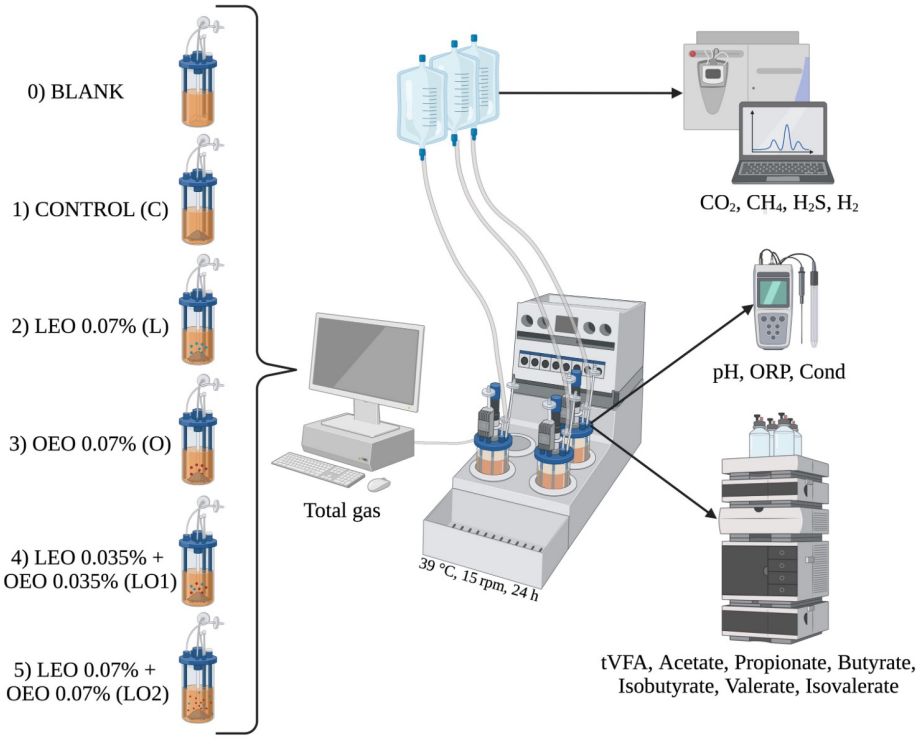


Fig. 1. Summary of the experimental design (ORP: oxidation reduction potential; Cond: conductivity; tVFA: total volatile fatty acids) created with BioRender.com.

2.2 Rumen Fluid Collection

Rumen fluid collection was conducted following the standardised method of Fortina et al. (2022). Briefly, for each run, the rumen fluid was collected in a slaughterhouse from the rumen of three clinically healthy,

multiparous dairy cows at the end of their productive lifespan. At the time of slaughter, the cows were not lactating and exhibited no clinical signs of metabolic disorders, infectious diseases, or nutritional deficiencies, confirming their overall health and ensuring that the rumen microbiota was not affected by any underlying health conditions. For each experimental run, 3 L of rumen fluid were collected from the rumen of each of three dairy cows, resulting in a total of 9 L, which were then thoroughly mixed. The rumen fluid was sampled immediately after slaughter and delivered to the laboratory within 30 min, maintaining it at 39 °C. It was then strained and kept at 39 °C under a CO_2 atmosphere. Filtered rumen fluid was mixed with the Menke et al. (1979) buffer (ratio 1:2, vol/vol).

2.3 Total Mixed Ration

The fermentation substrate was a TMR for dairy cows. The proportions of the ingredients and the chemical composition are detailed in Table 1. The chemical composition of the TMR was conducted according to AOAC standard procedures (AOAC, 2023). Dry matter (DM) (method 930.15) and ash content (method 942.05) using a Leco TGA801 gravimetric oven (Leco Instruments UK Ltd., Cheshire, UK); crude protein (CP) by Dumas combustion (method 990.03) using a LECO CN928 N Analyser (Leco Instruments UK Ltd., Cheshire, UK); ether extract (EE) was measured using Soxhlet extraction (method 920.39) (Fat Ex-tractor E-800, Büchi Labortechnik AG, Flawil, Switzerland); total starch content was determined using a total starch assay kit (Megazyme, International Ireland Ltd., Bray, Ireland) (method 996.11). Neutral detergent fibre (NDF) following the procedure of Mertens (2002) with the use of alpha amylase;

acid detergent fibre (ADF), was determined following the procedure of Van Soest et al. (1991). NDF and ADF were analysed using Ankom Delta Fibre Analyser (Ankom Technology Corporation, Fairport, NY). NDF and ADF content included ash.

Table 1.
Ingredients and chemical composition of total mixed ration used in the experiment.

Ingredient	**g kg^{-1} DM**
Corn flour	282
Meadow hay	260
Alfalfa hay	219.3
Wheat bran	130
Beet pulp	49.2
Soybean meal	43.5
Minerals and Probiotics mix	14
Monocalcium phosphate	1.5
Ammonium sulphate	0.5
Chemical composition	**(% of DM)**
DM (% fresh matter)	90.30
CP	14.80
EE	2.81
Ash	8.05
NDF	35.76
ADF	20.99
Total starch	22.79

DM: dry matter; CP: crude protein; EE: ether extract; NDF: neutral detergent fibre; ADF: acid detergent fibre.

2.4 Feed Additives

The feed additives used were the purified extracts of EOs derived from lemongrass (*Cymbopogon winterianus*) (LEO) and oregano (*Origanum vulgare*) (OEO). LEO and OEO are produced in leaves and flowers (Missopolinou et al., 2012; Mukarram et al., 2021). EOs yields as % of DM plant weight are 1-2% for LEO and 1-5% for OEO (Missopolinou et al., 2012; Ranade and Thiagarajan, 2015). These natural EOs were formulated into a powder by being absorbed onto a solid support comprising zeolite and silicon dioxide. Origin and main bioactive compounds composition

are detailed in Table 2. Additional information about the composition and formulation of LEO and OEO cannot be disclosed due to business confidentiality. These additives were incorporated into TMR, as detailed in the experimental design section, immediately before the incubation.

Table 2.
Lemongrass and oregano essential oils (LEO and OEO) origin and composition of main bioactive compounds.

	LEO	OEO
Species	*Cymbopogon winterianus*	*Origanum vulgare*
Family	Poaceae	Lamiaceae
Origin	Indonesia	Spain
Bioactive compounds	Citronellal (35 - 50%) Geraniol (15 - 25%) Citronellol (5 - 20%)	Carvacrol (60 - 75%) p-Cymene (4 - 15%) γ-Terpinene (4 - 10%) Thymol (0 - 5%)

2.5 *In Vitro* Fermentation and Total Gas Production Measurement

The *in vitro* fermentation was conducted according to the method of Braidot et al. (2023). Briefly, the apparatus used was the RITTER Biogas Batch Fermentation System composed of 18 RITTER PMMA fermentation bottles, each with 1 L of total available capacity. Each bottle was closed with an airtight cap equipped with a mixing system (rotation rhythm: 40 s of rotation followed by a 20 s stop at a speed of 15 rpm), connected with a flexible plastic tube (inner diameter 4 mm) to RITTER MilliGascounters and uniformly tempered at 39 °C in heating oven (Type BBFS-18). The total gas volume was automatically acquired from Biogas Batch Fermentation Systems through real time data logged in to Windows® software RIGAMO. In addition, each bottle was also connected with gas sampling bag (GSB-P/10, Dr.-Ing. Ritter Apparatebau GmbH & Co. KG, Germany) to collect and

quantify ruminal fermentation gases. Each bottle was filled with 750 mL of buffered rumen fluid plus 5.81 g of DM a combination of TMR and LEO or/and OEO additives at the percentages reported in the experimental design section and in Fig. 1.

2.6 Ruminal Gases Quantification: CO_2, CH_4, H_2S, H_2

After the *in vitro* fermentation, each gas sampling bag was analysed to quantify ruminal fermentation gases, in particular: CO_2, CH_4, H_2S and H_2, using the mass spectrometer (MS) (Hiden Analytical HPR-20 R&D). Calibration gases (single point calibration) consisting of CO_2 (69.78%), H_2 (1.96%), CH_4 (14.85%) and H_2S (0.101%) in argon were used to calibrate the MS using the software QGA (2.0.4) (Fig. 1).

2.7 pH, Oxidation Reduction Potential and Conductivity Measurements

After the incubation, the following parameters were measured: pH, oxidation reduction potential (ORP) and conductivity (Cond) using the XS pH 8 PRO Stirrer - Bench pHmeter with Electrode Standard DHS for pH and ORP and the XS COND 7 Vio portable conductivity metre with Cell 2301 T for Cond (Fig. 1).

2.8 Volatile Fatty Acids Quantification

After the incubation, 5 mL of buffered rumen fluid were collected and mixed with 5 mL of 0.01 N H_2SO_4. The samples were then stored at −20 °C until analysis.

To analyse VFA was followed the method applied by Spanghero et al. (2023). In brief, the samples were thawed at room temperature,

centrifuged at 9880 xg for 30 min at 10 °C and filtered through syringe filters (RC 0.45 µm, 25 mm; GVS North America Sanford, ME 04073 - USA). The filtrate was transferred into the autosampler vials and 20 µL were injected into high-performance liquid chromatography (HPLC) system. The system used was the Jasco Extrema LC-4000 including a Jasco PU-4180–HP pump, a Jasco UV-4075 UV-visible detector, a Jasco AS-4550 autosampler (20 µL loop) and a Jasco C0-4061 column oven (set at 40 °C). The HPLC separations were achieved using an Aminex HPX-87H column (300 mm × 7.8 mm) with a precolumn (Bio-Rad). Sulphuric acid 0.008 N was used as the mobile phase at a flow rate of 0.6 mL/min. Full spectra were recorded in the range of 190-400 nm and the optimum wavelength detection for all VFA was found to be 220 nm. Peaks of analytes were compared with the retention times of a standard mixture and quantification was based on the external standard method. VFA standards of acetate, propionate, butyrate, isobutyrate, isovalerate and valerate were obtained from Merck (Fig. 1) (Spanghero et al., 2023; Wang et al., 2021).

2.9 Statistical Analysis

Data from the two runs (total 6 repetitions for each treatment) were elaborated with RStudio 2022.07.2 and SAS 9.4. Statistical analyses were performed to evaluate the effects of feed additives (C, L, O, LO1, LO2) on gas production variables (total gas, CO_2, CH_4, H_2S, H_2), pH, ORP, Cond and VFA (tVFA, acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, acetate:propionate) using linear mixed-effects models (LME) with the 'lme' function from the 'nlme' package in R (version 3.1-161). The statistical model used was as follows: $Yij = \mu + Tj + Ri + eij$, where Yij is

each observation of the dependent variable (total gas, CO_2, CH_4, H_2S, H_2, pH, ORP, Cond or VFA), μ is the overall mean, *Tj* is the fixed effect of the feed additive treatment (C, L, O, LO1, LO2), *Ri* is the random effect of the run and *eij* is the residual error term. To verify the significance of the differences between feed additive treatments, post-hoc tests were conducted using the 'emmeans' and 'cld' functions from the 'emmeans' package (version 1.8.3). Estimated marginal means (EMMs) for each feed additive level were calculated, and pairwise comparisons were performed with Sidak adjustment for multiple comparisons. Experimental data were shown in the tables by means and standard deviation. Differences among means with $p < 0.05$ were represented as statistically significant differences.

To visualise and comprehensively analyse the effects of the tested additives, we employed Canonical Discriminant Analysis (CDA). This multivariate technique reduces dimensionality by finding linear combinations of quantitative variables that maximise separation between groups. Using feed additive levels as the classification variable, CDA generated canonical variables that captured the essential patterns of between-group variation. Prior to the CDA, we conducted Stepwise Discriminant Analysis (SDA) to identify the most significant parameters, streamlining the subsequent canonical analysis.

3. Results

3.1 Total Gas and Ruminal Gases Quantification: CO_2, CH_4, H_2S, H_2

Results of the total gas and ruminal gases quantification, as detailed in Table 3, indicate significant effects of the various treatments applied.

Specifically, the combination of the two EOs at both dosages, LO1 and LO2, resulted in a substantial reduction in total gas production compared to the C ($p = 0.001$). CO_2 emissions were significantly lower than that of the C for all the feed additives tested individually and in combination (L, O, LO1 and LO2) ($p < 0.001$). In contrast, CH_4 emissions were not significantly affected by any of the EOs treatments ($p = 0.192$). Furthermore, the production of H_2S remained unchanged across all the feed additive treatments ($p = 0.361$), while H_2 was reduced only by O when compared to C ($p = 0.022$).

3.2 pH, Oxidation Reduction Potential and Conductivity Measurements

Table 4 summarises the fermentation parameters after 24 h of the *in vitro* fermentation. In detail, pH was not affected by O, LO1 and LO2, while it was reduced by L ($p = 0.004$). Only O caused an increase of ORP which indicates greater oxidising capacity ($p = 0.004$). No significant differences in Cond were observed among the treatments ($p = 0.05$).

3.3 Volatile Fatty Acids Quantification

Table 5 reports the quantity of VFA measured after 24 h of the *in vitro* fermentation. No significant differences were observed for tVFA, acetate, butyrate, isobutyrate and valerate ($p > 0.05$). However, compared to C, LO2 caused a reduction in propionate ($p = 0.02$) and consequently an increase of the acetate:propionate ratio ($p = 0.04$). Moreover, with the same treatment, an increased production of isovalerate was recorded ($p = 0.03$).

Table 3.
Total gas and ruminal gases quantification: CO_2, CH_4, H_2S, H_2 (mL/g of feed dry matter) after 24 h of *in vitro* fermentation (N = 6).

	Treatment						
	C	**L**	**O**	**LO1**	**LO2**	**SEM**	**p VALUE**
Total gas	207 ± 21.8^{a}	194 ± 18.1^{ab}	200 ± 18.1^{ab}	188 ± 11.0^{b}	189 ± 13.6^{b}	6.6	0.001
CO_2	165 ± 22.0^{a}	151 ± 17.9^{b}	157 ± 19.2^{b}	147 ± 13.8^{b}	147 ± 15.4^{b}	5.4	<0.001
CH_4	40 ± 1.6	42 ± 0.9	42 ± 2.6	40 ± 3.3	41 ± 2.1	2.0	0.192
H_2S	0.5 ± 0.32	0.4 ± 0.36	0.7 ± 0.19	0.6 ± 0.12	0.6 ± 0.13	0.25	0.361
H_2	0.2 ± 0.04^{a}	0.2 ± 0.06^{ab}	0.1 ± 0.02^{b}	0.2 ± 0.02^{ab}	0.2 ± 0.01^{ab}	0.04	0.022

C = control; L = lemongrass 0.07%; O = oregano 0.07%; LO1 = lemongrass 0.035% and oregano 0.035%; LO2 = lemongrass 0.07% and oregano 0.07%. Different superscripts within a row indicate significant differences ($p < 0.05$). Results are expressed as mean ± SD.

Table 4.
Fermentation parameters: pH, oxidation reduction potential (mV) and conductivity (mS/cm) after 24 h of *in vitro* fermentation (N = 6).

	Treatment						
	C	**L**	**O**	**LO1**	**LO2**	**SEM**	**p VALUE**
pH	6.80 ± 0.060^{a}	6.76 ± 0.064^{b}	6.78 ± 0.076^{a}	6.81 ± 0.078^{a}	6.80 ± 0.082^{a}	0.004	0.004
ORP	-360 ± 6.3^{b}	-354 ± 5.8^{ab}	-345 ± 8.9^{a}	-349 ±1.9^{ab}	-349 ± 6.2^{ab}	1.2	0.004
Cond	12 ± 0.52	12 ± 0.68	12 ± 0.60	12 ± 0.42	12 ± 0.46	0.04	0.05

ORP = oxidation reduction potential; Cond = conductivity; C = control; L = lemongrass 0.07%; O = oregano 0.07%; LO1 = lemongrass 0.035% and oregano 0.035%; LO2 = lemongrass 0.07% and oregano 0.07%. Different superscripts within a row indicate significant differences ($p < 0.05$). Results are expressed as mean ± SD.

Table 5.
The quantity of volatile fatty acids after 24 h *in vitro* fermentation (tVFA as mg/mL; individual VFAs as % of tVFA) (N = 6).

	Treatment						
	C	**L**	**O**	**LO1**	**LO2**	**SEM**	**p VALUE**
tVFA	2.7 ± 0.41	2.9 ± 0.13	3.0 ± 0.11	3.2 ± 0.37	3.2 ± 0.46	0.06	0.07
Acetate	55.2 ± 2.28	55.8 ± 2.20	55.2 ± 1.82	55.3 ± 2.47	55.4 ± 2.28	0.09	0.50
Propionate	21.1 ± 1.2^{a}	20.4 ± 0.88^{ab}	20.4 ± 0.89^{ab}	20.1 ± 0.23^{ab}	19.8 ± 0.23^{b}	0.10	0.02
Butyrate	18.4 ± 1.53	17.9 ± 1.08	18.1 ± 0.71	18.2 ± 1.58	18.4 ± 1.50	0.16	0.79
Isobutyrate	0.98± 0.297	1.07 ± 0.158	1.16 ± 0.183	1.16 ± 0.152	1.16 ± 0.262	0.038	0.39
Valerate	2.90 ± 0.498	3.09 ± 0.441	3.03 ± 0.46	3.12 ± 0.495	3.12 ± 0.491	0.015	0.05
Isovalerate	1.43 ± 0.885^{b}	1.76 ± 0.832^{ab}	2.10 ± 0.883^{ab}	2.07 ± 1.056^{ab}	2.13 ± 1.096^{a}	0.087	0.03
Acetate:Propionate	2.63 ± 0.252^{b}	2.74 ± 0.217^{ab}	2.71± 0.203^{ab}	2.75 ± 0.154^{ab}	2.79 ± 0.136^{a}	0.013	0.04

C = control; L = lemongrass 0.07%; O = oregano 0.07%; LO1 = lemongrass 0.035% and oregano 0.035%; LO2 = lemongrass 0.07% and oregano 0.07%; tVFA = total Volatile Fatty Acids. Different superscripts within a row indicate significant differences ($p < .05$). Results are expressed as mean ± SD.

3.4 Canonical Discriminant Analysis of Selected Ruminal Fermentation Parameters

CDA was performed on 9 key ruminal fermentation parameters, selected from an initial set of 16 through Stepwise Discriminant Analysis (SDA). The selected parameters were pH, ORP, CO_2, H_2, tVFA, propionate (P), isobutyrate (IB), acetate:propionate ratio (AP) and isovalerate (IV).

Individual univariate analyses of these parameters showed limited discriminatory power, with R^2 values ranging from 0.05 to 0.45, with ORP showing the highest significance ($p < 0.01$). However, multivariate analysis revealed significant combined effects ($p < 0.0001$, Wilks' Lambda Test), suggesting strong interactions among parameters.

The first two canonical variables (Can1 and Can2) demonstrated robust discriminatory power, with squared canonical correlations of 0.89 and 0.83, respectively. Can1 accounted for 60.4% of the between-group variation, primarily distinguishing control (C) from lemongrass (L) treatments, while positioning the OEO treatments intermediately. Can2 explained an additional 36.3% of variation, separating both C and L groups from the OEO treatments. Together, these two canonical variables captured 96.7% of the total variation, providing a comprehensive representation of treatment effects.

The most influential discriminating parameters for Can1 were propionate, ORP, CO_2 and H_2, with pH, AP and IB also making a substantial contribution. As illustrated in Fig. 2 and 3, the multivariate relationships among these parameters revealed clear treatment-specific patterns. Notably, the ORP parameter showed that oregano's effects masked those

of lemongrass, suggesting a dominant influence of oregano in the combined treatments.

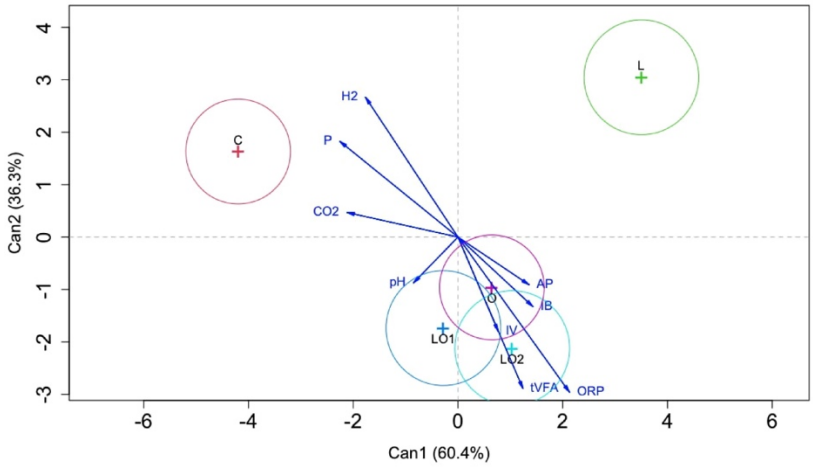


Fig. 2. Canonical discriminant analysis (CDA) of the nine selected ruminal fermentation parameters by a stepwise discriminant analysis: pH, ORP (oxidation reduction potential), CO_2, H_2, tVFA (total volatile fatty acids), P (propionate), IB (isobutyrate), AP (acetate:propionate), IV (isovalerate). C = control, L = lemongrass 0.07%, O = oregano 0.07%, LO1 = lemongrass 0.035% and oregano 0.035%, LO2 = lemongrass 0.07% and oregano 0.07%.

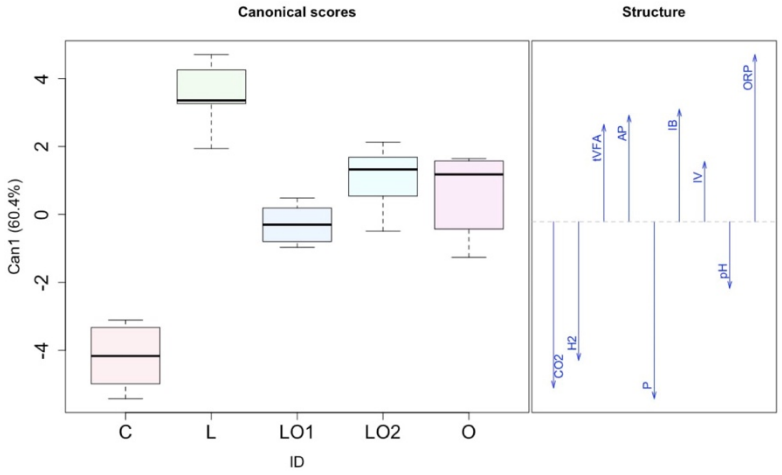


Fig. 3. Contribution of the 9 ruminal fermentation parameters to the first canonical variable (Can1). pH, ORP (oxidation reduction potential), CO_2, H_2, tVFA (total volatile fatty acids), P (propionate), IB (isobutyrate), AP (acetate:propionate), IV (isovalerate). C = control, L = lemongrass 0.07%, O = oregano 0.07%, LO1 = lemongrass 0.035% and oregano 0.035%, LO2 = lemongrass 0.07% and oregano 0.07%.

4. Discussion

In this study we investigated, for the first time, the combination of LEO and OEO at two dosages (LO1 and LO2) and we considered some generally overlooked ruminal fermentation parameters (CO_2, H_2S, H_2, ORP and Cond) to better understand the roles of LEO, OEO and their combination in the modulation of ruminal fermentation.

4.1 Total gas, Ruminal Gases: CO_2, CH_4, H_2S, H_2 and Volatile Fatty Acids Quantification

Total gas production was negatively affected by the combination of LEO and OEO at the two dosages LO1 and LO2, resulting in a decrease in total gas by about 9% compared to C, while no effect was observed for the additives used alone (L and O). A similar trend of reduction in total gas (9%) was showed by Joch et al. (2019) using, in an *in vitro* experiment, a blend of EOs (a mix of cresols, thymol, limonene, vanillin, guaiacol, eugenol and salicylates) at the dosage of 1000 mg/L (corresponding to 0.08% addition to TMR in each serum bottle), while no reduction was detected at lower doses. Also, Rossi et al. (2022) found a similar reduction in total gas (10%) caused by a combination of EOs (from cloves, coriander seed and geranium), tannins from chestnuts and flavonoids from olives at the dosage of 0.0025% on DM substrate (glucose) in an *in vitro* incubation. This result, obtained with lower dose at 0.0025% compared to our study at 0.07% (LO1) and 0.14% (LO2), could be due to the addition of tannins and flavonoids, both knows for their suppressive effects on ruminant GHG emissions (Min et al., 2020; Tedeschi et al., 2021). Alabi et al. (2023) tested, *in vitro*, four EO blends composed of four or five EOs at the dosage of 0.02% on TMR and registered a greater decrease of total gas ranging

from 12.7% (clove, oregano, peppermint, eucalyptus EOs) to 32% (garlic, lemongrass, cumin, lavender and nutmeg EOs). The higher reduction compared to our results could be due to the higher number of EOs used in the formulation of the blends (two in our study *vs* four/five in their work). Coşkuntuna et al. (2023) evaluated similar dosages of lavender essential oil: 0%, 0.05%, and 0.1% on dairy cow concentrate feed, observing no reduction in total gas production after 24 h of *in vitro* fermentation, consistent with our findings for LEO and OEO when not used in blends.

Both feed additives, whether used alone or in combination at two different dosages, demonstrated suppressive effects on CO_2 emissions compared to the C. Specifically, reductions averaged 9% for L, 5% for O, 11% for LO1 and 12% for LO2. Although there was a tendency toward greater effects with EO blends, the differences were not statistically significant. The reduction in CO_2 emissions contributes to lowering the carbon footprint of livestock production, which is a critical aspect in efforts to mitigate climate change (Kinley et al., 2020). The integration of these EOs is well aligned with the increasing consumer demand for sustainable and eco-friendly livestock production practices (Kamra et al., 2012). Similar, Coşkuntuna et al. (2023) observed reduction of 17% in CO_2 production at dosage 0.1% of lavender EOs on dairy cow concentrate feed. By contrast, in the same study, it did not find any CO_2 suppressive effect at the dosage of 0.05%. No CO_2 variation was found in the *in vitro* study of Günal et al. (2017) with LEO at the dosage of 500 mg/L of buffered rumen fluid (corresponding to 3.5% of LEO in diet) and in the *in vivo* study of Olijhoek et al. (2019) with OEO at dosages from 0.7% to 5.3%

of DM dietary. The discrepancies in CO_2 suppression effects among these studies could be attributed to variations in experimental condition (*in vitro vs in vivo*), animal diet (differences in TMR composition), EOs form and dosages.

LO1 and LO2 contribute to the reduction of total gas and CO_2 production. The potential mechanisms caused by LEO and OEO bioactive compounds could be:

i) inhibition of rumen microbiota: the hydrophobicity that is typical of EOs is responsible for the disruption of bacterial structures that leads to increased permeability due to an inability to separate the EOs from the bacterial cell membrane (Calsamiglia et al., 2007; Nazzaro et al., 2013);
ii) alteration of fermentation pathways: the bioactive compounds in LEO and OEO can interfere with enzymes involved in the decarboxylation reactions that lead to CO_2 production (Caroprese et al., 2023; Patra, 2011);
iii) selective bacterial activity: LO1 and LO2 may promote the activity of specific ruminal bacteria that produce less CO_2 and consequently reduce total gas (Alabi et al., 2023; Bokharaeian et al., 2023; Ike et al., 2024).

In our study, the EOs tested, both individually and in combinations, did not exhibit anti-methanogenic effects. These findings align with previous studies that evaluated the effect of LEO at dosages of 0.02, 0.04, 0.06% of TMR (Soares et al., 2023), 0.01, 0.02, 0.03% of TMR (Nanon et al., 2014) in *ex situ* and *in vitro* trials respecitivily; OEO at dosages of 0.005% of TMR

in vivo trial (Benchaar, 2020). In contrast, other studies have reported anti-methanogenic *in vitro* effects with LEO at 10, 20, 40, 80 µL/40 mL of buffered rumen fluid (corresponding to 0.002, 0.004, 0.009, 0.018% of DM diet, assuming the ISO 3217:2015 relative density of LEO = 0.872 − 0.897 g/mL) (Singh et al., 2018), 167, 333, 500, 667, 833 µL/L of buffered rumen fluid (corresponding to 0, 2, 5, 7, 10, 12% of DM diet, assuming the ISO 13171:2015 relative density of OEO = 0.930 − 0.955 g/mL) (Pawar et al., 2014); OEO at 91,130 mg/L of buffered rumen fluid (corresponding to 1.1% and 1.6% of OEO in feed) (Zhou et al., 2020) and 1.0, 1.5, 2.0 g/L of buffered rumen fluid (corresponding to 5, 10, 15, 20% of OEO in feed) (Cobellis et al., 2015). These differences suggest that the effectiveness of EOs in reducing CH_4 production could vary depending on different factors such as EOs form (liquid *vs* solid) and doses, animal diet (different composition of TMR or different substrate TMR *vs* concentrate), experimental conditions.

Monitoring the effects of feed additives on H_2S production is crucial, as high levels can cause toxic effects as polioencephalomalacia (Lévy, 2020). EOs are known to influence microbial populations; however, their impact on H_2S production has generally not been thoroughly investigated, despite their potential to affect sulfate-reducing bacteria and hydrogen-utilizing microbes (Patra, 2011; Shah et al., 2020). None of the tested additives showed an increase in H_2S levels compared to the C. Similarly, Coşkuntuna et al. (2023) did not observe any significant change in H_2S level with 0.05% lavender essential oil, while a reduction was observed at 0.1%. By contrast Alabi et al. (2023) found an increased production of H_2S in some EOs blends (mix of garlic, lemongrass, cumin, lavender and

nutmeg with and without fumaric acid at the dose of 0.02% of TMR), comparing to the control. The variation in H_2S production highlights the complexity of interactions between EOs and rumen microbial populations. Moreover, other parameters such as dietary sulphur levels, pH and dietary NDF content can also affect H_2S production (Shah et al., 2020).

EOs could inhibit hydrogen-producing bacteria such as *Lachnospira multiparus*, *Ruminococcus albus* and *Ruminococcus flavefaciens* and protozoa, resulting in a decreased production of H_2 and a consequent decrease of CH_4 (Cobellis et al., 2016a). In our study, only O influenced H_2 production, resulting in a 27% reduction compared to C. However, this reduction was not associated with changes in CH_4 emissions. The lack of correlation between reduced H_2 production and CH_4 emissions could be due to the complex and adaptive interactions within the rumen microbiota, where alternative metabolic pathways and microbial compensatory mechanisms can use H_2 as sink (*i.e.* reductive acetogens, sulphate-reducing bacteria, propionate producers, nitrate reducing bacteria) (Lan and Yang, 2019). The ineffectiveness of lemongrass in modulating H_2 is in agreement with the work of Günal et al. (2017) which used a dose of 500 mg/L of buffered rumen fluid (corresponding to 3.5% of LEO in diet).

In general, ruminal fermentation gases are closely linked to the production of total volatile fatty acids (tVFA) (Judd and Kohn, 2018). However, in our study, we observed a reduction in total gas and CO_2 production due to the inclusion of EOs, but this was not accompanied by

a corresponding decrease in tVFA levels, which remained unchanged. These findings align with previous studies on the role of EOs in ruminal fermentation. For example, Günal et al. (2017) reported that clove EOs reduced CO_2 and total gas production without affecting tVFA concentrations. Similarly, Alabi et al. (2023) found that a blend of EOs (including anise, clove, oregano, cedarwood and ginger) reduced total gas production without causing a decrease in tVFA. Additionally, in a study by Patra and Yu (2012), five EOs -clove, eucalyptus, garlic, oregano and peppermint- led to a reduction in fermentation gases, but had mixed effects on tVFA, with decreases observed for clove and oregano, no changes for garlic and peppermint and an increase for eucalyptus. The differing results regarding OEO between our study and the work of Patra and Yu (2012) (no reduction of tVFA *vs* reduction of tVFA) could be attributed to the different dosages used (0.07% *vs* 0.25, 0.5, and 1 g/L of buffered rumen fluid, corresponding to 2.5%, 5%, and 10% EOs, respectively). These findings emphasise the complex interactions between EOs and ruminal fermentation, highlighting that more research is needed to fully understand the underlying mechanisms and the varied effects of different EOs.

Individual VFA were not affected by any of the feed additives, with the notable exception of LO2. The ineffectiveness of EOs (except LO2) in modulating VFA concentrations indicate that they did not negatively impact ruminal fermentation. This observation aligns with the overall lack of impact on VFA described in the review of Cobellis et al. (2016b), in which similar results were observed with various EOs at the dosage of 0.1 g/L of buffered rumen fluid (0.8% of EOs in feed) including: *Ocimum*

basilicum, lemongrass, *Eucalyptus citriodora*, *Ocimum gratissimum*, *Laurus nobilis*, *Mentha piperita*, etc.

By contrast at the highest dosage, lemongrass and oregano EOs blend (LO2) significantly reduce propionate levels and increased isovalerate compared to the control. However, similar trends for propionate, though not statistically significant, are also observed in the individual EOs (L and O) and in the blend at the lowest dosage (LO1). This suggests that both EOs have the potential to inhibit propionate production, with the synergistic effect at the highest dose being significant compared to C. The reduction of propionate can be attributed to the selective antimicrobial effects of the essential oil compounds against bacteria that are key propionate producers, as *Selenomonas ruminantium* and *Megasphaera elsdenii* (Benchaar et al., 2007; Wang et al., 2012). Phenolic compounds, as thymol and caravacrol can disrupt the outer membranes of these Gram-negative bacteria, as demonstrated by Helander et al. (1998) and Dorman and Deans (2000), thereby impeding their growth and metabolism. Additionally, thymol has been shown to suppress the growth of *Streptococcus bovis* (Evans and Martin, 2000), a significant amylolytic bacterium involved in propionate production through starch fermentation (Benchaar et al., 2007). Citronellal and citronellol may exhibit antimicrobial properties similar to those of thymol and carvacrol. While their effectiveness has been demonstrated against *Escherichia coli* and *Staphylococcus aureus* (Lopez-Romero et al., 2015), their selective action against key propionate-producing bacteria remains to be explored.

The increase in isovalerate levels reflects a shift in fermentation pathways. Isovalerate is produced during the breakdown of branched-chain amino acids (decarboxylation and deamination) and the inhibition of propionate-producing microbes likely allows proteolytic bacteria to thrive (Allison, 1978; Apajalahti et al., 2019; Wallace et al., 1997).

According to our results, Patra and Yu (2012) observed a decrease in propionate and an increase in AP with oregano and peppermint EOs at the dosage of (0.25, 0.5, 1 g/L of buffered rumen fluid, corresponding to 2.5, 5, 10% EOs in feed).

These changes in the VFA profile could have important implications for dairy cattle. The higher AP could improve the fat content of dairy products (National Research Council (U.S.), 1988). However, the reduction in propionate levels, observed with LO2 supplementation, may impair glucose availability, potentially leading to decreased milk yield (García-Roche et al., 2021). Therefore, dairy nutritionists should carefully evaluate the inclusion of LO2 in feed formulations, aiming to balance the benefits for milk composition with the potential impacts on overall milk production.

4.2 pH, Oxidation Reduction Potential and Conductivity Measurements

To assess rumen health and ensure proper metabolic function, monitoring pH, ORP and Cond is essential. Despite their importance, few studies have thoroughly examined these parameters in relation to EOs (Fang, 2023).

In our study, rumen pH ranged from 6.76 to 6.80, consistently within the ideal range of 5.5 to 7.0 for optimal rumen functions (Fregulia et al.,

2021). Only LEO caused a slight reduction in pH, but the change was less than 1%. This slight reduction is unlikely to cause adverse effects on rumen health, as it remains well within the physiological range required for maintaining microbial balance and preventing acidosis (Fregulia et al., 2021). Our findings are consistent with Soares et al. (2023), who found similar pH values with LEO (0.02, 0.04, 0.06% of TMR - *in vivo* study), ranging between 6.68 to 6.77 without any statistical differences in comparison to the control. Benchaar (2020), also stated that OEO and its main bioactive compound, carvacrol, (0.005% of TMR - *in vivo* study) did not affect ruminal pH. The absence of LEO effect on pH suggests that these additives do not disrupt the rumen's acid-base balance, which is critical for ensuring efficient fermentation (Riond, 2001).

ORP values of rumen fluid typically shows negative readings, reflecting the strong reducing power of the rumen environment caused by microbial fermentation activity (Dijkstra et al., 2020). In our study ORP values ranged from -360 mV in the C to -345 mV in the O treatment, remaining within the physiological range for ruminants (-430 mV to -115 mV) according to the review of Huang et al. (2018). These values reflect a stable reducing environment conducive to effective microbial activity, which is important for maintaining the health of rumen microorganisms and supporting overall digestive function (Huang et al., 2018).

Cond of rumen fluid is rarely investigated despite its potential role in better understanding the nutritional elements and ion activities during the fermentation process (Fang, 2023). In our study Cond was 12 mS/cm and did not differ among the treatments. This value was in the range

found in few previous studies from 11.4 to 18.3 mS/cm (Mayberry et al., 2009; Peñafiel and Ticona, 2015)

4.3 Canonical Discriminant Analysis of Selected Ruminal Fermentation Parameters

CDA revealed distinct clustering patterns, clearly separating the treatments into three groups: C, L and a cluster containing O, LO1 and LO2. Can1 captured the majority of the discrimination (60.4%). The key parameters driving this discrimination were propionate (P), ORP, CO_2 and H_2. Although each of these variables showed statistically significant differences when analyzed individually, none alone reproduced the clear treatment separation achieved by the combined canonical analysis. This highlights the strength of CDA in capturing complex, multidimensional patterns not apparent in univariate assessments. These parameters reflect changes in microbial activity and fermentation pathways induced by the EOs.

The C group represents typical ruminal fermentation, with balanced microbial activity and normal fermentation profiles. In contrast, treatments involving LEO, OEO and their combinations (LO1, LO2) displayed distinct shifts in fermentation patterns. Specifically, oregano-based treatments were particularly effective in reducing propionate production, lowering CO_2 and H_2 emissions and altering ORP. These effects are likely due to the antimicrobial properties of bioactive compounds in oregano, such as carvacrol and thymol, which target specific ruminal microbes involved in CO_2 and propionate production. This suggests that OEO treatments influence fermentation pathways by modulating the

microbial community, particularly those microbes responsible for the production of these gases and fermentation end-products.

Previous studies have applied multivariate techniques as CDA and Canonical Correspondence Analysis (CCA) primarily to explore the effects of different diets on various parameters, including fermentation kinetics (Gannuscio et al., 2024), the relationship between bacterial community structure and ruminal fermentation (Belanche et al., 2019), dimethylacetals in rumen fluid (Cappucci et al., 2018), volatile organic compounds in beef meat (Vasta et al., 2011) and the fatty acid profile of muscle (Cama-Moncunill et al., 2021). These studies highlight the growing importance of multivariate approaches in understanding complex biological processes. However, to the best of our knowledge, our study is the first to apply CDA specifically to examine the impact of EOs on ruminal fermentation parameters.

5. Conclusions

LEO and OEO, both individually and in combinations significantly modulated ruminal fermentation and reduced GHGs, specifically CO_2, although their anti-methanogenic activity was not confirmed. The VFA profile was slightly affected only by the combination of the EOs at the highest dose, resulting in a reduction in propionate and an increase in AP and isovalerate. Moreover, the addition of EOs did not influence pH, ORP and Cond, that remained within the physiological range for ruminants. The CDA confirmed distinct effects of the EOs, highlighting their potential as natural feed additives for improving ruminal fermentation and mitigating the environmental impact of ruminant livestock production.

Based on these outcomes, we would recommend LO2 as the most promising treatment for future studies aiming to further investigate EOs as natural feed additives for reducing the environmental impact of ruminant livestock production. Future studies should include feed degradability and microbiota analysis to gain a deeper understanding of the effects of LEO and OEO on ruminal fermentation. Furthermore, investigating potential synergies with other feed additives and assessing the economic feasibility of incorporating these EOs into ruminant diets would be highly beneficial.

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Published Papers and Conference Presentations

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1. Fortina, R., **Glorio Patrucco, S.**, Barbera, S., Tassone, S., 2022. Rumen Fluid from Slaughtered Animals: A Standardized Procedure for Sampling, Storage and Use in Digestibility Trials. Methods Protoc. 5, 59. https://doi.org/10.3390/mps5040059.
2. Tassone, S., Mabrouki, S., Barbera, S., **Glorio Patrucco, S.**, 2022. Laboratory Analyses Used to Define the Nutritional Parameters and Quality Indexes of Some Unusual Forages. Animals 12, 2320. https://doi.org/10.3390/ani12182320.
3. Mabrouki, S., Brugiapaglia, A., **Glorio Patrucco, S.**, Tassone, S., Barbera, S., 2023. Texture profile analysis of homogenized meat and plant-based patties. Int. J. Food Prop. 26, 2757–2771. https://doi.org/10.1080/10942912.2023.2255758.
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5. **Glorio Patrucco, S.**, Rivoira, L., Bruzzoniti, M.C., Barbera, S., Tassone, S., 2024. Development and application of a novel extraction protocol for the monitoring of microplastic contamination in widely consumed ruminant feeds. Sci. Total Environ. 947, 174493. https://doi.org/10.1016/j.scitotenv.2024.174493.
6. Mabrouki, S., Abid, K., Kaihara, H., **Glorio Patrucco, S.**, Tassone, S., Barbera, S., 2024. Assessing texture profile analysis in natural state

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8. Tassone, S., Barbera, S., Kaihara, H., **Glorio Patrucco, S.**, Abid, K., 2024. First Evidence of the Effects of Polyethylene Terephthalate Microplastics on Ruminal Degradability and Gastro-Intestinal Digestibility of Mixed Hay. Animals 14, 2139. https://doi.org/10.3390/ani14152139.
9. Tassone, S., Kaihara, H., Barbera, S., **Glorio Patrucco, S.**, Issaoui, R., Abid, K., 2025. Low-Density Polyethylene Microplastics in the Rumen: Implications for Rumen Fermentation Dynamics and Utilization of ConcentrateFeed. Animals 15,297. https://doi.org/10.3390/ani15030297.
10. **Glorio Patrucco, S.**, Lotto, A., Dorigo, M., Fornaciari, R., Sagliano, A., Martinelli, N., Cosani, A., Abid, K., Barbera, S., Kaihara, H., Tassone, S., 2025. Influence of lemongrass and oregano essential oils and their combination on in vitro ruminal fermentation and greenhouse gas emissions in total mixed ration for dairy cows. Ital. J. Anim. Sci. 24, 266–280. https://doi.org/10.1080/1828051X.2025.2450509.

Conference Presentations and Posters:

1. IEEE International Workshop on Measurements and Applications in Veterinary and Animal Sciences (IEEE MeAVeAS), Naples, Italy, 26-28 April 2023: Rapid detection of microplastics in feeds using NIR Spectroscopy.
2. Italian Animal Science and Production Association (ASPA) Congress, Monopoli, Italy, 13-16 June 2023: Microplastic contamination in dairy cows' total mixed ration (Awarded as one of the seven best posters).
3. International Conference on Micro and Nano-Plastics in the Agri-Food Chain (AgriFoodPlast), Piacenza, Italy, 10-12 September 2023: 1) Microplastic contamination in ruminant feeds: an emerging issue for livestock sector; 2) Microplastics detection in mixed hays using NIR spectroscopy; 3) Preliminary results on the degradation of three microplastic polymers by rumen microbiota.
4. IEEE International Workshop on Measurements and Applications in Veterinary and Animal Sciences (MeAVeAS), Grugliasco, Italy, 22-24 April 2024: 1) Assessment of ANKOM Daisy^{II} Incubator for measuring microplastic degradability in rumen environment; 2) *In vitro* gas production technique applied to measure the impact of low-density polyethylene microplastics on lamb ruminal activity; 3) Assessment of microplastic contamination levels in mixed hay for dairy cows.
5. European Federation of Animal Science (EAAP) Congress, Florence; Italy, 1-5 September 2024: 1) Impact of low-density polyethylene microplastic on ruminal degradability of feeds; 2) Seasonal nutritional values of *Rubus fruticosus* leaves as a sustainable alternative feed for goat in mountain regions.

Acknowledgments

First of all, I would like to thank my supervisor, Sonia Tassone, I remember that August day in 2021 as if it were yesterday. As soon as you found out that I was looking for a job, you immediately told me to come to the University because there might be an opportunity for me. Shortly after, I embarked on my PhD journey. You have supported me not only professionally but also on a personal level, always prioritizing my well-being above everything else. You truly have a big heart, and we have always understood each other because we are so alike even in our hypochondria! Jokes aside, thank you for always being by my side and guiding me through this journey. You never made me feel under pressure, always gave me the time I needed, and, most importantly, believed in me. I am deeply grateful for that.

Thank to Salvatore Barbera, although not officially, you have practically been my co-Supervisor! You were always by my side, despite my pessimism and my natural tendency to find problems rather than solutions. I really hope to improve in that regard! Thank you for showing me that work should be driven by passion. I have always seen in you a deep enthusiasm for your work and an innate curiosity, fundamental qualities for a researcher. You and Sonia, the founders of the BesTeam, have always treated me and the other members as equals, fostering an open and collaborative environment where our ideas were truly heard. I will definitely miss our BesTeam meetings! Last but not least, you have constantly encouraged me to stay active, challenging me and my colleagues with training competitions - which we almost always lost!

Thank to Luca Rivoira and Maria Concetta Bruzzoniti for introducing me to the world of MP extractions and guiding me through it. I am truly grateful for your guidance and support. I had the pleasure of working with you for half of my PhD, and together we achieved excellent results.

Continuing my acknowledgments at University, I would like to thank my fellow travelers on this journey, my collegues! The first colleagues I had the luck of working alongside were Sabah and Carmine. Every single day with you at work was filled with smiles! One of my favourite memories with you is the conference in Monopoli. Hatsumi and Khalil, you are both part of the BesTeam with me! Thank you for sharing the microplastics research with me. Hatsumi, you taught me what it means to work in Japan! You are focused, precise, impeccable - and you drink a lot of tea! Khalil, you are truly passionate about what you do and an absolute workaholic! Thank you both for working with me and for all the help you gave me in my research. Thank also to all the other colleagues I had the pleasure to meet: Hideaki, Neyrouz, Flavia, Damiano, and Roberto. Our coffee breaks and birthday celebrations at work will always remain among the most enjoyable moments I experienced at the University.

During my PhD, I had the pleasure of working at Nutristar - what can I say? Thank you! From day one, I felt at home at Nutristar. Thanks to Nicola, especially for our talks while analyzing milk! Beyond that, thank you for your incredible professionalism; it was truly a pleasure working alongside you, and I am grateful for all the support you gave me in my research. Thank you, Antonio, it was a joy working with you! You supported me in my research and always managed to make me smile. A big thank also to Martina, Alessandra, Sara, Simone, Raffaele, Alice, Arianna, Tommaso,

and Surendra (sorry if I misspelled it!). Most importantly, thank you to Alessandro Lotto for giving me the opportunity to do my industry internship at Nutristar.

Moving on to personal thanks, I would like to start by thanking my family. Thank you, Mom, for always supporting me, for always believing in me, and most importantly, for always allowing me and my brother Kevin to choose what to do with our lives, always standing by us and ready to help. You are the most sensitive and at the same time the strongest person I know. If one day I will become a mother, I would to be at least half the mum you are. I love you so much, Mamy! Thank you to my little brother, no longer little, Kevin. Over these years, I have seen you grow into a man, and I am so proud to be your sister. I will always be there for you! Thanks to my amazing Grandma Bilella, no words are enough to describe you. I will just say that you are the pillar of our family. Thank you for everything! Thanks to Olly for treating me as if I were your daughter and for always being there for me, even for all the moves I have had to do during these years of my PhD!

A special thanks goes to my boyfriend, Matteo. The most beautiful gift this PhD has given me is the chance to live with you every day. We have shared the entire PhD journey together, supporting each other, in addition to having followed the Master's Degree together, which gave us the opportunity to meet and fall in love. My luck and your misfortune were that we had our office windows right in front of each other! Joking aside, thank you because from the very first day we got together, you have always made me laugh, feel protected, and most importantly, loved. With the PhD, we had the opportunity to live together, a step that has

strengthened our love even more and recently allowed us to expand our family with our little Tamarindo. I hope to live by your side through all our future personal and professional achievements. I love you so much.

Thanks to my storic friends: Paola, Marta, Chiara and Giorgia.

I want to dedicate the final part of my acknowledgements to two persons who, unfortunately, passed away during my PhD.

Iolanda, my boyfriend's grandmother. I truly felt like your granddaughter. You were and still are like a grandmother to me. The Sundays spent having coffee with you and Matteo before going to Grugliasco are the most beautiful memories I hold in my heart. You had a gold heart, and you passed it to your grandson, Matteo. I am grateful for the honor of having known you and spent time with you. I love you, "*Sagrinte Nen*", I will take care of your grandson.

Dear nonno Sergio, "*Il mio nonno col bastone*", I wish I could have celebrated this success with you, but I know you are still with me. Losing you was the most painful moment of this PhD and my entire life. I miss going to Diano and seeing you sitting in the chair at Roseto near the turtle pond... I miss having lunch with you, I miss you. You always told me, "*Sara sarà famosa*", and you loved me so much because, after all, I am your first granddaughter… I hope in my life to achieve even just a fifth of what you were able to obtain. You taught me to be non-conformist and, most importantly, as always you said "*Prima faccio, poi parlo e poi penso*". I hope in life to make you proud.

A te nonno, dedico questo traguardo e tutti quelli che raggiungerò.
Sei il faro in mezzo al mare che mi guiderà per sempre nella mia vita.