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DEVELOPMENT OF QUALITATIVE AND QUANTITATIVE ANALYSIS FOR THE ASSESSMENT OF MICROPLASTICS IN FOOD PRODUCTS

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DECLARATION

I declare that the present thesis has not been previously submitted as an exercise for a degree at the University of Padova (Italy), or any other university.
I further declare that this thesis is my own work.

CONTENTS

ABSTRACT

The presence of microplastics in the environment has increasingly attracted the attention of the scientific community due to their widespread distribution, persistence, and potential adverse effects on human health. Although numerous studies have investigated microplastics in water and seafood, information on animal-based foods remains limited, despite their crucial role in human diet.

With this background, the objectives of the present Ph.D. thesis were: i) to investigate the occurrence, characteristics, and potential sources of microplastic contamination in milk and dairy products; ii) to assess microplastic contamination in skim-milk powders; and iii) to characterize microplastics in beef hamburgers.

The Chapter 1 addressed microplastic contamination in milk, fresh cheese, and ripened cheese, with the dual aim of qualitatively and quantitatively characterizing the microplastics present and comparing contamination levels across these different dairy matrices.

The Chapter 2 extended the investigation to skim-milk powders used for cheesemaking across European countries, applying a specific analytical protocol to identify plastic particles and evaluate the extent of contamination in this widely used food ingredient.

Finally, the Chapter 3 expanded the investigation to beef hamburgers, aiming to provide insights into the occurrence and heterogeneity of microplastics in processed animal-based foods.

The results of this doctoral thesis confirm, as expected for most food products, that microplastics are also widespread in foods of animal origin, including

processed dairy and meat products. Considering the variability in their presence, morphology, and polymer types, the findings highlight the need for further research aimed at validating the outcomes of this study, investigating the main sources of contamination, and identifying effective strategies to reduce the occurrence of microplastics arising from the processing of raw materials into marketed products.

RIASSUNTO

La presenza di microplastiche nell'ambiente ha suscitato crescente interesse nella comunità scientifica a causa della loro diffusione ampia e persistente e dei potenziali effetti negativi sulla salute umana. Sebbene numerosi studi abbiano indagato la presenza di microplastiche nelle acque e nei prodotti ittici, le indagini sugli alimenti di origine animale rimangono limitate, nonostante il loro riconosciuto importante ruolo nella dieta umana.

Gli obiettivi del presente progetto di dottorato sono stati: i) indagare la presenza, le caratteristiche e le possibili fonti di contaminazione da microplastiche in latte e prodotti lattiero-caseari; ii) valutare la contaminazione da microplastiche in campioni di latte in polvere scremato; iii) analizzare la presenza e le caratteristiche delle microplastiche in hamburger di manzo.

Il Capitolo 1 ha indagato la contaminazione da microplastiche in latte, formaggi freschi e formaggi stagionati, con il duplice obiettivo di caratterizzare qualitativamente e quantitativamente le microplastiche presenti e di confrontarne i livelli tra le diverse matrici lattiero-casearie.

Il Capitolo 2 ha esteso l'indagine a campioni di latte in polvere scremato utilizzato nei processi di caseificazione industriale, applicando un protocollo analitico dedicato per identificare le particelle plastiche e valutare l'entità della contaminazione in questo ingrediente di diffuso utilizzo.

Infine, il Capitolo 3 ha esteso l'indagine ai prodotti a base di carne, attraverso uno studio preliminare sugli hamburger di manzo, al fine di fornire indicazioni sulla

presenza e sull'eterogeneità delle microplastiche negli alimenti trasformati di origine animale.

I risultati del presente lavoro di tesi confermano, come atteso per la maggior parte degli alimenti, che le microplastiche sono diffuse anche negli alimenti di origine animale, inclusi i prodotti trasformati lattiero-caseari e i prodotti trasformati a base di carne. Considerando la variabilità nella presenza, nella morfologia e nella tipologia dei polimeri, i risultati evidenziano la necessità di ulteriori ricerche volte a confermare i risultati della presente tesi di dottorato, di approfondire le principali fonti di contaminazione e all'identificazione di strategie efficaci per ridurre la presenza di microplastiche derivanti dai processi di trasformazione delle materie prime in prodotti commercializzati.

GENERAL INTRODUCTION

Definition and Classification of Microplastics

In recent decades, plastic pollution has emerged as one of the most urgent environmental issues worldwide. Due to their durability, versatility, and relatively low cost, plastics have been extensively used in almost every sector of human activity, including packaging, construction, textiles, agriculture, and healthcare. However, their environmental persistence, combined with the continuous increase in global plastic production, has led to an extraordinary accumulation of plastic debris in natural ecosystems. In 2021, global plastic production exceeded 390 million tons, and projections suggest that this amount will continue to rise in the coming years if current trends remain unchanged (PlasticsEurope, 2022).

Among the different categories of plastic pollution, microplastics (MP) have received particular scientific and public attention. Microplastics are typically defined as plastic particles smaller than 5 mm in size (Thompson et al., 2004; EFSA, 2016). Microplastics are highly heterogeneous, differing in size, morphology, color, chemical composition, and origin. Based on their origin, MP are usually classified into two major categories: primary MP and secondary MP. Primary MP are intentionally manufactured in small sizes used in personal care products, industrial abrasives, and production pellets (Cole et al., 2011; Andrady, 2017). Secondary MP, by contrast, originate from the degradation and fragmentation of larger plastic items through physical, chemical, and biological processes, including UV radiation, mechanical abrasion, and microbial activity (Cole et al., 2011; Andrady, 2017).

The diversity of MP in terms of polymer type and morphology plays a key role in determining their environmental fate and potential health effects. For example, the type of polymer affects whether MP float or sink, how quickly they break down, and how they interact chemically in the environment, while their size and shape influence how easily they are ingested by organisms and how they accumulate in the food chain (Zhang et al., 2024; Kumar et al., 2025). The most commonly identified polymers in environmental and food samples include polyethylene, polypropylene, polyethylene terephthalate, polystyrene, and polyvinyl chloride, which indeed are also among the most widely used plastics globally (Rocha-Santo & Duarte, 2015; Revel et al., 2018). Their shape varies from fragments, fibers, films, and pellets, depending on the degradation process and the original plastic material. Importantly, MP can adsorb environmental pollutants, heavy metals, and pathogenic microorganisms, acting as vectors of potentially hazardous substances (Kinigopoulou et al., 2022).

The combination of persistence, ubiquity, and capacity to interact with biological systems has raised concerns regarding the impacts of MP, not only on ecosystems but also in respect to food safety and human health (Rahman et al., 2021). Consequently, investigating their occurrence, transport, and biological effects has become a multidisciplinary research priority across environmental, toxicology, and food sciences.

Microplastics in the Environment

Microplastics are now recognized as ubiquitous pollutants, having been detected in nearly all natural environments. Their widespread distribution is mainly due to their resistance to biodegradation, their ability to be transported over long distances by air and water currents, and their continuous input from human activities.

Marine environments represent the most studied ecosystem with respect to MP. Since the first reports by Thompson et al. (2004), MP have been detected in oceans (do Sul & Costa 2014), inland waters (Free et al., 2014), sediments (Van Cauwenberghe et al., 2015a; Zhang et al., 2018), and even in marine organisms (Van Cauwenberghe & Janssen, 2014; Van Cauwenberghe et al., 2015b). The accumulation of plastic debris in oceans highlights the persistence and mobility of these particles (Kinigopoulou et al., 2022). Moreover, marine biota, such as fish, crustaceans, and molluscs are particularly exposed through ingestion and trophic transfer, raising concerns about biodiversity and seafood safety (Van Cauwenberghe & Janssen, 2014; Van Cauwenberghe et al., 2015b).

Freshwater ecosystems have also been identified as important areas of accumulation and transport pathways for MP. Rivers, lakes, and artificial basins receive MP from rainwater, wastewater, industrial activities, and the atmosphere, and act as pathways transporting MP from land to the oceans. Studies have demonstrated the presence of MP in riverine sediments and freshwater organisms, suggesting that contamination is widespread and not limited to marine ecosystems (Li et al., 2018).

Soils and terrestrial environments have attracted growing attention as potential hotspots for MP. Agricultural soils may accumulate MP through the use of organic fertilizers, plastic films used to cover crops, and irrigation with contaminated water (Corradini et al., 2019; Fan et al., 2023).

Finally, the atmosphere has been increasingly recognized as an important pathway for the dispersion of MP. Airborne MP, particularly fibers and small fragments, originate from synthetic textiles, tire wear, and urban dust, and have been detected in both indoor and outdoor environments, including remote areas such as mountainous and polar regions (Allen et al., 2019; Singh, 2024).

The omnipresence of MP in the environment raises concerns about their potential to enter food chain and ultimately reach humans. The fact that MP have been detected in diverse environments, from oceans to agricultural soils and the atmosphere, suggests that exposure is almost inevitable than multifaceted. The scientific literature has encouraged increasing research into the mechanisms of human exposure and implications for food safety and public health (Rahman et al., 2021).

Human Exposure

Humans are exposed to MP through multiple pathways, the most relevant being i) ingestion of contaminated food and water, ii) inhalation, and iii) direct dermal contact through personal care products, textiles, or dust (Prata, 2018; Kutralam-Muniasamy et al., 2023). The complexity of these exposure routes reflects the ubiquity of MP in the environment, in consumer products, and along the food chain.

Ingestion is still considered the primary pathway for human exposure to MP. Numerous studies have demonstrated the occurrence of MP in a wide range of food and beverages, including drinking water (Oßmann et al., 2018; Pivokonsky et al., 2018), fish and seafood (Akhbarizadeh et al., 2020; Diaz-Basantes et al., 2022), honey (Mühlschlegel et al., 2017; Diaz-Basantes et al., 2020), table salt (Yang et al., 2015; Iñiguez et al., 2017; Karami et al., 2017), beer (Lachenmeier et al., 2015; Kosulth et al., 2018; Li et al., 2022a), and other beverages (Shruti et al., 2020). Microplastics have also been detected in nutritionally important foods, such as human breast milk (Ragusa et al., 2022; Liu et al., 2023); powdered milk (Da Costa Filho et al., 2021; Zhang et al.,2023), raw milk (Da Costa Filho et al., 2021; Rbaibi Zipak et al., 2024), and packaged drinking milk (Kutralam-Muniasamy et al., 2020; Kiruba et al., 2022; Basaran et al., 2023). Once ingested, the fate of MP within the human gastrointestinal tract depends on particle size, morphology, and surface chemistry. Smaller particles, especially those below 150 μm, may cross the intestinal barrier through transcytosis or paracellular transport, entering systemic circulation and potentially accumulating in organs and tissues (Pironti et al., 2021; Ramsperger et al., 2023). The ingestion of MP is also concerning because they may carry chemical additives and pollutants, which can be released in the gastrointestinal tract and transferred into body tissues (Wright & Kelly, 2017).

Inhalation represents another pathway for human exposure to MP. Once inhaled, MP can reach different regions of the respiratory tract, where their persistence may trigger adverse health effects (Wright & Kelly, 2017). The accumulation of MP in the lungs has been associated with inflammatory

responses, oxidative stress, and potential chronic respiratory conditions (Prata, 2018). Moreover, MP can act as carriers of chemical contaminants and microorganisms, further enhancing their potential toxicity (Wright & Kelly, 2017). Although less studied compared to ingestion, this route of exposure raises increasing concern as airborne MP have been detected in various environments (Ageel et al., 2022).

Dermal contact is generally regarded as a less significant pathway of human exposure to MP. However, primary MP used in personal care products, including hand detergents, face washes, masks, and toothpastes, come into direct contact with human skin. The presence of MP in cosmetics and personal care products has been associated with localized inflammation and cytotoxic effects, raising concerns about their potential contribution to skin damage (Sharma & Chatterjee, 2017).

Although these exposure pathways have been identified, the toxicological consequences of MP in humans remain insufficiently understood. Laboratory studies on animal models and in vitro experiments suggest that MP can induce inflammatory responses, alter the gut microbiota, generate oxidative stress, and act as vectors for chemical contaminants and pathogens (Wright & Kelly, 2017; Prata, 2018). Still, direct evidence in humans is scarce, partly due to limitations in detecting and quantifying MP in biological samples (Ramsperger et al., 2023). This knowledge gap underscores the importance of studying MP in food products and consumer exposure.

Microplastics in Animal-Based Food Products

The occurrence of MP in food is a growing concern, as they have been detected across a wide range of agricultural and processed products, including drinking water, beer, honey, table salt, and beverages (Toussaint et al., 2019). However, this thesis focuses specifically on animal-based foods, given their global dietary relevance and the potential implications for food safety and human health (Rahman et al., 2021). While initial studies focused on marine environments and seafood as the major MP source, subsequent research has highlighted the presence of MP across a wider range of food categories.

Among animal-based foods, dairy products, powdered milk, and meat are of particular interest due to their widespread consumption, nutritional importance, and the relatively long and complex production chain, which may increase their potential exposure to MP accumulation.

Dairy products such as liquid milk, cheese, and yogurt are highly consumed worldwide and are nutritionally important (Górska-Warsewicz et al., 2019). These matrices are particularly vulnerable due to the complexity of the dairy supply chain and the extensive use of plastics at multiple stages (Kaseke et al., 2023). Contamination may occur at the farm level, through animal feed, milking equipment, or protective clothing, as well as during industrial processing, packaging, and storage, where additives, machinery, and plastic-based protective gear can further contribute to increasing MP accumulation (Diaz-Basantes et al., 2020; Sobhani et al., 2020). Nevertheless, only limited number of studies have investigated MP contamination in dairy products (Rbaibi Zipak et al., 2022;

Buyukunal et al., 2023; Banica et al., 2024). Considering the widespread consumption of these products, comprehensive and systematic characterization of MP is advisable to better assess potential human exposure and associated health risks.

Milk powders represent another critical matrix, particularly due to their use in infant nutrition and industrial food formulations (Zhang et al., 2023). Although early studies have confirmed the presence of MP in milk powders, comprehensive qualitative and quantitative data remain scarce (Kutralam-Muniasamy et al., 2020; Rahman et al., 2021). The detection of MP in this matrix is of high relevance given the vulnerability of infant consumers and other sensitive groups relying on powdered milk.

Meat products, particularly processed and cured meat such as beef hamburgers, sausages, salami, and mortadella, also merit attention. These products undertake multiple stages of handling, grinding, and packaging, which may increase the risk of contamination. Hamburgers are widely consumed globally, raising concerns about exposure through this pathway. Preliminary investigations have confirmed the presence of MP in meat products (Kedzierski et al., 2020; Habib et al., 2022; Bilal et al., 2023), yet the sources of contamination, whether from feed, slaughtering, processing, packaging, or cooking, are poorly investigated (Katsara et al., 2022). This highlights the need for robust and standardized studies to clarify both levels and sources of contamination.

Honey has also been reported to contain MP, although research on this matrix remains limited (Liebezeit & Liebezeit, 2013; 2015; Mühlschlegel et al., 2017; Diaz-

Basantes et al., 2020; Basaran et al., 2024). Microplastics are a borderless pollutant, and due to their ubiquitous distribution, honeybees can interact with them through air, water, flowers, and pollen, subsequently transporting particles to the hive (Liebezeit & Liebezeit, 2015; Alma et al., 2023). Microplastics can be dispersed over long distances by wind and are known to contaminate atmospheric environments (Boccia et al., 2024). In addition, the presence of MP attached to the bodies of honeybees has been observed, especially in relation to urban settlements within their foraging areas (Edo et al., 2021). Other sources of contamination include feeds used in apiculture, plastic beehives, synthetic fibers from beekeepers' clothing, and plastic-based equipment (Mühlschlegel et al., 2017). Furthermore, MP may migrate into honey during packaging, storage, or transportation, as a consequence of physical abrasion of plastic-based materials. These findings highlight the vulnerability of honey to MP contamination and its potential role as a bio indicator of environmental pollution.

Eggs have also emerged as a potential route of human exposure to MP, although current evidence is still scarce (Liu et al., 2022). Contamination may occur primarily through animal feed, which has been identified as a key vector for MP ingestion by poultry. Additional sources include plastic-based housing systems, litter materials, and synthetic fibers from workers' clothing and equipment used during collection and processing. Once ingested, MP may accumulate in the gastrointestinal tract and potentially translocate to edible tissues or eggs, although research in this area is still at an early stage. The limited number of

studies available underscores the need for further investigations to determine both the extent of contamination and the potential implications for food safety.

Overall, current evidence shows that milk and dairy products, milk powders, meat, honey, and eggs are all susceptible to MP contamination, although the extent and sources vary across matrices. These findings highlight the importance of expanding research on animal-based food products to better assess potential exposure risks for consumers.

Analytical Methods and Current Challenges

The detection and characterization of MP in food is challenging, mainly due to their small size and to the complexity of food matrices. Over the years, several analytical approaches have been developed, typically involving three main steps: sample preparation, polymer identification, and quantification.

The preparation of samples represents a critical step to eliminate interfering matrix components, including proteins, lipids, carbohydrates, and minerals, that are inherently present in food matrices.

Common strategies include chemical digestion using alkaline solutions such as potassium hydroxide, oxidative treatments like hydrogen peroxide, enzymatic digestion with proteases, lipases, or amylases, and extraction methods using hot water or alcohol (Gerhard et al., 2022; Li et al., 2022b). To enhance the separation of MP from organic compounds, density separation is commonly applied, allowing particles with higher density to resuspend and improving the efficiency of the isolation procedures. Following digestion and separation, MP are generally

collected through filtration, with the pore size of the filter determining the lower detection limit.

Polymer identification is essential to distinguish MP from other non-plastic particles. Microscopy-based techniques, such as scanning and transmission electron microscopy, provide information on particle size, shape, and color, but cannot confirm chemical composition. In contrast, spectroscopic techniques, including Fourier-transform infrared (FTIR) and Raman spectroscopy, are sensitive techniques that enable polymer identification in food samples while preserving particle integrity during measurement (Bai et al., 2022; Kadac-Czapska et al., 2023). On the other hand, thermal methods, such as pyrolysis-gas chromatography coupled with mass spectrometry, allow both identification and quantification of polymers but are destructive since the sample is decomposed during analysis (Di Fiore et al., 2024). Comparing results reported in the literature is challenging due to the wide variety of methods used to identify and quantify MP contamination in food, as well as the lack of standardized protocols, agreed definitions, consistent quality control procedures, and uniform reporting metrics. In particular, the utilization of different methodologies makes it difficult to directly compare studies (Gerhard et al., 2022). Furthermore, limitations inherent in certain analytical techniques, combined with the difficulty of distinguishing between plastic polymer itself and chemical additives incorporated into plastics, such as plasticizers, stabilizers, or pigments, can lead to substantial variability in the reported quantities of MP across different studies (Guo et al., 2022).

Quantification is typically expressed as the number of MP particles per unit of mass or volume. However, this step can be affected by particle loss during digestion, filtration, and handling, which is often corrected by calculating the recovery. Detection limits and variability across different food matrices also contribute to uncertainty. Laboratory contamination from air, equipment, or clothing is another crucial concern, and samples are therefore handled under strict quality controls in cleanroom conditions.

Overall, careful sample preparation, accurate polymer identification, and controlled quantification are key to producing reliable data. These steps are particularly important for complex animal-based foods, such as milk powders, dairy products, and beef hamburgers, which are the focus of this doctoral research thesis.

Limits and Actual Regulations on Microplastics

Despite the increasing body of scientific evidence demonstrating the presence of MP within the food chain, specific legislation governing MP contamination in food products has not yet been established. Current regulatory frameworks at both international and national levels remain fragmented, as most provisions address plastics in general rather than MP as distinct contaminants (Casella et al., 2024).

The European Food Safety Authority (EFSA) has acknowledged the potential risks associated with MP ingestion, particularly through seafood consumption. Nevertheless, EFSA highlighted that the current paucity of robust toxicological

evidence precludes the definition of tolerable daily intake levels (EFSA, 2016). In parallel, both the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have stressed the urgent need for standardized methodologies to quantify MP in food, along with more comprehensive evaluations of human exposure (WHO, 2019).

At the European Union level, regulatory initiatives have recently targeted intentionally added MP, such as those used in cosmetic and industrial products, under the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation (EC No. 1907/2006). Nevertheless, these restrictions do not extend to unintentionally generated MP or those found in food matrices (Catone et al., 2024). On the other hand, the United States Food and Drug Administration (FDA) has not yet issued specific guidelines on MP in food, although it continues to monitor developments in the scientific literature (Cox et al., 2019).

One of the major limitations to establishing clear regulatory thresholds lies in the absence of harmonized and validated analytical protocols capable of reliably detecting, characterizing, and quantifying MP across different food matrices, such as milk, meat, fish, honey, and eggs. Furthermore, the toxicological implications of chronic dietary exposure remain largely unknown, particularly regarding particle size, polymer type, and the influence of plastic additives or adsorbed environmental pollutants (Shopova et al., 2020; Guo et al., 2022).

Taken together, these considerations reveal a significant regulatory gap. While awareness of MP as emerging food contaminants is steadily increasing, the lack of harmonized methodologies, risk assessment frameworks, and internationally

coordinated policies represents a major challenge. Addressing these aspects will be crucial for ensuring consumer safety and for developing effective strategies of risk communication with respect to animal-based foods.

Although current regulations do not specifically target MP in food, broader legislative measures aimed at reducing the overall use of plastics, such as restrictions on single-use plastics and incentives for sustainable packaging, may contribute indirectly to limiting MP contamination along the food chain. In this context, general plastic reduction policies can act as a proactive and preventive strategy, helping to mitigate the spread of MP in food products even in the absence of dedicated food-specific regulations.

Scientific Interest and Research Perspectives on Microplastics

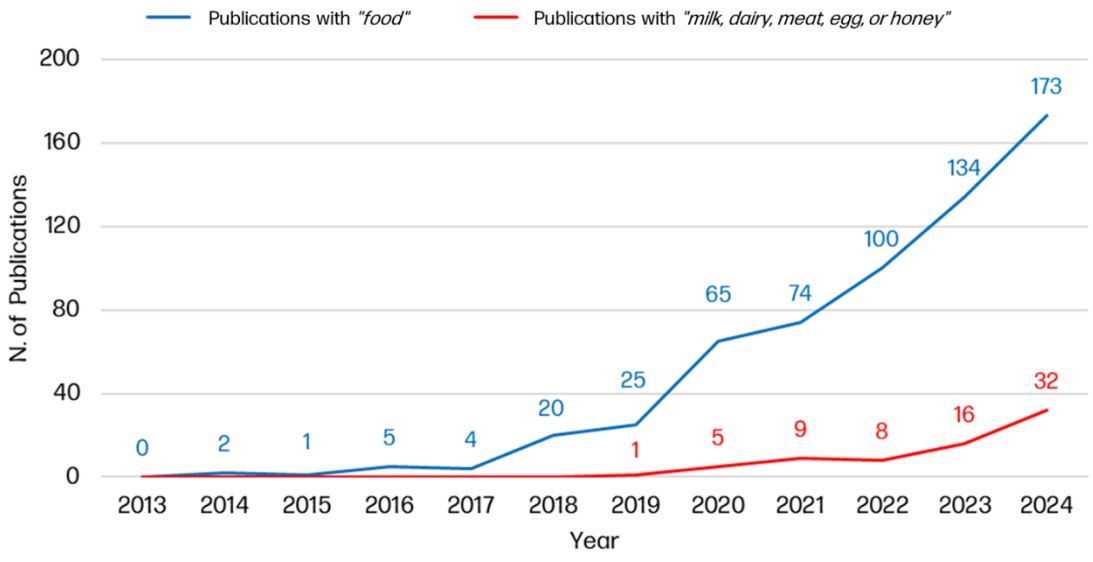
In recent years, MP have emerged as a global concern covering multiple disciplines, from environmental sciences to food safety and toxicology (Prata et al., 2020). Their ubiquitous presence in different ecosystems and potential impacts on human health have stimulated a rapid growth of scientific interest. Despite this growing interest, significant knowledge gaps remain, particularly concerning the occurrence and implications of MP in animal-based foods such as milk, dairy products, meat, honey, and eggs (Toussaint et al., 2019). These products are highly relevant in human diet, yet remain underexplored compared to other matrices, such as seafood, which dominate the current literature (Rahman et al., 2021).

To provide a comprehensive overview of the scientific output on MP in animal-based foods, a bibliometric analysis was conducted using Scopus

(https://www.scopus.com/), covering the period 2013-2024. Publications were retrieved based on the keywords *"microplastic"* and *"food"* or *"microplastic"* combined with *"milk", "dairy", "meat", "egg", or "honey".* The resulting dataset was then analyzed to highlight temporal trends and keyword co-occurrences.

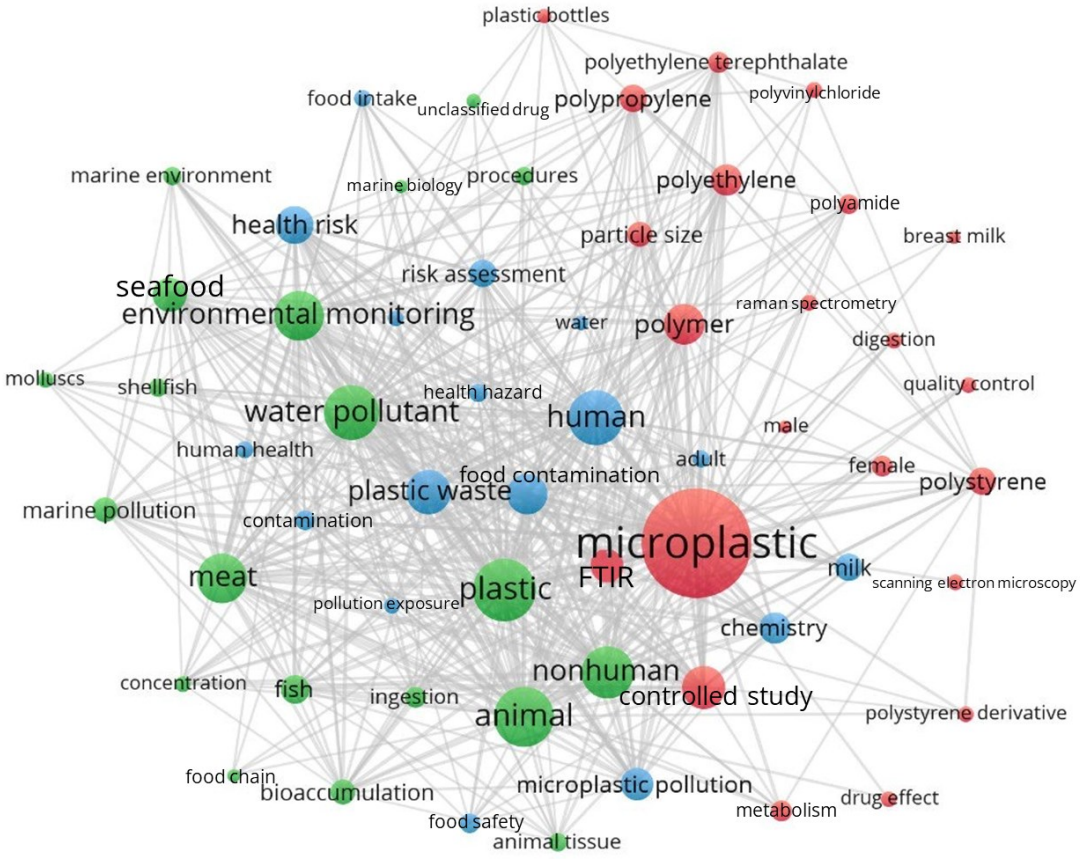
The temporal distribution of publications (Figure 1) reveals a sharp increase in the number of studies addressing MP in food in general, with an exponential growth after 2019 and a peak of 173 papers in 2024. In contrast, works specifically focused on animal-based foods remain scarce, although they show a progressive rise, reaching 32 publications in 2024.

Figure 1. Temporal distribution of publications including the keywords *"microplastic"* and *"food"* (blue line) or *"microplastic"* in combination with *"milk", "dairy", "meat", "egg", or "honey"* (red line) in title or author keywords fields in Scopus (https://www.scopus.com/), covering the period 2013-2024.



The keyword co-occurrence network (Figure 2) further illustrates the broad thematic scenery of this research area, identifying three distinct clusters. The first red colored cluster includes analytical techniques and polymer types such as FTIR, Raman spectrometry, polypropylene and polyethylene. The second green cluster covers environmental sources and contaminants including keywords like water pollutants, seafood, and marine pollution. The third blue cluster includes food safety and health implications, represented by keywords such as human, food contamination, and health risk. This structure highlights the inherently interdisciplinary nature of MP research, which integrates chemical, environmental, and toxicological perspectives.

Figure 2. Keyword co-occurrence network based on bibliographic information of 145 articles published between 2013 and 2024. The search included *"microplastic"* in the title or as author keywords, alone or in combination with *"milk", "dairy", "meat", "egg" or "honey"* appearing in the title, abstract or keywords. The network displays the 60 authors keywords with an occurrence ≥ 10.



Overall, the bibliometric evidence underscores the rapid expansion of this field while pointing to the limited but growing attention toward animal-based food products. This context provides the basis for the present Ph.D. thesis, which aims to address this knowledge gap in animal-based foods and to develop a qualitative and quantitative analytical method for the characterization of MP in various animal-based food products.

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AIMS OF THE THESIS

The overall objective of this thesis was to investigate MP contamination in animal-based food products. The specific aims were:

i) to evaluate the qualitative and quantitative presence of MP in liquid milk, fresh cheese, and ripened cheese, with a focus on differences in contamination levels across these dairy products.

ii) to characterize the occurrence and polymeric composition of MP in skim-milk powder samples collected from different European countries;

iii) to preliminary assess the abundance and characteristics of MP contamination in beef hamburgers produced by Italian beef companies.

CHAPTER 1

Assessing microplastic contamination in milk and dairy products

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Assessing microplastic contamination in milk and dairy products

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The presence of microplastics in food has raised growing concern due to potential health risks. While many studies have investigated microplastics in water and seafood, limited data are available for dairy products. This study qualitatively and quantitatively characterizes microplastics in milk, fresh cheese, and ripened cheese, assessing concentration levels and polymer composition through the analysis of 28 dairy samples using Fourier-transformed infrared micro-spectroscopy in attenuated total reflectance mode. Poly(ethylene terephthalate) was the most frequent, followed by polyethylene and polypropylene. Most microplastics were smaller than 150 µm, with 51–100 µm being the most common (33.8%). Irregular fragments (77.4%) and grey particles (68.4%) were predominant. Ripened cheese exhibited the highest microplastic concentration (1857 MP/kg), followed by fresh cheese (1280 MP/kg) and milk (350.0 MP/kg). Results confirm widespread microplastic contamination in dairy products and highlight the importance of further research into contamination pathways and strategies to reduce microplastic exposure in the dairy chain.

The presence of microplastics (MP) in the environment has become a growing concern due to their widespread distribution, persistence, and potential adverse effects on human health. Microplastics are defined as plastic fragments ranging in size from 0.1 µm to 5,000 µm[1,2]. Microplastics have been detected in various natural environments, including oceans[3], inland waters[4,5], sediments[6,7], and biota[8,9]. The accumulation of MP in natural environments raises urgent concerns about its long-term impacts[10]. Due to their small size, MP can accumulate through different trophic levels and enter the food chain, with potential impacts on both ecological systems and human health[11].

The dietary pathway represents the main route through which MP enters the human body[12–14]. Indeed, MP have been detected in various food categories, including drinking water[15–17], fish and seafood products[18,19], honey[20,21], table salt[22–24], beer[25–27], and beverages[28]. The ingestion of MP through contaminated food raises important questions about their bioavailability, accumulation, and potential health risks, especially from a long-term exposure perspective[29].

Dairy products have received limited attention in respect to the characterization of their potential MP content. Products such as powdered milk, liquid milk, cheese, and yogurt may be contaminated with MP at various stages along the supply chain[30,31]. Contamination may occur at the farm level, through contaminated feed[32], milking equipment, or clothing[20]. Contamination may also happen at the plant level, during processing, again arising from clothing and protective gear, including hairnets, disposable laboratory coats, and gloves[20,33]. Moreover, contamination can also arise from food additives containing MP residues[34], as well as from processing machinery, transportation, and storage[35]. Given the complexity of the dairy sector and the extensive use of plastic materials along the entire production chain, understanding the pathways through which MP enter dairy products is crucial for ensuring food safety and assessing potential health risks[30].

Existing MP studies have focused on human breast milk[36,37], powdered milk[33,38,39], raw milk[33,40], and packaged drinking milk[20,33,41–43], probably due to their relatively simple detection in terms of analytical procedures, with particular regard to the digestion and filtration steps required for samples[44,45]. However, only a limited number of studies have investigated MP contamination in dairy products. Rbaibi Zipak[46] assessed the presence of MP in yogurt during individual production steps. Similarly, Buyukunal[47] investigated MP presence along the production of milk-based beverages, detecting MP in all examined samples. Additionally, Banica[48] analyzed high-fat dairy products, including butter and sour cream samples, reporting significant contamination also in these products. The presence of MP in such products highlights the need for comprehensive studies to assess contamination levels and potential health risks associated with MP ingestion through various dairy products.

This specific research gap highlights the need for further investigation into the contamination of MP in dairy products. Thus, this study aims (i) to qualitatively and quantitatively characterize MP contamination in milk,

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fresh cheese, and ripened cheese, and (ii) to assess differences in contamination levels across these different products.

Results and discussion

Characterization of MP in dairy products: size, shape, and color

Descriptive statistics for sizes of MP detected in dairy products are presented in Table 1. Sizes ranged from a minimum of 24.0 µm to a maximum of 4817 µm, with an overall average size of 243.7 µm. Among polymers, polyacrylate exhibited the largest average size (1048 µm), whereas polyvinylidene fluoride and ethylene vinyl acetate had the smallest (32.0 µm and 37.5 µm, respectively). As the most frequently detected MP, poly(ethylene terephthalate), polypropylene, and polyethylene had average sizes of 775.8, 114.4, and 109.0 µm, respectively, with relatively high coefficients of variation (112.5, 127.8, and 55.2%, respectively). As shown in Fig. 1A, 33.8% of the identified MP had sizes comprised between 51 µm and 100 µm, followed by MP in the range between 3 µm and 50 µm (19.9%), and by MP in the range between 101 µm and 150 µm (19.6%). Larger MP fragments, ranging from 201 µm to 250 µm and from 251 µm to 300 µm, were less frequent, representing 3.76% and 2.26% of the total, respectively. The predominance of small MP is consistent with previous studies that reported a similar size distribution, with the majority of particles measuring below 150 µm[46,47]. In a study on MP contamination during the yogurt production process, Rbaibi Zipak[46] reported that 43.3% of the detected MP had sizes between 1 µm and 150 µm. Buyukunal[47] analyzed MP contamination along the production of milk-based beverages and reported that 37.4% of detected MP were also in the 1–150 µm range. The similarity between our results and those reported for yogurt and milk-based beverages suggests that MP contamination mechanisms in dairy products are consistent, even across different production processes. The predominance of MP below 150 µm suggests contamination from processing equipment, plastic packaging degradation, or the deposition of airborne particles during production[49].

Data regarding the shapes of detected MP are presented in Table 2 and Fig. 1B. Beads were found exclusively within the copolymers class, representing for 0.38% of the total MP (Fig. 1B). Fibers of poly(ethylene terephthalate) (89.4%), polyacrylate (75.0%), polypropylene (16.0%), and polyethylene (3.95%) were observed (Table 2), collectively accounting for 22.2% of the total MP detected (Fig. 1B). These findings point to synthetic textiles as a likely source of fiber contamination, potentially introduced through filtration systems, protective clothing (such as lab coats, gloves, or hairnets in laboratory or food processing settings), remnants of synthetic materials, or airborne fibers settling during sample processing[50]. Irregular shapes were predominant across most polymers (77.4% of the total MP; Fig. 1B). The high occurrence of irregular fragments is likely due to mechanical degradation of plastic materials, potentially originating from packaging, processing equipment, or the wear and tear caused by friction between machine components and surfaces during the production process[50]. These results are consistent with those reported by Buyukunal[47], who also found that fragments and fibers were the predominant MP shapes. However, their study on milk-based beverages revealed a different proportion in terms of shapes, with fibers representing the most abundant MP morphology (49.8%), followed by fragments (14.7%), and spheres (12.4%).

The frequencies of MP color detected in dairy products are shown in Table 2. Gray MP was predominantly observed in polyethylene (84.2%), polystyrene (83.3%), polyvinyl chloride (83.3%), and polypropylene (82.0%). Brown and black MP were detected in smaller proportions, being identified in 13 and 11 out of the 20 identified polymers, respectively. Transparent MP was exclusively found in poly(ethylene terephthalate) (4.26%), which may be attributed to specific packaging components. As shown in Fig. 1C, about 93% of the MP identified in this study were pigmented, with gray (68.4%), brown (15.0%), and black (9.77%) being the most prevalent colors. However, Buyukunal[47] reported a different color distribution, with blue (19.8%), red (17.9%), and transparent (15.0%) being

Table 1 | Descriptive statistics for sizes (µm) of MP detected in dairy products

MP	Detected MP, *n*	Mean	SD	CV, %	Minimum	Maximum
Chlorinated polyethylene	5	87.6	28.4	32.4	51.0	124.0
Copolymers[a]	16	79.4	52.4	66.0	31.0	212.0
Ethylene vinyl acetate	2	37.5	12.0	32.1	29.0	46.0
Nylon	5	138.8	124.6	89.8	42.0	352.0
Polyacrylate	8	1,048	1,561	149.0	73.0	4,817
Polybutadiene	1	40.0	–	–	–	–
Polyester	4	68.3	27.3	40.0	42.0	106.0
Polyethylene	76	109.0	60.2	55.2	35.0	325.0
Poly(ethylene terephthalate)	47	775.8	873.1	112.5	41.0	3,766
Polyisoprene	5	60.0	17.3	28.9	40.0	81.0
Polyoxymethylene	1	40.0	–	–	–	–
Polyphenylene sulfide	1	95.0	–	–	–	–
Polypropylene	50	114.4	146.2	127.8	24.0	903.0
Polystyrene	18	77.4	36.1	46.6	34.0	143.0
Polytetrafluoroethylene	5	98.0	40.8	41.6	31.0	131.0
Polyurethane	2	86.0	76.4	88.8	32.0	140.0
Polyvinyl chloride	6	78.2	36.6	46.8	38.0	141.0
Polyvinylidene fluoride	1	32.0	–	–	–	–
Resin	1	70.0	–	–	–	–
Silicone	12	91.9	34.0	37.0	40.0	145.0

[a]Copolymers class comprises: acrylonitrile–butadiene–styrene, chlorinated polyethylene–polystyrene copolymer, polyacrylonitrile–butadiene–styrene copolymer, polyethylene–nylon copolymer, polyethylacrylate–polystyrene–polyacrylamide copolymer, polystyrene–polyacrylate copolymer, polystyrene–polybutadiene copolymer, silicone–polyisoprene copolymer, and styrene–ethylene–butylene–styrene.

SD standard deviation, *CV* coefficient of variation.

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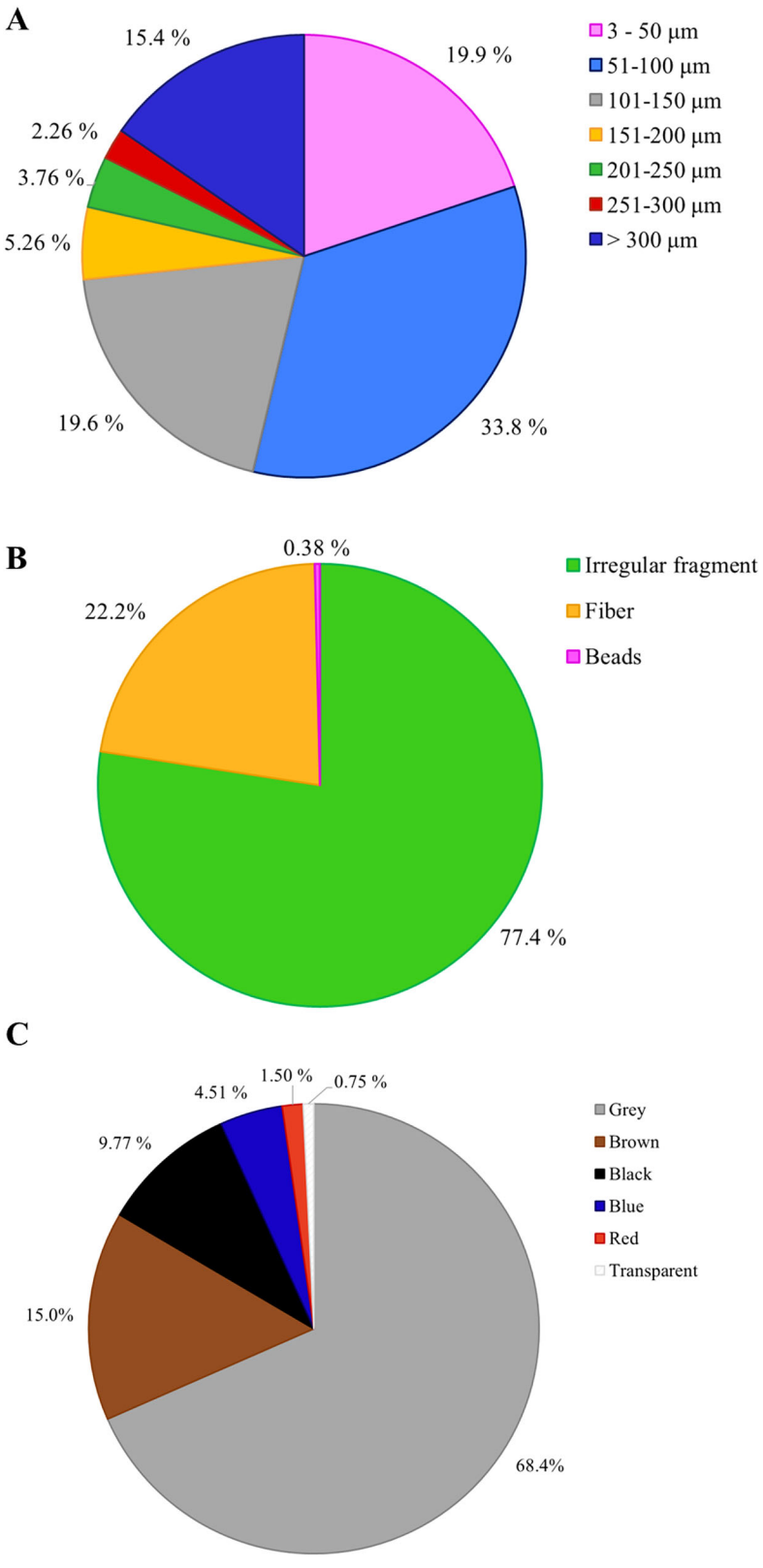

Fig. 1 | Qualitative characteristics of MP in dairy products. Frequencies (%) of size classes (**A**), shape (**B**), and color (**C**) of MP identified in dairy products.

the most abundant, followed by black (11.2%). Moreover, it is important to consider that various color pigments can be added to polymer mixtures during the production process. The colors observed under the microscope may therefore result from the pigments and additives used in the manufacturing of plastic packaging[46]. For this reason, Fourier-transformed infrared micro-spectroscopy confirmation is essential for a conclusive identification. The predominance of gray MP observed in our study supports the hypothesis that the primary sources of contamination in dairy products are related to food-contact plastics and packaging materials. Gray MP is commonly associated with industrial plastic components used in food packaging, such as films, liners, and closures, which often contain neutral pigments[30]. Their presence may result from mechanical abrasion, aging, or degradation of these materials during processing, handling, or storage, rather than from environmental contamination[35,41].

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Table 2 | Frequency of different shapes and colors of MP detected in dairy products

MP	Detected MP, *n*	Shape, %			Color, %					
		Beads	Irregular fragment	Fiber	Black	Blue	Brown	Gray	Red	Transparent
Chlorinated polyethylene	5	–	100.0	–	40.0	20.0	–	–	40.0	–
Copolymers[a]	16	6.25	93.8	–	31.3	6.25	6.25	56.3	–	–
Ethylene vinyl acetate	2	–	100.0	–	–	–	50.0	50.0	–	–
Nylon	5	–	100.0	–	20.0	–	–	80.0	–	–
Polyacrylate	8	–	25.0	75.0	25.0	25.0	25.0	25.0	–	–
Polybutadiene	1	–	100.0	–	–	–	100.0	–	–	–
Polyester	4	–	100.0	–	50.0	–	–	50.0	–	–
Polyethylene	76	–	96.1	3.95	1.32	–	13.2	84.2	1.32	–
Poly(ethylene terephthalate)	47	–	10.6	89.4	19.2	12.8	10.6	53.2	–	4.26
Polyisoprene	5	–	100.0	–	–	20.0	80.0	–	–	–
Polyoxymethylene	1	–	100.0	–	–	–	–	100.0	–	–
Polyphenylene sulfide	1	–	100.0	–	100.0	–	–	–	–	–
Polypropylene	50	–	84.0	16.0	2.00	2.00	12.0	82.0	2.00	–
Polystyrene	18	–	100.0	–	5.56	–	11.1	83.3	–	–
Polytetrafluoroethylene	5	–	100.0	–	–	–	60.0	40.0	.–	–
Polyurethane	2	–	100.0	–	50.0	–	50.0	–	–	–
Polyvinyl chloride	6	–	100.0	–	–	–	16.7	83.3	–	–
Polyvinylidene fluoride	1	–	100.0	–	–	–	–	100.0	–	–
Resin	1	–	100.0	–	–	–	–	100.0	–	–
Silicone	12	–	100.0	–	–	–	25.0	75.0	–	–

[a]Copolymers class comprises: acrylonitrile–butadiene–styrene, chlorinated polyethylene–polystyrene copolymer, polyacrylonitrile–butadiene–styrene copolymer, polyethylene–nylon copolymer, polyethylacrylate–polystyrene–polyacrylamide copolymer, polystyrene–polyacrylate copolymer, polystyrene–polybutadiene copolymer, silicone–polyisoprene copolymer, and styrene–ethylene–butylene–styrene.

Quantification of MP in dairy products

Descriptive statistics of MP concentration in dairy products are presented in Table 3. A total of 28 dairy product samples were analyzed for MP contamination, with MP detected in 26 of them. Overall, 266 plastic particles were identified, belonging to 20 different polymer classes. Poly(ethylene terephthalate) was found in 19 samples, being the most frequently identified polymer, followed by polyethylene (found in 15 samples), polypropylene (found in 12 samples), and copolymers (found in 12 samples). Polyethylene exhibited the highest average concentration (640.0 MP/kg), followed by polypropylene (516.7 MP/kg) and poly(ethylene terephthalate) (357.9 MP/kg). Notably, some polymers were detected in isolated cases; these included polybutadiene, polyoxymethylene, polyphenylene sulfide, polyvinylidene fluoride, and resin, which were found in single samples. Silicone exhibited the greatest coefficient of variation (127.3%), suggesting significant variability in the analyzed samples. In contrast, polyacrylate, polyvinyl chloride, and polypropylene showed the lowest coefficients of variation (35.0, 38.3, and 38.6%, respectively), indicating a more homogeneous distribution across contaminated samples.

Similar MP concentrations have been observed in other studies. For instance, in a study on MP in Turkish milk-based beverages, Buyukunal[47] reported MP concentrations ranging from 3 to 43 MP/100 mL, with the highest contamination levels detected in samples collected after the homogenization and pasteurization steps. The same authors reported an average concentration of 19 MP/100 mL in raw milk, suggesting that the contamination in the raw matrix plays a crucial role in determining the overall MP content in the final product. Rbaibi Zipak et al.[46], in their investigation of MP contamination throughout the yogurt production process reported concentrations ranging from 2 to 58 MP/100 mL, with the lowest contamination found in the early stages of processing (in the bulk tank milk), and the highest contamination detected in the final stages (during the yogurt cup filling process). Da Costa Filho et al.[33] identified MP concentrations in different types of milk, including raw milk, whole and skimmed liquid milk, and powdered milk products, ranging from 204 to 1004 MP/100 mL, with polyethylene and polypropylene being the predominant polymers, which is consistent with our findings. In contrast, Kutralam-Muniasamy[41] and Basaran[43] found considerably lower MP concentrations in commercial liquid milk from different brands, ranging from 1–16 MP/L to 3–11 MP/L, respectively. The large variability of MP levels detected across different studies can be attributed to differences in sample characteristics—such as type of dairy product, fat content, and origin—as well as to the specific stage of the dairy production process sampled along with the MP measurement protocols employed. Common sources of MP include plastic-based packaging, food-contact materials used during processing, and synthetic fibers from operators' clothing[30,31].

Similar levels of contamination have been observed in other food products, particularly in meat. For instance, Visentin[51] identified a total of 898 plastic particles in beef hamburgers, belonging to 18 different polymer classes. The detection of different polymer classes, including polycarbonate, polyethylene, and polypropylene, highlights the high variability of plastic contaminants in meat products, which is consistent with the polymer diversity observed in the present study. Kedzierski et al.[52] demonstrated that plastic particles from polystyrene trays used in the commercial packaging of chicken meat adhered to the meat surface even after rinsing, highlighting a potential risk for consumers. Contamination pathways identified along the meat supply chain could also apply to the dairy sector, with packaging and processing equipment as possible common sources of MP contamination across different food matrices.

Variability of MP contamination among dairy products

Data points and least square means (LSMs) of the overall MP concentration detected in milk, fresh cheese, and ripened cheese samples are

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Table 3 | Descriptive statistics of MP concentration (MP/kg) in dairy products

MP	*n* of contaminated samples	Mean	SD	CV, %	Minimum	Maximum
Chlorinated polyethylene	3/28	333.3	230.9	69.3	200.0	600.0
Copolymers[a]	12/28	283.3	232.9	82.2	200.0	1000
Ethylene vinyl acetate	2/28	200.0	–	–	–	–
Nylon	5/28	200.0	–	–	–	–
Polyacrylate	6/28	233.3	81.7	35.0	200.0	400.0
Polybutadiene	1/28	200.0	–	–	–	–
Polyester	3/28	266.7	115.5	43.3	200.0	400.0
Polyethylene	15/28	640.0	442.1	69.1	200.0	2,000
Poly(ethylene terephthalate)	19/28	357.9	171.0	47.8	200.0	800.0
Polyisoprene	3/28	333.3	230.9	69.3	200.0	600.0
Polyoxymethylene	1/28	200.0	–	–	–	–
Polyphenylene sulfide	1/28	200.0	–	–	–	–
Polypropylene	12/28	516.7	199.2	38.6	200.0	800.0
Polystyrene	9/28	311.1	202.8	65.2	200.0	800.0
Polytetrafluoroethylene	5/28	200.0	–	–	–	–
Polyurethane	2/28	200.0	–	–	–	–
Polyvinyl chloride	5/28	240.0	89.4	38.3	200.0	400.0
Polyvinylidene fluoride	1/28	200.0	–	–	–	–
Resin	1/28	200.0	–	–	–	–
Silicone	4/28	550.0	700.0	127.3	200.0	1,600

[a]Copolymers class comprises: acrylonitrile–butadiene–styrene, chlorinated polyethylene–polystyrene copolymer, polyacrylonitrile–butadiene–styrene copolymer, polyethylene–nylon copolymer, polyethylacrylate–polystyrene–polyacrylamide copolymer, polystyrene–polyacrylate copolymer, polystyrene–polybutadiene copolymer, silicone–polyisoprene copolymer, and styrene–ethylene–butylene–styrene.
SD standard deviation, *CV* coefficient of variation.

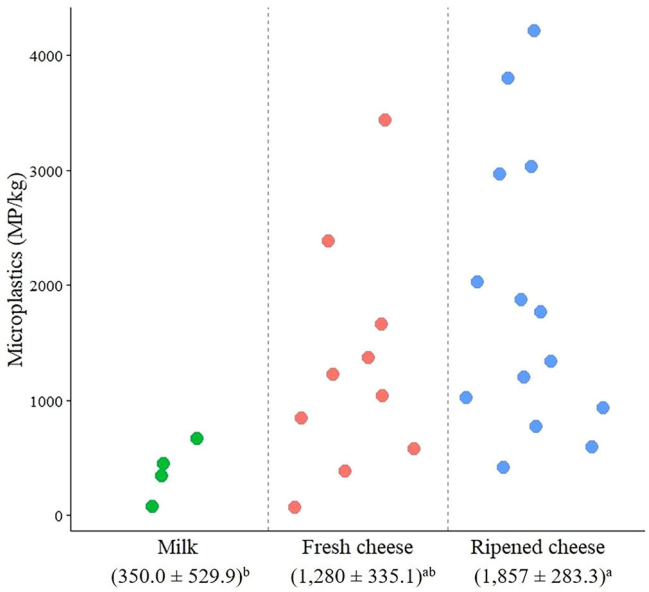

Fig. 2 | Variability of MP in dairy products. Data points and LSMs were calculated as the sum of the individual MP concentrations (MP/kg) identified in each sample, grouped into three types of dairy products. Means with different superscript letters differ significantly ($P < 0.05$).

presented Fig. 2. The analysis of variance highlighted that ripened cheese had the highest MP concentration (1857 MP/kg), followed by fresh cheese (1280 MP/kg), and milk (350.0 MP/kg; $P < 0.05$). Current literature provides scarce information on the variability of MP contamination in dairy products, particularly in relation to different processing stages and different cheese types. Kutralam-Muniasamy et al.[41] analyzed 23 milk samples from various brands in Mexico, including whole milk, lactose-free milk, and low-fat milk. They found that MP were present in all the analyzed samples, with processed milk (such as lactose-free and low-fat varieties) exhibiting higher concentrations of MP compared to whole milk. In a study on MP contamination in milk products, Da Costa Filho[33] analyzed 8 samples, including 2 raw milk samples, 3 whole milk samples, 1 skim milk sample, and 2 skim milk powder samples. They found that powdered milk contained the highest concentration of MP, with contamination linked to ubiquitous environmental plastics, packaging materials, and processing (particularly the use of polymeric membranes during filtration and drying). Badwanache et al.[53] analyzed 21 milk samples, including 7 raw milk samples directly transferred from the farm to the laboratory into steel containers, 7 milk samples from different dairy companies packed in polyethylene plastic bags, and 7 commercially branded milk samples in polyethylene plastic bottles. Their findings revealed that the commercially branded milk samples exhibited the highest MP concentration, which was primarily attributable to the plastic packaging. In contrast, milk collected directly from farms or dairies showed lower contamination levels, confirming the contribution of packaging to increased MP contamination.

In the present study, cheese was found to have higher levels of MP compared to milk. This is likely due to the cheese-making process, as a procedure that removes whey, thereby reducing total mass and concentrating solid components, including any MP fragments[41]. In other words, it is likely that MP are not preferentially removed through the whey fraction, resulting in their concentration in the curd. This phenomenon is particularly evident in ripened cheese, where moisture loss during aging and additional processing steps may further contribute to MP accumulation. To the best of the authors' knowledge, no studies have specifically investigated MP contamination in cheese. However, Banica[48] investigated the occurrence of MP in high-fat dairy products, including

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11 butter samples from various brands (both conventional and organic), as well as 7 sour cream samples from different producers. Their findings indicated that conventional butter had the highest MP concentration (7875 MP/kg), followed by sour cream (5600 MP/kg), and organic butter (2500 MP/kg). These findings are in line with the results of the present study, where dairy products undergoing more intensive processing and containing higher fat levels—such as cheese—were found to have higher MP concentrations than less processed low-fat products such as liquid milk. This supports the hypothesis that intensive processing and high-fat content may facilitate MP accumulation.

Moreover, the consistent detection of MP across different products suggests that multiple contamination sources – introduced at various stages of the production chain – likely contribute to the differences in MP concentrations observed among dairy products. Cheese making involves multiple steps, such as curd cutting, molding, pressing, and aging, all of which can increase MP exposure due to prolonged contact with processing surfaces, equipment, and food-contact materials[54]. For example, plastic molds, storage containers, and packaging films commonly used during cheese production and maturation could represent potential sources of MP contamination. The prolonged maturation time of ripened cheese could further contribute to MP accumulation, as extended storage in plastic materials may lead to the gradual release of MP particles. The differences in MP contamination levels among the three product categories analyzed in this study—liquid milk, fresh cheese, and ripened cheese—suggest that ripened cheeses are more susceptible to MP contamination, likely due to the complexity of their production processes and the materials used throughout their supply chains.

It can be concluded that MP contamination varies considerably among different dairy products. The 28 analyzed samples, including liquid milk, fresh cheese, and ripened cheese, highlighted a wide variability of MP in terms of size, shape, and color. Detected MP ranged from 24.0 to 4817 µm in size, with an average of 243.7 µm. Irregular fragments were the predominant morphology (77.4%), and the vast majority of MP were pigmented, with gray (68.4%), brown (15.0%), and black (9.77%) being the most frequent colors. The most frequently detected polymers were poly(ethylene terephthalate), polyethylene, and polypropylene, found in 19, 15, and 12 samples, respectively. Significant differences in MP concentrations were observed across dairy products, with ripened cheese exhibiting the highest MP levels (1857 MP/kg), followed by fresh cheese (1280 MP/kg) and milk (350.0 MP/kg). These findings emphasize the importance of considering the specific dairy product type, when assessing the extent of MP contamination in the dairy sector. Both the production process and the packaging materials used may influence the level of contamination, and specific mitigation strategies should be developed accordingly. Future research should be conducted at the plant level, encompassing all stages of the production process to identify specific sources of MP contamination and to support the development of effective mitigation strategies.

Methods

Sample collection

A total of 28 samples were purchased from large-scale retail markets, including 4 ultra-high temperature milk samples (1 L each), 10 fresh cheese samples (500 g each; less than 1 month of aging), and 14 ripened cheese samples (500 g each; more than 4 months of aging). Commercial names are not disclosed to maintain objectivity and avoid potential conflicts of interest.

After purchase, samples were transported to the laboratory of the European Center for the Sustainable Impact of Nanotechnology (ECSIN, Padova, Italy), as part of Mérieux NutriSciences Company (Chicago, USA), where they were frozen upon arrival.

Reagents and equipment

Sample pretreatment, including quartering, digestion, microfiltration, and MP detection, was conducted in a dedicated clean room adhering to ISO 14644-1:2015 Class 7 standards (ISO, 2015).

All solutions were micro-filtered through a silver membrane filter (3.0 µm pore size, 25 mm diameter; Sterlitech Corporation, Auburn, United States). Ultrapure water (resistivity: 18.3 MΩ/cm at 25 °C) was generated using a Zeneer Power III system (Human Corporation, Garak-ro, Republic of Korea). Work surfaces were thoroughly cleaned prior and during experimental procedures to avoid sample contamination. Glassware was washed in five cycles with distilled water, followed by five rinses with ultrapure water, and oven-dried before use.

The laboratory environment was carefully managed to prevent contamination, maintaining strict control over airborne particulates, temperature, and humidity. The laboratory was equipped with a cleanroom environment with a controlled ISO class 7 standard, ensuring that High-Efficiency Particulate Air (HEPA) filters were used to maintain air quality, and positive pressure was maintained to prevent the ingress of contaminants from outside the cleanroom. Operators wore antistatic lab coats and avoided using materials, such a latex or nitrile gloves, that could release particles or residues potentially interfering with MP analyses. Hair covers were also excluded to further minimize contamination risks. To protect equipment and maintain sample purity, all tools used during sample preparation were covered with aluminum foil.

Sample quartering, digestion, and microfiltration

Before analysis, milk samples were thawed overnight at room temperature and gently inverted five times to promote fat and solids homogenization. Therefore, an aliquot of 100 mL was used for the subsequent analytical phases. Fresh and ripened cheeses were thawed overnight, and removed from the packaging. Afterwards, a quartering step was performed to obtain a representative cheese aliquot. This procedure involved dividing each sample into four equal parts, discarding two quarters, and recombining the remaining portions. The process was repeated by further quartering the recombined portion, until a final sample weight of 10 g was achieved.

To remove organic components, a digestion protocol based on the methods described by Dehaut[44] and EFSA[1] was employed, involving both enzymatic and chemical digestion steps. As for the enzymatic digestion protocol, 100 mL of ultrapure water and 20 mL of 5% trypsin solution (Merck KGaA, Darmstadt, Germany) were added to 100 mL of milk and 10 g of cheese in a glass flask. The mixture was stirred at 37 °C overnight using a Shake'n Bator (EuroClone, Milan, Italy). As for the chemical digestion, 100 mL of 10% potassium hydroxide (KOH) solution was added to each sample. The mixture was stirred at 60 °C overnight in the Shake'n Bator. Finally, 100 mL of 10% ethylenediaminetetraacetic acid (EDTA) solution was added, and the mixture was stirred at 60 °C for 2 h in the Shake'n Bator.

After digestion, all samples were micro-filtered through silver membrane filters (3.0 µm pore size; Sterlitech Corporation, Auburn, United States). Density separation of MP in milk and cheese samples was performed using an oversaturated NaCl solution (Merck KGaA, Darmstadt, Germany) as flotation solution, which enabled to suspension also heavy MP fragments. This step was repeated three times to improve the separation efficiency. Throughout the microfiltration step, the filtering funnel was covered with aluminum foil to minimize contamination. Finally, the filter membrane was dried in an oven at 70 °C for 24 h.

Identification and quantification of MP through µ-FTIR-ATR

Filter membranes were analyzed using an FTIR Spectrometer Frontier coupled to a microscope Spotlight 400 (PerkinElmer Italia Spa, Milan, Italy). Polymers were identified via Fourier-transformed infrared microspectroscopy, with spectral detection wavelengths in the range between 4000 cm^{-1} and 650 cm^{-1}. Each detected MP particle underwent four repeated µ-FTIR-ATR scans, until the final average spectrum was acquired. Background spectra were recorded using air as the reference medium. To minimize errors, spectra obtained for each MP particles were corrected by subtracting the corresponding air background spectra. Therefore, spectra were compared against a reference library using

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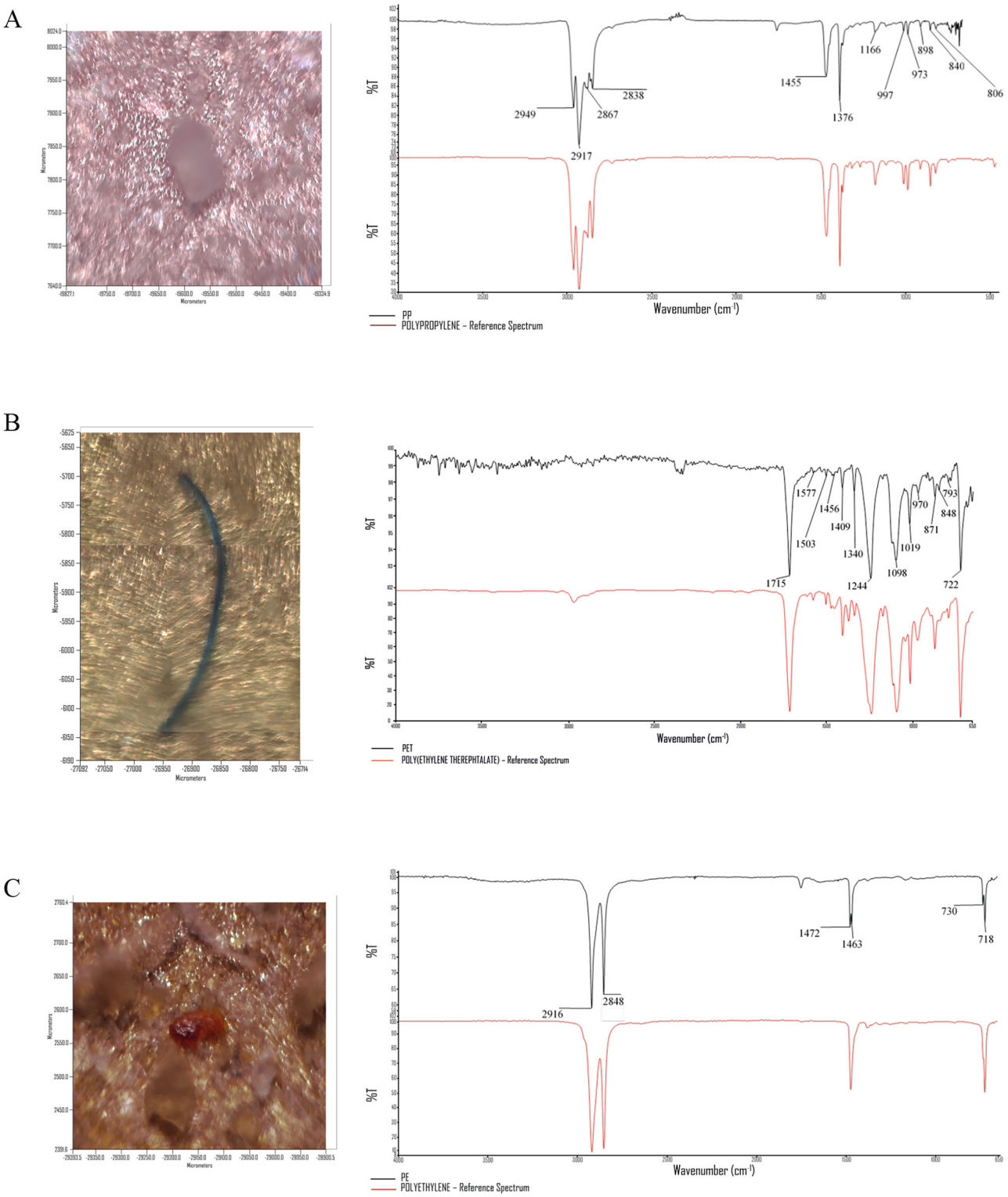

Fig. 3 | Identification of MP using μ-FTIR-ATR spectroscopy. Images and Fourier-transformed infrared micro-spectroscopy in attenuated total reflectance mode analysis (μ-FTIR-ATR) spectra of three identified MP with different shapes and colors: **A** polypropylene, **B** poly(ethylene terephthalate), and **C** polyethylene (black line for MP spectra from dairy products, red line for MP spectra from reference libraries).

Spectrum 10 software (PerkinElmer Italia Spa, Milan, Italy) (Fig. 3). Polymers were considered as correctly determined when the matching was greater than 80%. The most significant peaks are listed in Table 4, together with the associated vibrational modes.

High-resolution images of MP were electronically stored using Spectrum 10 software. The size, color, and shape of each MP particle were determined through ImageJ software (ImageJ 2022). Size was measured on the maximum length of each MP particle.

Blanks and method recovery

To assess potential contamination arising from the analytical procedure, blanks were prepared simultaneously along with each sample, following the

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Table 4 | Characteristics bands with assigned vibrational modes from μ-FTIR-ATR spectra of polypropylene[55], poly(ethylene terephthalate)[56], and polyethylene[57]

Wavenumber (cm^{-1})	Vibrational mode
Polypropylene	
~2949	CH_3 asymmetric stretching
~2917	CH_2 asymmetric stretching
~2867	CH_3 symmetric stretching
~2838	CH_2 symmetric stretching
~1455	CH_3 asymmetric bending
~1376	CH_3 symmetric bending
~1166	CH_2 twisting and CH wagging
~997	CH bending and wagging, CH_3 rocking
~973	CH_3 rocking, CH_2 wagging, and CH bending
~898	C–C chain symmetric stretching
~840	CH_2 rocking and C-CH_3 stretching
~806	C–C chain symmetric stretching and CH_2 rocking
Poly(ethylene terephthalate)	
~1715	Stretching vibration of the carbonyl ester group C=O
~1577, ~1503	Vibrations aromatic skeleton with stretching C=C
~1456, ~1409, ~1340	Stretching of the C–O group, deformation of the O–H group, and bending and wagging vibrational modes of the ethylene glycol segment
~1244	Terephthalate group ($OOCC_6H_4$–COO)
~1098, ~1019	CH_2 group and vibrations of the ester C–O bond
~970, ~871, ~848	Aromatic rings 1, 2, 4, 5; Tetra replaced
~793	Vibrations of the adjacent two aromatic H in p-substituted compounds and aromatic bands
~722	Out-of-plane vibration of the benzene ring
Polyethylene	
~2916	CH_2 asymmetric stretching
~2848	CH_2 symmetric stretching
~1472, ~1463	CH_2 symmetric bending
~730, ~718	CH_2 bending and rocking

same sample pretreatment procedures (i.e., digestion and microfiltration) and μ-FTIR-ATR analysis. Blanks were deemed acceptable if they contained no more than 10 MP fragments. If a blank exceeded this threshold (i.e., ≥11 MP fragments), the analysis of the corresponding sample was considered invalid and repeated. The number of MP contaminants detected in blanks was subtracted from the total MP fragments detected in the corresponding sample.

The recovery rate of MP was evaluated by spiking ultrapure water samples with a commercial standard of polystyrene (Cospheric LLC, Santa Barbara, CA). Spiked samples were processed following the entire digestion and filtration procedures. Ten spiking levels, ranging from 22 MP/kg to 207 MP/kg of water, were tested. Recovery rates ranged from 66% to 122%, with an average recovery of 84%.

Statistical analyses

For each sample, the total MP concentration (MP/kg) was calculated as the sum of the concentrations of all MP classes. The total MP concentration was normally distributed, and no outliers were detected. Data were then analyzed through R Software (v. 4.1.3), according to the following linear model described in Eq. (1):

$$y_i = \mu + \text{dairy product}_i + e \quad (1)$$

were y_i is the dependent variable (i.e., the total MP concentration detected in each sample); μ is the overall intercept of the model; dairy product$_i$ is the fixed effect of the *ith* type of dairy product (three classes: milk, fresh cheese, and ripened cheese); and e is the random error. Multiple comparisons of LSMs were performed using the Bonferroni adjustment, with the significance threshold set at $P \leq 0.05$.

Data availability

Data is provided within the manuscript.

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Author contributions

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Competing interests

The authors declare no competing interests.

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CHAPTER 2

Characterization of microplastics in skim-milk powders

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Characterization of microplastics in skim-milk powders

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ABSTRACT

The diffusion of microplastics in the food supply chain is prompting public concern as their impact on human health is still largely unknown. The aim of this study was to qualitatively and quantitatively characterize microplastics in skim-milk powder samples (n = 16) from different European countries (n = 8) through Fourier-transform infrared microspectroscopy in attenuated total reflectance mode analysis. The present study highlights that the use of hot alkaline digestion has enabled the efficacious identification of microplastics in skim-milk powders used for cheesemaking across European countries. The adopted protocol allowed detection of 29 different types of polymeric matrices for a total of 536 plastic particles. The most abundant microplastics were polypropylene, polyethylene, polystyrene, and polyethylene terephthalate. Microplastics were found in skim-milk powders in 3 different shapes (fiber, sphere, and irregular fragments) and 6 different colors (black, blue, brown, fuchsia, green, and gray). Results demonstrate the presence of microplastics in all skim-milk powder samples, suggesting a general contamination. Results of the present study will help to evaluate the impact of microplastics intake on human health.
Key words: microplastics, analysis, Fourier-transform infrared microspectroscopy, dairy

INTRODUCTION

Microplastics (**MIPL**) are defined as plastic fragments, fibers, and beads with a diameter smaller than 5 mm, deriving from the degradation of larger plastic debris (SAPEA, 2019). The presence of MIPL in the environment is an increasingly relevant topic. The fact that plastic particles are present in food products has led to their inevitable exposure to humans, which has raised particular concern. In particular, MIPL have been observed in several food products, such as fish (Bessa et al., 2018; Peters et al., 2018), meat (Kedzierski et al., 2020), dairy products (Kutralam-Muniasamy et al., 2020; Da Costa Filho et al., 2021), water (Oßmann et al., 2018), energy drinks, and soft drinks (Shruti et al., 2020).

The effects of MIPL on human health depend on the way in which the plastic particles enter the body but also on the source of exposure. Scientific evidence has shown that humans can be exposed to MIPL through ingestion of contaminated food and water, inhalation, and direct dermal contact through personal care products, textiles, or dust (Prata, 2018; Kutralam-Muniasamy et al., 2023). Still, the ingestion of contaminated food represents the primary route of entry for MIPL in the human intestine (Van Cauwenberghe and Janssen, 2014). Microplastics are also able to reach the respiratory tract through the ciliary movements of the mucosa after inhalation (Salim et al., 2014). The uptake of MIPL through inhalation represents a risk for human health due to inflammation, chemical toxicity, and infection by microorganisms introduced to the body via MIPL (Wright and Kelly, 2017). The accumulation of particles in the respiratory system can cause acute release of proinflammatory chemotactic factors that can induce chronic inflammation, known as dust overload (Prata, 2018). Other health risks may arise from the dermal contact of MIPL used in hand detergents, face washes, face masks, and toothpastes. In fact, the presence of MIPL in personal care products has been associated with skin damage due to local inflammation and cytotoxicity (Sharma and Chatterjee, 2017). However, due to the limited information on the potential uptake and effects of MIPL at the human health level, tolerance limits are still not defined (Kirstein et al., 2021).

Considering the potential toxicity of MIPL and their absorption into human cells, it is important to investigate sources, quality, and quantity of plastics in food to protect the consumer (Kadac-Czapska et al., 2023). Despite an increasing public concern over the presence of MIPL in the food chain (SAPEA, 2019), consumer awareness is

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The list of standard abbreviations for JDS is available at adsa.org/jds-abbreviations-24. Nonstandard abbreviations are available in the Notes.

mainly focused on marine ecosystems and fewer studies have investigated food products other than fish or crustaceans (Bessa et al., 2018; Peters et al., 2018). This is partially due to the lack of standard methods for processing MIPL in food (e.g., dairy products, meat, honey, and soft drinks). In respect to sample preparation, different digestion solvents are used to remove food's organic and inorganic compounds to facilitate MIPL observation and identification, the most common being alkaline digestion with potassium hydroxide (KOH; Dehaut et al., 2016; Guo et al., 2022). As concerns the analytical phase, several identification methods exist, but they either lack adequate sensitivity for correct polymer identification or specificity to correctly discriminate OM from MIPL, thereby increasing the risk of false positives (Bai et al., 2022). These methods are microscopy-based techniques such as scanning electron microscopy and transmission electron microscopy. Instead, spectroscopic techniques such as Fourier-transform infrared microspectroscopy (**μ-FTIR**) and Raman spectroscopy are alternative analytical methods that are sensitive and nondestructive, allowing for the identification and quantification of different polymer residues in food samples (Bai et al., 2022; Kadac-Czapska et al., 2023).

Milk and dairy products play a key role in human nutrition and development throughout life (Thorning et al., 2016). Such products are routinely tested for the presence of pathogens and other chemical substances that could harm human health. Nevertheless, the extent of MIPL contamination in milk and dairy products and their effect on human health remain largely unknown (Kutralam-Muniasamy et al., 2020; Rahman et al., 2021). Microplastics contamination may occur at different stages along the dairy supply chain, with milking procedures, technological treatments, and packaging representing the major points of contamination risk. This raises concern about the possible implications of MIPL on human health as ingested via milk and dairy products (Diaz-Basantes et al., 2020).

With respect to milk powders, only Da Costa Filho et al. (2021) and Zhang et al. (2023) determined MIPL in 2 cow milk powders and 13 infant milk powders, respectively. There is a lack of studies that attempted to characterize MIPL in skim-milk powder samples on a broader scale. Therefore, the aim of this study was to qualitatively and quantitatively characterize MIPL in skim-milk powder samples from different European countries.

MATERIALS AND METHODS

Sample Collection

Procedures adopted in this study are excluded from animal ethics evaluation because they do not reach thresholds established in the Directive 2010/63/EU (Art. 1) of the European Parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes.

Skim-milk powder samples (n = 16) were produced and collected from 8 different European countries, including 1 sample from Austria, 1 sample from Belgium, 1 sample from Germany, 9 samples from France, 1 sample from Ireland, 1 sample from Italy, 1 sample from the Netherlands, and 1 sample from Poland. In particular, the 9 samples from France were produced in 9 different French dairy plants. All samples (2 kg of net weight) were collected using a multilayer paper bag with a polyethylene inner bag.

Sample preparation and analyses of MIPL were carried out in the laboratory of the European Center for the Sustainable Impact of Nanotechnology (ECSIN, Padova, Italy), as part of Mérieux NutriSciences Company (Chicago, IL).

Reagents

All the procedures involving reagents filtration, reagents handling, and glass washing were carried out in a dedicated clean room, according to the guidelines described in the ISO 14644–1:2015 Class 7 (ISO, 2015). Ultrapure water (18.3 MΩ/cm resistivity at 25°C) was obtained through Zeneer Power III (Human Corporation, Garak-ro, Republic of Korea). All solutions used during the preparation phase were previously microfiltered using a silver membrane filter (3.0-μm pore-size, 25-mm diameter; Sterlitech Corporation, Auburn, WA) and kept in glass containers to avoid any contamination. Glassware was washed 5 times using liquid dishwashing detergent, then rinsed 5 times with deionized water, and finally rinsed 5 more times with ultrapure water.

Sample Quartering, Digestion, and Microfiltration

All the procedures adopted for sample quartering, digestion and filtration were carried out in a dedicated clean room, according to the guidelines described in the ISO 14644–1:2015 Class 7 (ISO, 2015). Before digestion and microfiltration steps, sample quartering was performed to obtain a representative aliquot. In order to remove organic compounds from skim-milk powder samples, a digestion protocol was set up, adapted from Dehaut et al. (2016) and EFSA (2016). For all skim-milk powders, 15 g of sample were weighed in a 1,000-mL glass flask. Then, 100 mL of ultrapure water and 10% KOH were added. To start the alkaline digestion process, samples were placed in a Shake'n Bator (EuroClone, Milan, Italy) and heated to 60°C overnight. Thereafter, 75 mL of 10% EDTA solution were added and samples were

incubated in the Shake'n Bator at 60°C for 2 h. Digested skim-milk powders were microfiltered through a 3.0-µm pore-size silver membrane filter (Sterlitech Corporation, Auburn, WA) using a vacuum pump connected to a glass filter funnel. To enhance the separation of MIPL from organic compounds, a density separation step was applied. This action was aimed at resuspending MIPL characterized by higher density. This step was performed by using an oversaturated solution of sodium chloride (NaCl) added to the same glass flask used for the filtration of the sample. After the density separation step of each sample, the solution containing MIPL was subjected to vacuum filtration. During filtration, the filtering funnel was covered with aluminum foil to minimize contamination. All filters were stored in previously decontaminated glass Petri dishes to prevent contamination. Finally, filters were left to dry at 70°C in an oven until analysis carried out with µ-FTIR in attenuated total reflectance (**ATR**) mode.

Detection and Identification of MIPL by µ-FTIR-ATR

Detection and identification of MIPL were carried out in a dedicated clean room, according to the guidelines described in the ISO 14644–1:2015 Class 7 (ISO, 2015). The analyses to determine the polymeric matrix, the size (µm), the color, and the concentration (MIPL/kg) of MIPL fragments were performed through FTIR Spectrometer Frontier coupled to a microscope Spotlight 400 (Perkin Elmer Italia Spa, Milan, Italy) in µ-FTIR-ATR analysis. The spectrum range was set between 4,000 and 650 cm^{-1} with 4 repeated scans for each measurement. The polymeric matrix of the detected particles was identified by comparing the collected µ-FTIR-ATR spectra with spectra reference libraries using Spectrum 10 software (Perkin Elmer Italia Spa, Milan, Italy). Each MIPL particle was considered correctly identified when the match between MIPL spectra from skim-milk powders and MIPL spectra from reference libraries was >80%. In particular, identified MIPL residues included acrylonitrile butadiene styrene, ethylene-propylene diene monomer, ethylene vinyl acetate, nylon, nylon: resin copolymer, polyacrylate, poly(chlorostyrene), polyester, polyethylene (**PE**), polyethylene chlorinated (**CPE**), PE-polyamide (**PA**) copolymer, PE:CPE, polyethylene terephthalate (**PET**), polyisoprene, polyoxymethylene, polypropylene (**PP**), PP-polybutylene terephthalate copolymer, polystyrene (**PS**), PS-polyacrylate copolymer, PS:CPE copolymer, PS-polyurethane (**PU**) copolymer, polytetrafluoroethylene, PU, polyvinyl chloride, polyvinylidene fluoride, resin, silicone, styrene-butadiene-styrene, and styrene-ethylene-butylene-styrene. High-resolution images of MIPL fragments were electronically stored through Spectrum 10 software. The size of each MIPL was evaluated using ImageJ software (ImageJ, 2022). In particular, fibers were measured along their length and fragments characterized by irregular or spherical shapes were measured in their greater dimension. The identified MIPL exhibited a variety of colors such as black, blue, brown, fuchsia, green, and gray. No transparent MIPL particles were found in the skim-milk powder samples.

Blanks and Recovery

Blanks were prepared along with each skim-milk powder sample and according to the same protocol, including digestion, filtration, detection, and identification steps. Blanks were considered valid up to 10 MIPL fragment contaminants (that is, whenever 11 MIPL fragment contaminants were retrieved in a blank, the analysis of the corresponding sample was considered invalid and was repeated). In the analyzed blank filters, a maximum of 3 MIPL fragment contaminants was found. Polyethylene terephthalate was the most frequent polymer in the blank samples, representing 25% of total MIPL contaminants, followed by PP, PE, and polyisoprene (each representing 19% of total MIPL contaminants) and ethylene vinyl acetate, styrene-butadiene-styrene, and polyvinyl alcohol to an even lesser extent (each representing 6% of total MIPL contaminants). The number of MIPL contaminants detected in blanks was subtracted from the total MIPL fragments detected in the corresponding skim-milk powder sample.

The recovery of the method was performed by spiking ultrapure water with commercial standard of PS (Cospheric LLC, Santa Barbara, CA). Ten different spiking levels were tested, ranging from 22 to 207 MIPL/kg of water. Particle recovery ranged from 66% to 122%, with average recovery (SD) of 84% (19.90 MIPL/kg of water).

RESULTS AND DISCUSSION

Qualitative Features of Identified Microplastics

Results of the present study highlight a widespread presence of plastic particles in skim-milk powder samples from different European countries. Details about the identified MIPL, including dimensions, morphology, and colors are reported in Supplemental Table S1 (see Notes). Half of the identified MIPL were ≤60.50 µm; approximately 30% of the identified MIPL were in the range of 60.50 to 99.00 µm, and approximately 20% of them were >99.00 µm with a maximum size of 1,444.00 µm. Such dimensions are considerably smaller compared to those reported by Kutralam-Muniasamy et al. (2020), who detected the majority of MIPL in milk samples with a size <500 µm (40%), followed by sizes between 500 and 1,000 µm (28%) and between 1,000 and 2,000 µm

(25%) using Raman spectroscopy. In general, the size distribution of MIPL in food is affected by the different methods used for observing and measuring them. Difficulties also arise when comparing results from various studies because bias may be introduced by different laboratory procedures and environments (Filella, 2015). In addition, it is possible to infer that physical processes adopted during skim-milk powder production may lead to the fragmentation and miniaturization of MIPL originally contained in the starting liquid milk. Although the majority of the particles identified in the present study consisted of irregular fragments (88%), spheres, and fibers were also observed (7% and 5%, respectively). This somewhat differs from the results reported by Kutralam-Muniasamy et al. (2020), who observed that fiber was the most represented shape (97.5%), and fragments accounted for only 2.5% in the 23 milk products analyzed. Figure 1 shows some µ-FTIR-ATR spectra and images of major species of MIPL found in skim-milk powder samples. All MIPL fragments characterized in the present study were pigmented, with brown, gray, black, and blue being the most common colors (about 51%, 40%, 6%, and 2.4% respectively; Supplemental Table S1). In particular, blue MIPL in food may be at least partly attributed to the work clothes and masks worn by personnel during the food production processes (Bai et al., 2022).

Quantitative Extent of MIPL Contamination

Table 1 reports count and descriptive statistics of MIPL in skim-milk powders. The most widespread MIPL characterized in the analyzed samples (Table 1) were PP (5,764.71 MIPL/kg), PS (1,081.00 MIPL/kg), and PE (466.00 MIPL/kg); their presence in skim-milk powder samples may be due to farm environment, milking procedures, milking apparatuses (Diaz-Basantes et al., 2020), contamination from worker uniforms (generally made of PET) and hygiene caps and masks (commonly made of PP; Da Costa Filho et al., 2021). Other sources of MIPL contamination in milk can include pipes made of plastic materials commonly used for milk transport and storage at the dairy industry level (Kutralam-Muniasamy et al., 2020).

Although MIPL were detected in all analyzed samples, MIPL types varied greatly among skim-milk powders, ranging from a minimum of 2 to a maximum of 13 MIPL species within a sample (Figure 2). Additionally, 29 different types of polymeric matrices were identified (Figure 2) for a total count of 536 particles (Supplemental Table S1) in the 16 analyzed skim-milk powders. Such results suggest a greater variability of polymeric matrices and a greater number of plastic particles compared with the results reported by Zhang et al. (2023). The same authors detected only PE, PP, PET, PA, and polyvinylchloride in infant milk powder using µ-FTIR. Furthermore, skim-milk powders analyzed in the present study originated from different countries, wherefore transport and the use of different packaging materials is a source of contamination to be taken into consideration. Results of the present study also highlighted that skim-milk powder samples from different countries had various types and levels of MIPL contamination (Figure 2). This could be due to different and specific conditions adopted for milk powders production, which include pretreatment (skimming, homogenization, and pasteurization), concentration, and drying (including air filtering and energy supply; Moejes and Van Boxtel, 2017). In fact, the presence of a greater number of MIPL in powdered milk compared to liquid milk may be due to the release of plastic particles from polymeric membranes or filters with reduced performance used during the different phases of milk powder production (Kumar et al., 2013). A similar contamination is due to the degradation of PET milk bottles exposed to chemical or physical agents (particularly applied to reusable PET bottles), which indeed is presumed to lead to the release of plastic particles (Schymanski et al., 2018; Sobhani et al., 2020). Therefore, the exact knowledge of all the activities performed during milk powder production may help to shed light on the main sources of MIPL contamination in powdered milk samples.

Method Feasibility and Advantages

The digestion protocol used in this study, after sample solubilization through water addition, consisted of a hot alkaline digestion, which allowed removal of organic and inorganic residues (i.e., proteins, lipids, minerals, and cellulose), thus facilitating the identification and the quantification of MIPL in skim-milk powder samples. Overall, KOH, which is alkaline in nature, is the most common digestion solution used in food matrices (Guo et al., 2022), with less common acid digestion based on either hydrogen peroxide (H_2O_2) or nitric acid (HNO_3). These strong acids can destroy or damage polymers with a low pH tolerance, such as PS and PA (Cole et al., 2014). Their application in the analysis of MIPL is thus limited (Cole et al., 2014). In addition, in a study aiming at the characterization of MIPL in seafood, Dehaut et al. (2016) confirmed that KOH-based extraction is an effective and practical method to separate plastic particles from OM.

To date, the µ-FTIR technique is the most frequently used approach in MIPL identification and quantification (Chen et al., 2020; Bai et al., 2022) and represents a promising tool for automated MIPL analysis. It allows concurrent identification and quantification of polymer types (Sridhar et al., 2022), while also inferring information on their chemical characteristics and structure (Bai et al., 2022). Although the analysis time required for

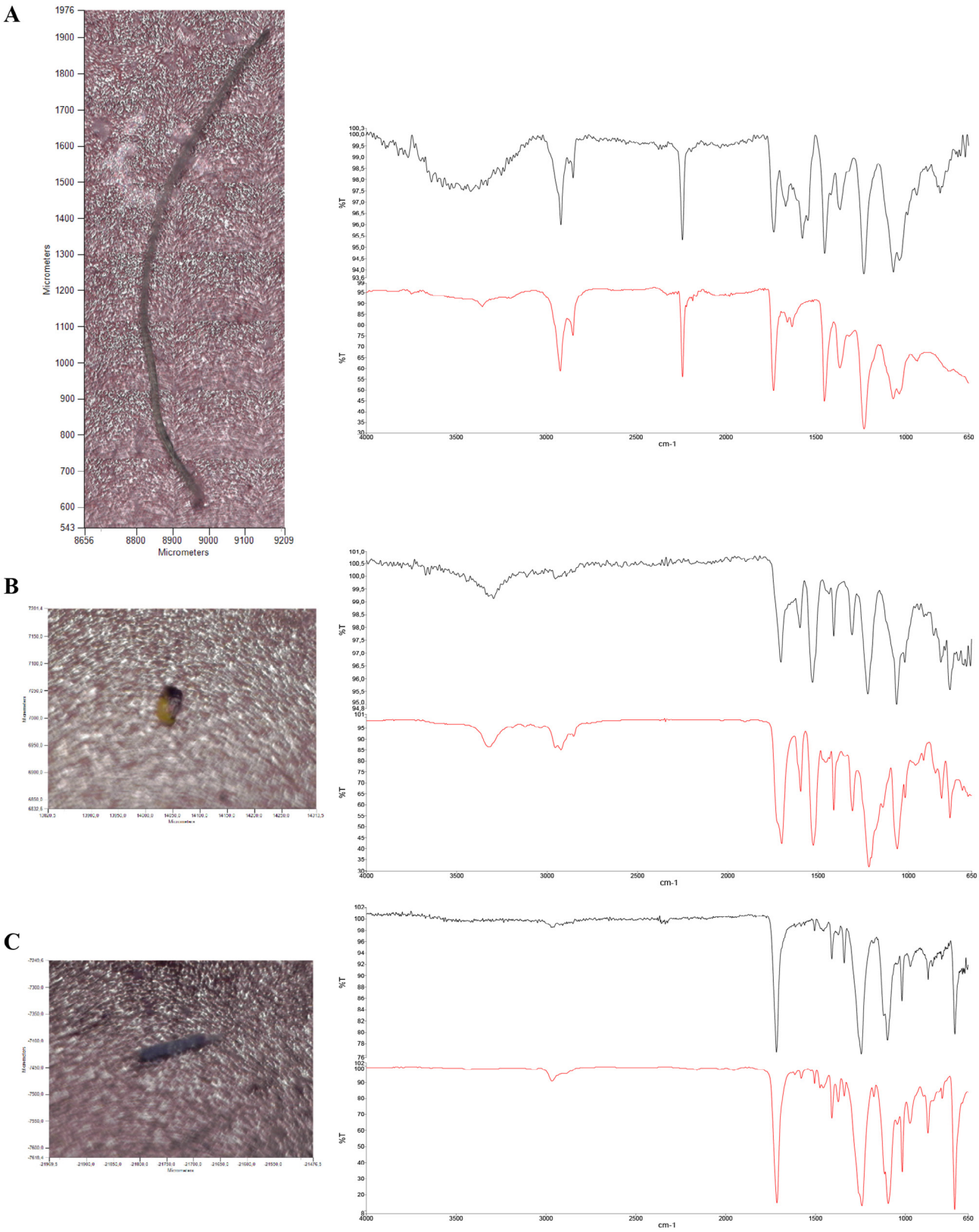

Figure 1. Images and μ-FTIR-ATR spectra (black line = microplastic spectra from skim-milk powders; red line = microplastic spectra from reference libraries) of the major species of microplastics in skim-milk powders: (A) polyacrylate, (B) PU, (C) PET, (D) polyisoprene, (E) PP, (F) PS, and (G) styrene-ethylene-butylene-styrene.

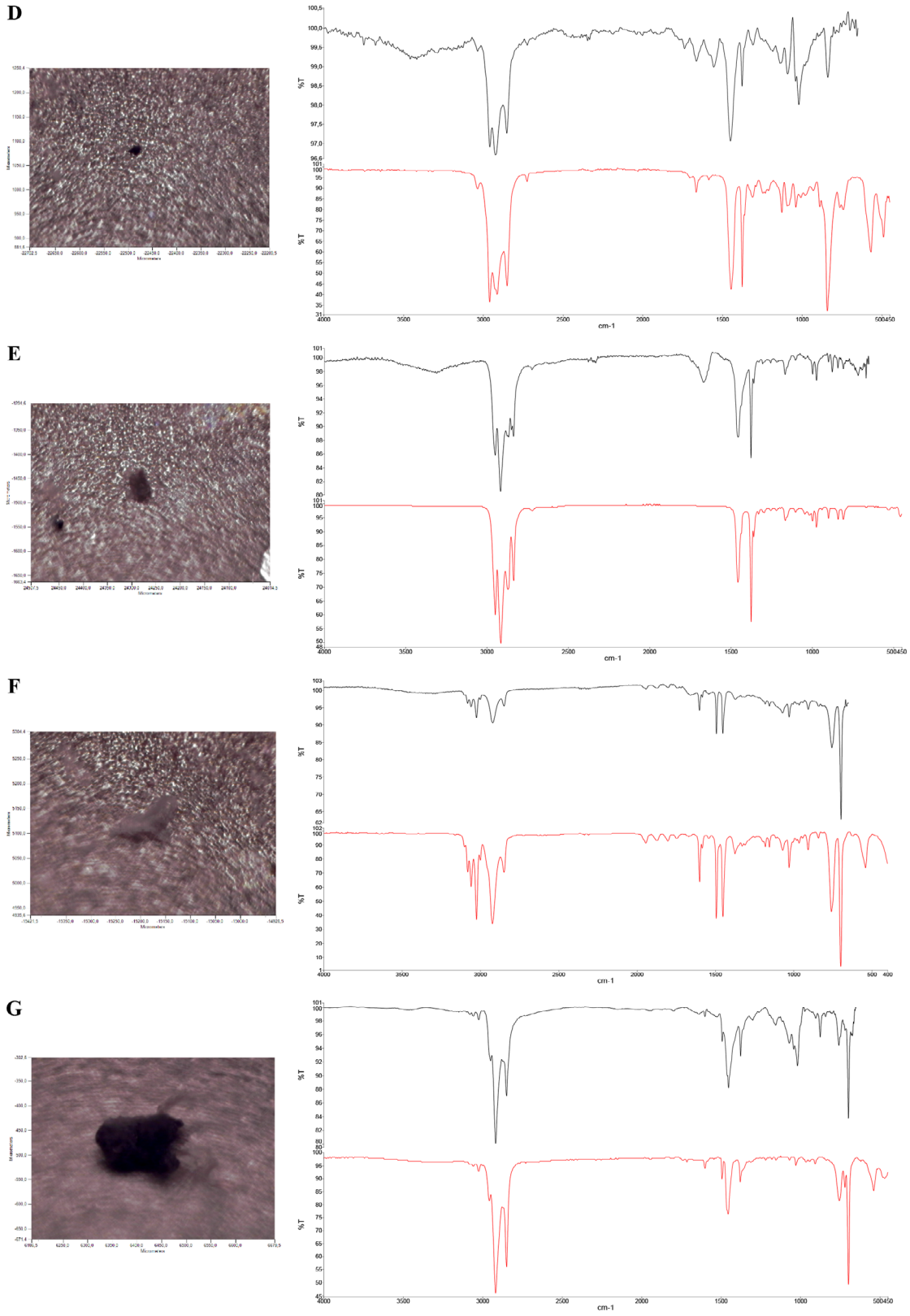

Figure 1 (Continued). Images and μ-FTIR-ATR spectra (black line = microplastic spectra from skim-milk powders; red line = microplastic spectra from reference libraries) of the major species of microplastics in skim-milk powders: (A) polyacrylate, (B) PU, (C) PET, (D) polyisoprene, (E) PP, (F) PS, and (G) styrene-ethylene-butylene-styrene.

Table 1. Count and descriptive statistics of MIPL in skim-milk powders (detected in at least 2 samples)[1]

MIPL	MIPL count	Mean	SD	Minimum	Maximum
Ethylene vinyl acetate	7	219.83	198.84	67.00	562.00
Nylon	11	276.00	149.10	133.00	562.00
Polyacrylate	2	119.50	19.09	106.00	133.00
Polyester	8	370.50	341.80	106.00	843.00
Polyethylene	33	466.00	454.39	133.00	1,785.00
Polyethylene chlorinated	2	231.50	177.48	106.00	357.00
Polyethylene terephthalate	23	368.40	229.19	133.00	714.00
Polyisoprene	13	239.33	194.08	40.00	581.00
Polypropylene	334	5,764.71	12,837.49	67.00	47,765.00
Polystyrene	43	1,081.00	2,052.00	133.00	7,071.00
Polyurethane	9	227.43	216.62	133.00	714.00
Polyvinyl chloride	4	240.75	94.90	133.00	357.00
Polyvinylidene fluoride	9	578.50	237.52	357.00	843.00
Resin	6	441.50	252.38	133.00	714.00
Silicone	5	172.40	103.85	106.00	357.00
Styrene-butadiene-styrene	11	343.25	356.19	106.00	867.00

[1]The MIPL count was calculated as the sum of MIPL retrieved in all analyzed filters. The mean, SD, minimum, and maximum values are expressed as number of MIPL per kilogram of sample.

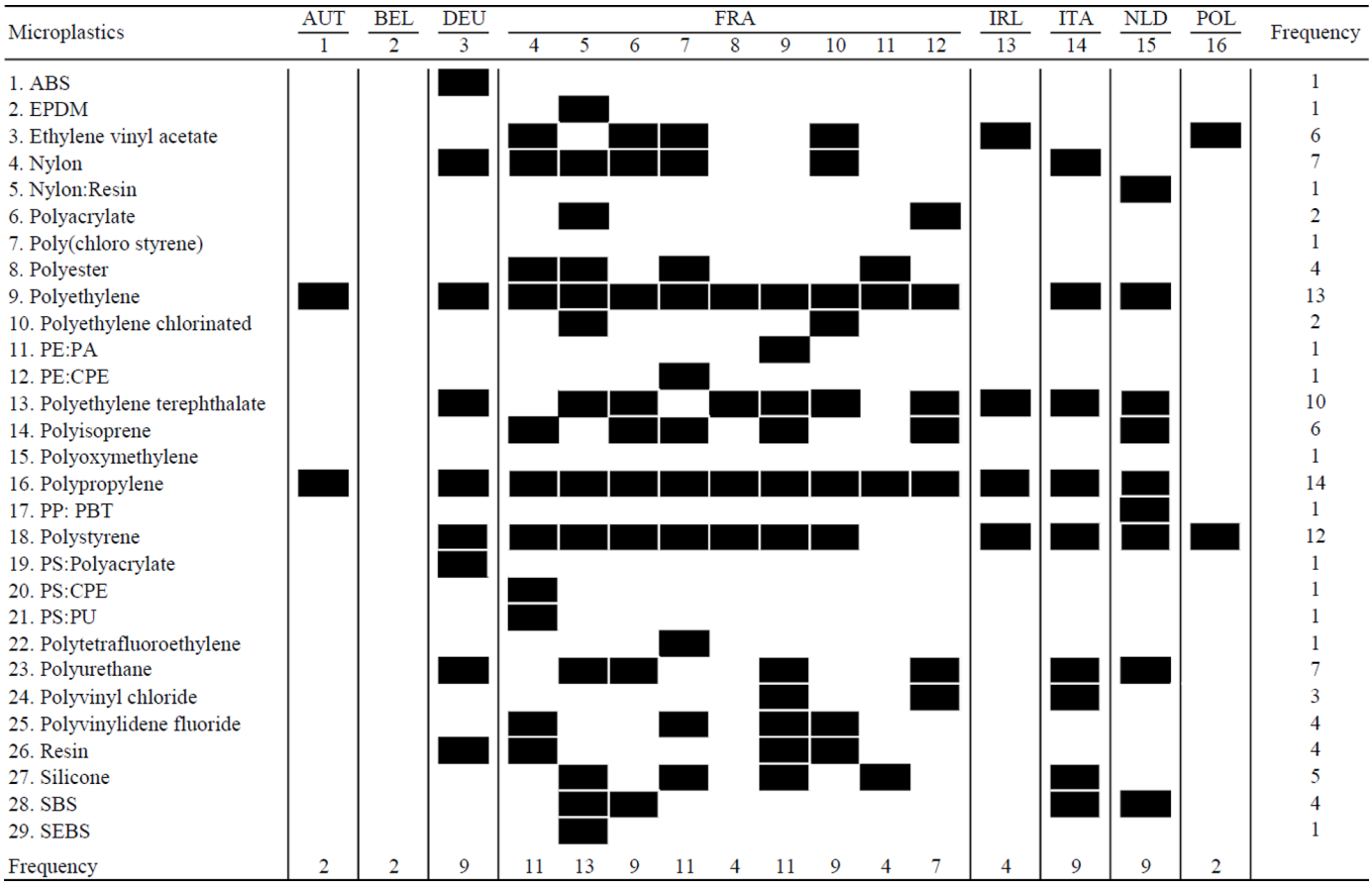

Figure 2. Microplastics characterized in 16 skim-milk powder samples from 8 different European countries. The "Frequency" column on the right refers to each type of microplastic across samples; frequency numbers listed across the bottom refer to numbers of microplastics particles within each sample. The number listed under each country is the sample number. Microplastics: ABS = acrylonitrile butadiene styrene; EPDM = ethylene-propylene diene monomer; PE:PA = polyethylene-polyamide copolymer; PE:CPE = polyethylene-polyethylene chlorinated copolymer; PP:PBT = polypropylene-polybutylene terephthalate copolymer; PS:Polyacrylate = polystyrene-polyacrylate copolymer; PS:CPE = polystyrene-polyethylene chlorinated copolymer; PS:PU = polystyrene-polyurethane copolymer; SBS = styrene-butadiene-styrene; SEBS = styrene-ethylene-butylene-styrene. Countries: AUT = Austria; BEL = Belgium; DEU: =Germany; FRA = France; IRL = Ireland; ITA = Italy; NLD = the Netherlands; POL = Poland.

each filter obtained at the end of sample preparation was long (about 2 working days per filter), it is undisputed that this technique allows more accurate and efficacious detection of microsized particles compared with other microscope-based techniques (Song et al., 2015; Lee and Chae, 2021). The µ-FTIR technique requires good sample-filter-surface conditions in terms of wrinkles, folds, and organic compounds because the MIPL may otherwise be covered or trapped by sample fibers, leading to an underestimation of their quantification.

Further research should be devoted to improving automated analysis methodology and to reduce the identification time. In addition, it is imperative to standardize appropriate methods of identification and characterization of MIPL in foods using reliable protocols with strict quality assurance and blank control. This would provide repeatable, sensitive, and accurate results to guarantee a relationship of trust between the food industry and consumers.

CONCLUSIONS

To our knowledge, this is the first contribution addressing a qualitative and quantitative characterization of MIPL in skim-milk powder samples from different European countries. Skim-milk powder samples used for cheesemaking in different European countries were analyzed to detect and identify MIPL through µ-FTIR-ATR. Results demonstrated a wide diffusion of MIPL in the analyzed samples, which may be transferred to cheese products and, ultimately, ingested by consumers. A great variability of polymeric particles exists, both in terms of quality and quantity. Based on the presence of these MIPL across countries, the most frequent are PP (n = 14), PE (n = 13), PS (n = 12), and PET (n = 10). In conclusion, due to the uncertainty surrounding the effect of MIPL on human health, it is of most importance to identify the main MIPL contamination in skim-milk powder, so a selective reduction in the use of plastic along the supply chain can be made. This will ultimately prevent, or at least minimize, the amount of MIPL ingested by consumers.

NOTES

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Nonstandard abbreviations used: ABS = acrylonitrile butadiene styrene; ATR = attenuated total reflectance; AUT = Austria; BEL = Belgium; CPE = polyethylene chlorinated; DEU: =Germany; EPDM = ethylene-propylene diene monomer; FRA = France; IRL = Ireland; ITA = Italy; MIPL = microplastics; µ-FTIR = Fourier-transform infrared microspectroscopy; NLD = the Netherlands; PA = polyamide; PE = polyethylene; PE:PA = polyethylene-polyamide copolymer; PE:CPE = polyethylene-polyethylene chlorinated copolymer; PET = polyethylene terephthalate; POL = Poland; PP = polypropylene; PP:PBT = polypropylene-polybutylene terephthalate copolymer; PS = polystyrene; PS:CPE = polystyrene-polyethylene chlorinated copolymer; PS: Polyacrylate = polystyrene-polyacrylate copolymer; PS:PU = polystyrene-polyurethane copolymer; PU = polyurethane; SBS = styrene-butadiene-styrene; SEBS = styrene-ethylene-butylene-styrene.

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Supplemental Table S1. Morphological and chemical features of the identified microplastics[1].

Microplastics	Count of microplastics	Size (± SD)[2], µm	Shape			Color						Country
			Fiber	Irregular	Sphere	Black	Blue	Brown	Fuchsia	Green	Gray	
1. ABS	1	72.00	-	1	-	1	-	-	-	-	-	Germany
2. EPDM	1	131.00	-	1	-	1	-	-	-	-	-	France
3. Ethylene vinyl acetate	2	99.00 (± 48.08)	-	2	-	-	-	1	-	-	1	France
	1	25.00	-	1	-	1	-	-	-	-	-	France
	1	23.00	-	1	-	1	-	-	-	-	-	France
	1	62.00	-	1	-	-	-	1	-	-	-	France
	1	85.00	-	1	-	-	-	1	-	-	-	Ireland
	1	68.00	-	1	-	1	-	-	-	-	-	Poland
4. Nylon	2	53.50 (± 0.71)	-	2	-	-	-	-	-	-	2	Germany
	2	48.50 (± 3.54)	-	2	-	-	-	2	-	-	-	France
	2	62.00 (± 21.21)	-	2	-	1	-	1	-	-	-	France
	1	100.00	-	-	1	-	-	1	-	-	-	France
	2	49.50 (± 31.82)	-	2	-	-	-	2	-	-	-	France
	1	93.00	-	1	-	-	-	-	-	-	1	France
	1	129.00	-	1	-	-	-	-	-	-	1	Italy
5. Nylon:Resin	1	102.00	1	-	-	1	-	-	-	-	-	Netherlands
6. Polyacrylate	1	85.00	1	-	-	-	1	-	-	-	-	France
	1	1444.00	1	-	-	-	-	-	-	1	-	France
7. Poly(chloro styrene)	1	43.73	-	1	-	1	-	-	-	-	-	Belgium
8. Polyester	3	217.00 (± 169.58)	1	2	-	-	-	3	-	-	-	France
	1	47.00	-	1	-	1	-	-	-	-	-	France
	3	195.00 (±169.14)	2	1	-	1	-	2	-	-	-	France
	1	171.00	-	1	-	-	-	1	-	-	-	France
9. Polyethylene	1	81.92	-	1	-	-	-	-	-	-	1	Austria
	3	73.33 (± 7.77)	-	3	-	-	-	2	-	-	1	Germany
	3	300.67 (± 378.09)	1	2	-	1	-	2	-	-	-	France

	3	101.50 (± 57.28)	-	2	1	1	-	2	-	-	-	France
	1	242.00	-	1	-	-	-	1	-	-	-	France
	2	30.50 (± 16.26)	-	2	-	-	-	2	-	-	-	France
	1	84.00	-	1	-	-	-	-	-	-	1	France
	2	49.50 (± 2.12)	-	2	-	-	-	2	-	-	-	France
	5	70.20 (± 31.09)	-	5	-	-	-	-	-	-	5	France
	4	78.75 (± 23.99)	-	4	-	-	-	4	-	-	-	France
	4	51.50 (± 25.21)	-	4	-	-	-	-	-	-	4	France
	3	124.00 (± 77.08)	-	2	1	-	-	-	-	-	3	Italy
	1	69.00	-	1	-	-	-	1	-	-	-	Netherlands
10. Polyethylene chlorinated	1	34.00	-	1	-	-	-	1	-	-	-	France
	1	78.00	-	1	-	1	-	-	-	-	-	France
11. PE:PA	1	115.00	-	-	1	-	-	-	-	-	1	France
12. PE:CPE	1	32.00	-	1	-	-	-	1	-	-	-	France
13. Polyethylene terephthalate	2	290.50 (± 277.89)	1	1	-	-	-	-	-	-	2	Germany
	2	27.50 (± 4.95)	-	2	-	-	1	1	-	-	-	France
	5	197.40 (± 165.88)	5	-	-	-	3	2	-	-	-	France
	1	899.00	1	-	-	-	-	-	-	-	1	France
	1	94.00	-	1	-	-	-	-	-	-	1	France
	1	356.00	1	-	-	-	-	-	-	-	1	France
	1	58.00	-	1	-	-	-	-	-	-	1	France
	2	45.00 (± 18.38)	1	1	-	-	-	1	-	-	1	Ireland
	3	63.00 (± 24.28)	-	3	-	-	-	1	-	-	2	Italy
	5	169.00 (± 177.65)	5	-	-	-	2	1	-	-	2	Netherlands
14. Polyisoprene	1	23.00	-	1	-	-	-	1	-	-	-	France
	2	35.50 (± 7.78)	-	1	1	1	-	1	-	-	-	France
	1	27.00	-	1	-	-	-	1	-	-	-	France
	2	56.50 (± 28.99)	-	2	-	2	-	-	-	-	-	France
	3	72.00 (± 25.32)	-	3	-	-	-	3	-	-	-	France
	4	43.67 (± 13.05)	-	4	-	1	-	3	-	-	-	Netherlands
15. Polyoxymethylene	1	108.92	-	1	-	-	-	-	-	-	1	Belgium
16. Polypropylene	1	68.58	-	1	-	-	-	-	-	-	1	Austria
	8	50.63 (±17.08)	-	8	-	-	-	2	-	-	6	Germany
	7	80.29 (± 93.01)	1	4	2	1	-	6	-	-	-	France

	13	61.54 (± 34.46)	-	12	1	-	2	11	-	-	-	France
	1	38.00	-	-	1	-	-	1	-	-	-	France
	1	214.00	-	1	-	-	-	-	-	-	1	France
	3	150.33 (± 174.67)	-	3	-	-	-	2	-	-	1	France
	25	44.84 (± 16.48)	1	20	4	-	-	25	-	-	-	France
	134	62.17 (± 48.29)	-	134	-	-	-	-	-	-	134	France
	112	59.11 (± 26.36)	1	102	9	-	-	112	-	-	-	France
	4	86.25 (± 52.39)	-	4	-	-	-	1	-	-	3	France
	1	51.00	-	1	-	-	-	-	-	-	1	Ireland
	20	88.25 (± 59.58)	2	16	2	-	-	-	-	-	20	Italy
	4	84.75 (± 63.47)	-	4	-	-	-	4	-	-	-	Netherlands
17. PP:PBT	1	30.00	-	1	-	1	-	-	-	-	-	Netherlands
	2	60.50 (± 30.41)	-	1	1	-	-		-	-	2	Germany
	2	29.00 (± 15.56)	-	2	-	-	-	2	-	-	-	France
	3	24.67 (± 12.74)	-	3	-	2	-		1	-	-	France
	2	116.50 (± 55.86)	-	2	-	-	-	2	-	-	-	France
	23	46.91 (± 21.32)	-	22	1	-	-	23	-	-	-	France
	2	33.50 (± 3.54)	-	2	-	-	-	2	-	-	-	France
18. Polystyrene	1	47.00	-	1	-	-	-	-	-	-	1	France
	3	82.00 (± 21.28)	-	3	-	-	-	1	-	-	2	France
	2	39.00 (± 1.41)	-	2	-	-	-	2	-	-	-	Ireland
	1	45.00	-	1	-	-	-	1	-	-	-	Italy
	1	58.00	-	1	-	-	-	1	-	-	-	Netherlands
	1	49.00	-	1	-	1	-	-	-	-	-	Poland
19. PS:Polyacrylate	3	35.67 (± 32.39)	-	3	-	-	3	-	-	-	-	Germany
20. PS:CPE	1	64.00	-	-	1	-	-	1	-	-	-	France
21. PS:PU	1	32.00	-	-	1	1	-	-	-	-	-	France
22. Polytetrafluoroethylene	1	41.00	-	1	-	-	-	1	-	-	-	France
	1	76.00	-	1	-	-	-	1	-	-	-	Germany
	2	42.00 (± 2.83)	-	2	-	1	-	1	-	-	-	France
23. Polyurethane	1	45.00	-	1	-	-	-	1	-	-	-	France
	2	43.00 (± 9.90)	-	2	-	2	-	-	-	-	-	France
	1	66.00	-	1	-	-	-	1	-	-	-	France
	1	37.00	-	1	-	-	-	-	-	-	1	Italy

	1	49.00	-	1	-	1	-	-	-	-	-	Netherlands
24. Polyvinyl chloride	1	55.00	-	1	-	-	-	1	-	-	-	France
	1	41.00	-	1	-	-	-	-	-	-	1	France
	2	39.00 (± 5.66)	-	2	-	-	-	-	-	-	2	Italy
25. Polyvinylidene fluoride	3	74.33 (± 40.45)	-	3	-	-	-	3	-	-	-	France
	3	39.67 (± 6.03)	-	3	-	-	-	3	-	-	-	France
	1	43.00	-	1	-	1	-	-	-	-	-	France
	2	86.00 (± 38.18)	-	2	-	-	-	2	-	-	-	France
26. Resin	1	50.00	-	1	-	-	-	-	-	-	1	Germany
	2	29.50 (± 6.36)	-	-	2	-	-	2	-	-	-	France
	2	45.50 (± 33.23)	-	1	1	1	-	1	-	-	-	France
	1	42.00	-	1	-	-	-	-	-	-	1	France
27. Silicone	1	69.00	-	1	-	-	-	1	-	-	-	France
	1	36.00	-	1	-	-	-	1	-	-	-	France
	1	51.00	-	1	-	-	-	1	-	-	-	France
	1	82.00	-	1	-	-	-	1	-	-	-	France
	1	151.00	-	1	-	-	-	1	-	-	-	Italy
28. SBS	1	33.00	-	1	-	1	-	-	-	-	-	France
	2	30.50 (± 4.95)	-	-	2	-	-	2	-	-	-	France
	7	44.14 (± 8.21)	-	2	5	2	-	-	-	-	5	Italy
	1	63.00	-	1	-	1	-	-	-	-	-	Netherlands
29. SEBS	2	108.50 (± 105.36)	1	1	-	1	-	1	-	-	-	France

[1]ABS: Acrylonitrile-butadiene-styrene; EPDM: Ethylene-propylene diene monomer; PE:CPE: Polyethylene-polyethylene chlorinated copolymer; PE:PA: Polyethylene-polyamide copolymer; PP:PBT: Polypropylene-polybutylene terephthalate copolymer; PS:Polyacrylate: Polystyrene-polyacrylate copolymer; PS:CPE: Polystyrene-polyethylene chlorinated copolymer; PS:PU: Polystyrene-polyurethane copolymer; SBS: Styrene-butadiene-styrene; SEBS: Styrene-ethylene-butylene-styrene.

[2]If more than 1 sample, it is indicated as mean ± standard deviation.

CHAPTER 3

Preliminary characterization of microplastics in beef hamburgers

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ABSTRACT

The diffusion of microplastics in meat products is an emerging topic, as their impact on animal and human health is still largely unknown. The present study aimed to preliminarily determine the number and the quality of microplastics diffusion in beef hamburgers ($n = 10$) through Fourier-transformed infrared micro-spectroscopy in attenuated total reflectance mode analysis. Microplastics were detected in all analyzed samples. The abundance of microplastics ranged from 200.00 to 30,300.00 MP/kg. Microplastics observed in the analyzed samples were mainly characterized by irregular shapes (95.99%), grey color (70.16%), and dimensions comprised between 51 and 100 μm (57.46%). Eighteen different polymers were detected, with polycarbonate (30,300.00 MP/kg), polyethylene (1580.00 MP/kg) and polypropylene (750.00 MP/kg) being the most abundant classes. Results demonstrate an extensive diffusion of microplastics in the analyzed samples, which may be originated from various sources, including animal body, industrial processing, and packaging. Findings from this study will aid in pinpointing the source of microplastics contamination, enabling the creation of targeted guidelines to mitigate microplastics spread in processed meat food.

1. Introduction

Meat and processed meat products play an important role in human nutrition and health. According to Stewart et al. (2021), the most recent data on red meat and processed meat consumption has been estimated to be 23.7 and 26.8 g/day, respectively. Particularly, red meat contributes to the intake of high-quality proteins, essential vitamins, and minerals, such as zinc, selenium, calcium, and iron (De Smet & Vossen, 2016). Especially noted for its high heme-iron content, beef meat is acknowledged among the best sources of iron in human nutrition (Kongkachuichai et al., 2002), which makes it important in a healthy diet and crucial in preventing nutritional iron deficiency (Abbaspour et al., 2014; World Health Organization, 2001). Meat is also a relevant source of B-complex vitamins including thiamin, riboflavin, niacin, biotin, vitamins B_6 and B_{12}. In particular, among the aforementioned vitamins, the last cannot be found in vegetables and plant-based foods (Food and Agriculture Organization of the United Nations (FAO), 2002; Watanabe, 2007).

Despite these favorable nutritional traits, it is worthy to report that in several circumstances meat can become a source of potentially dangerous substances, such as heavy metal traces (i.e. arsenic, cadmium, lead, nickel, and mercury) (Demirezen & Uruç, 2006; Emami et al., 2023) and antibiotic residues (Elsayed et al., 2023). For instance, the accumulation of heavy metals in meat may result from industrial activities and agricultural practices, including the use of contaminated animal feed, fertilizers, or pesticides (Hossain et al., 2024). On the other hand, the overuse and misuse of veterinary antibiotics can lead to the presence of active ingredient residues in animal organs and tissues (Redwan Haque et al., 2023).

The term '*microplastic*' was introduced by Thompson et al. (2004), who first characterized plastic particles smaller than 5 mm in marine sediments. Humans are exposed to microplastics (**MP**) through two main pathways, including inhalation into the respiratory tract and ingestion into the gastrointestinal tract (Pironti et al., 2021; Ramsperger et al., 2023). Several studies conducted in three fish species from the Northeast Atlantic Ocean (Barboza et al., 2020) and in shrimps and zebrafish embryos (Galloway et al., 2017), have investigated the ingestion and subsequent translocation of MP in different animal tissues. Conclusions drawn from these studies have raised concerns also in respect to the toxicological effects that MP may pose for human health (Prata et al., 2020; Wright & Kelly, 2017). Indeed, several studies already described risks associated with MP ingestion, including inflammatory response, alteration of composition, and function of gut microbiota and transfer of contaminants to body tissues and organs (Prata, 2018; Wright & Kelly,

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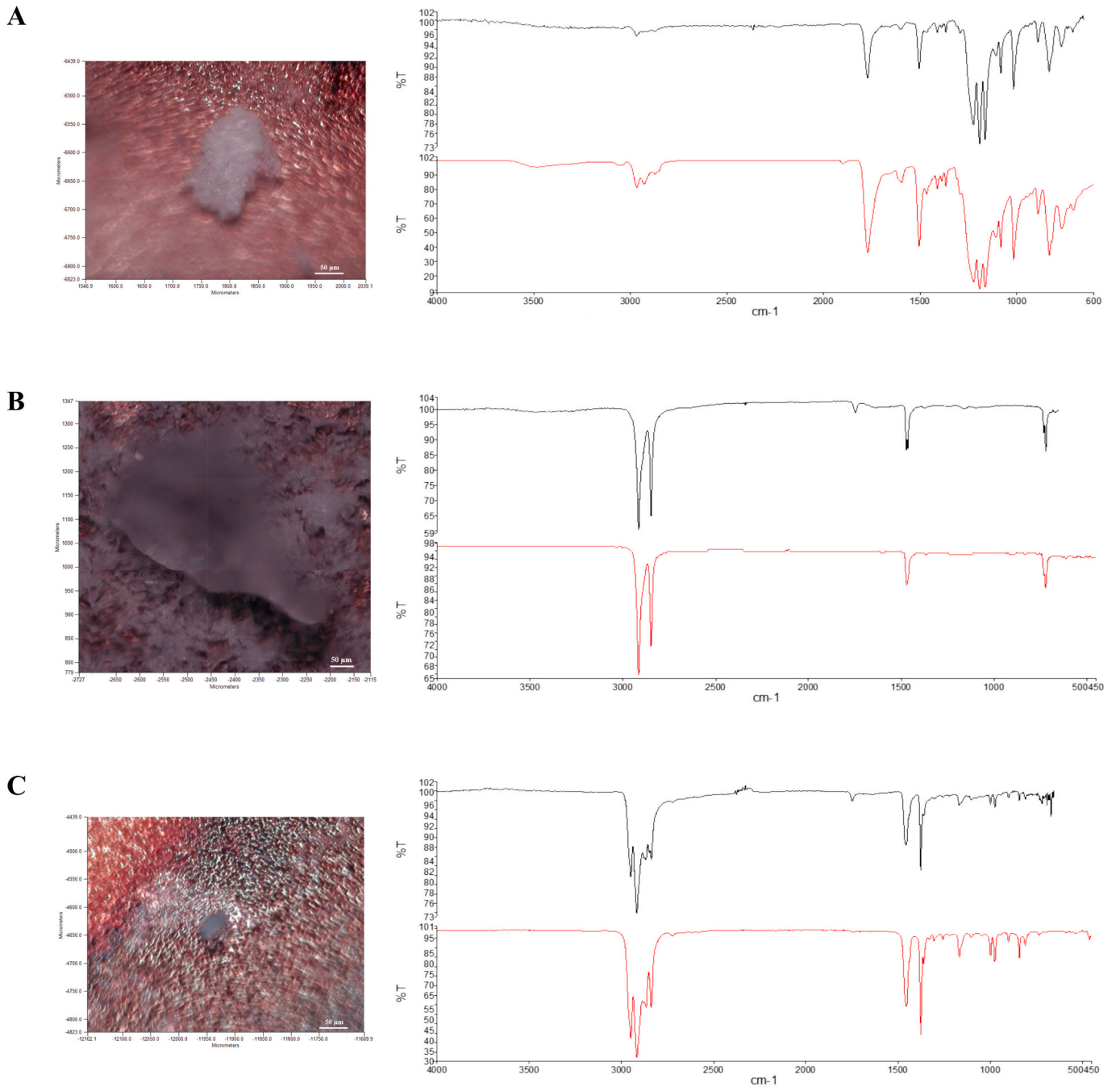

Fig. 1. Images and Fourier-transformed infrared micro-spectroscopy in attenuated total reflectance mode analysis (μ-FTIR-ATR) spectra of the most abundant identified microplastics: (**A**) polycarbonate, (**B**) polyethylene, and (**C**) polypropylene (black line for microplastics spectra from beef hamburgers, red line for microplastics spectra from reference libraries). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2017). In this scenario, the diffusion of MP in animal body and derived meat products is an emerging issue, which is gaining the attention of scientific community since associated effects on animal and human health are still largely unknown (Al Mamun et al., 2023; Lin et al., 2022).

In the recent years, a growing number of studies have addressed the exposure of humans to MP through the ingestion of contaminated food (Rahman et al., 2021). However, most of the literature is focused on the occurrence of MP in drinking water (Oßmann et al., 2018; Pivokonsky et al., 2018; Zhang et al., 2020), beverages (Shruti et al., 2020), honey (Diaz-Basantes et al., 2020; Mühlschlegel et al., 2017), fish (Kumar et al., 2021; Nalbone et al., 2021), milk, and dairy products (Da Costa Filho et al., 2021; Kutralam-Muniasamy et al., 2020; Visentin et al., 2024). To date, few studies investigating the presence of MP in meat have been published (Kedzierski et al., 2020; Habib, Kindi, et al., 2022; Bilal et al., 2023). Recently, Huang et al. (2020) proposed a rapid method based on mid infrared spectroscopy combined with chemometric techniques to identify the level of MP contamination in chicken meat. Bilal et al. (2023) provided evidence for MP in poultry which may originate from contaminated feed. Kedzierski et al. (2020) highlighted that MP on the surface of chicken meat mainly derived from food packaging. Habib, Kindi, et al. (2022) concluded that the primary source of MP contamination in chicken meat was the polythene-based plastic cutting board used for meat preparation. Katsara et al. (2022) observed MP migration

from food packaging to the surface of cured meat products stored at refrigeration temperatures (4 °C). To authors knowledge, only Habib, Poulose, et al. (2022) and Van der Veen et al. (2022) have attempted the characterization of MP in red meat by analyzing samples of beef meat through Fourier-transformed infrared spectroscopy and pyrolysis-gas chromatography coupled with mass spectrometry, respectively. Even less information is available in respect to processed beef meat (i.e., to authors knowledge, there are no studies on processed beef meat).

Therefore, the aim of this study was to preliminarily determine the number and the quality of MP contamination in beef hamburgers produced by Italian beef companies.

2. Materials and methods

2.1. Sample collection

Ten different beef hamburgers produced by 2 Italian companies were purchased in a Large-Scale Retail Market. Out of the 10 samples, 8 were produced by "Company 1" (**C1**) and 2 were produced by "Company 2" (**C2**). Seven hamburgers (5 from C1 and 2 from C2) were characterized by polyethylene skin-packaging. The remaining 3 hamburgers (all from C1) were characterized by polyethylene flow-packaging. After purchase, hamburgers were transported in the laboratory of European Center for the Sustainable Impact of Nanotechnology (ECSIN, Padova, Italy), as part of Mérieux NutriSciences Company (Chicago, USA) and frozen at −20 °C until analyses were performed.

2.2. Reagents and laboratory environment

All solutions used during digestion and microfiltration steps were previously micro-filtered using a silver membrane filter (3.0 μm pore-size, 25 mm diameter; Sterlitech Corporation, Auburn, United States) and kept in glass containers. Ultrapure water (18.3 MΩ/cm resistivity at 25 °C) was produced through Zeneer Power III (Human Corporation, Garak-ro, Republic of Korea). Work surfaces were accurately cleaned before and during the experimental procedures. Before use, all glassware was washed 5 times with distilled water, then rinsed 5 more times with ultrapure water and oven dried.

All procedures adopted for sample pretreatment (i.e. quartering, digestion, and microfiltration) and Fourier-transformed infrared micro-spectroscopy in attenuated total reflectance mode analysis (**μ-FTIR-ATR**) were carried out in a dedicated clean room, according to the guidelines described in the ISO 14644-1:2015 Class 7 (ISO (International Organization for Standardization), 2015).

2.3. Sample pretreatment

2.3.1. Quartering

Prior to the analysis, samples were thawed overnight at room temperature (22 °C). After thawing, the packaging was removed, and a quartering step was performed in order to obtain a representative aliquot from each hamburger. In particular, each sample was divided into quarters, whereupon two opposite quarters were discarded. The remaining 2 quarters were recombined and further divided into quarters. The quartering step was repeated until obtaining the desired sample weight (10 g).

2.3.2. Digestion

A digestion protocol adapted from Dehaut et al. (2016) and EFSA Panel on Contaminants in the Food Chain (CONTAM) (2016), comprising both an enzymatic and a chemical digestion step, was performed in order to remove sample organic matter.

As for the enzymatic digestion, 10 g of sample were weighed in a glass flask, added with 184 mL of ultrapure water and 1 mL of cellulase solution (Merck KGaA, Darmstadt, Germany). The mixture was stirred at 37 °C overnight in Shake'n Bator (EuroClone, Milan, Italy). The subsequent day, 20 mL of 5% trypsin solution (Merck KGaA) were added and again samples were stirred at 37 °C overnight in Shake'n Bator.

As for the chemical digestion, each sample was added with 100 mL of 20% potassium hydroxide (KOH). The mixture was stirred at 60 °C overnight in Shake'n Bator. Finally, samples were added with 100 mL of 10% ethylenediaminetetraacetic acid (EDTA) solution and stirred at 60 °C for 2 h in Shake'n Bator.

2.3.3. Microfiltration and density separation

Sample microfiltration was carried out through a vacuum filtration system connected to a glass filtering funnel and to a silver membrane filter (3.0 μm pore-size; Sterlitech Corporation, Auburn, United States). After this step, the fat-soluble residues were dissolved by repeated rinsing by using isopropanol until no traces of fat and organic matter were left on the filter.

Successively, as proposed by Guo et al. (2022), an oversaturated brine solution of sodium chloride (NaCl) was used during the density separation step. During the entire microfiltration phase, the filtering funnel was covered with aluminum foil to minimize contamination. All filters were stored in cleaned glass Petri dishes to prevent contamination. Finally, filters were dried at 70 °C for 2 h until subsequent μ-FTIR-ATR analyses.

2.4. Characterization of microplastics

2.4.1. Fourier-transformed infrared micro-spectroscopy analyses

Fourier-transformrd infrared micro-spectroscopy analyses were performed using an FTIR Spectrometer Frontier coupled to a microscope Spotlight 400 (PerkinElmer Italia Spa, Milan, Italy). The spectrum range was set between 4000 and 650 cm^{-1}. Four repeated μ-FTIR-ATR scans were performed for all detected MP particles, until the final average spectrum was acquired. The polymeric matrix of the detected particles was identified by comparing the collected μ-FTIR-ATR spectra with reference library spectra using Spectrum 10 software (PerkinElmer Italia Spa, Milan, Italy) (Fig. 1). Following instrument manufacturer instructions, each MP particle was considered correctly identified when the matching between MP spectra from beef hamburgers and MP spectra from reference libraries was >80%. Identified MP particles included ethylene vinyl acetate (**EVA**), nylon, polyacrylate (**PA**), poly butyl methacrylate (**PBMA**), polycarbonate (**PC**), polyester (**PES**), polyethylene (**PE**), polyethylene chlorinated (**CPE**), polyethylene terephthalate-polycarbonate copolymer (**PET:PC**), polyethylene terephthalate (**PET**), polyisoprene (**PI**), polyoxymethylene (**POM**), polypropylene (**PP**), polystyrene (**PS**), polyvinylidene chloride-poly(methyl methacrylate) copolymer (**PVDC:PMMA**), polyurethane (**PU**), resin, and silicone.

2.4.2. Size, shape, and color

High resolution images of MP were electronically stored through Spectrum 10 software. The size of each MP was measured by using ImageJ software (ImageJ, 2022). Fibers were measured along their length whereas fragments characterized by irregular or spherical shapes were measured along their greatest dimension. Visual inspection allowed for the identification of 7 different MP colors, including black, blue, brown, green, grey, yellow, and transparent.

2.5. Blanks and method recovery

Blanks were prepared along with each meat sample and according to the same protocol, thus including digestion, microfiltration, and μ-FTIR-ATR analysis. Blanks were considered valid up to 10 MP fragment contaminants (in other terms, whenever 11 MP fragments were retrieved in a blank, the analysis of the corresponding sample was considered invalid and repeated again). In the analyzed blanks, a maximum of 4 MP fragments was found. Microplastics contaminants detected in the blanks included only PA, EVA, and PC. The number of

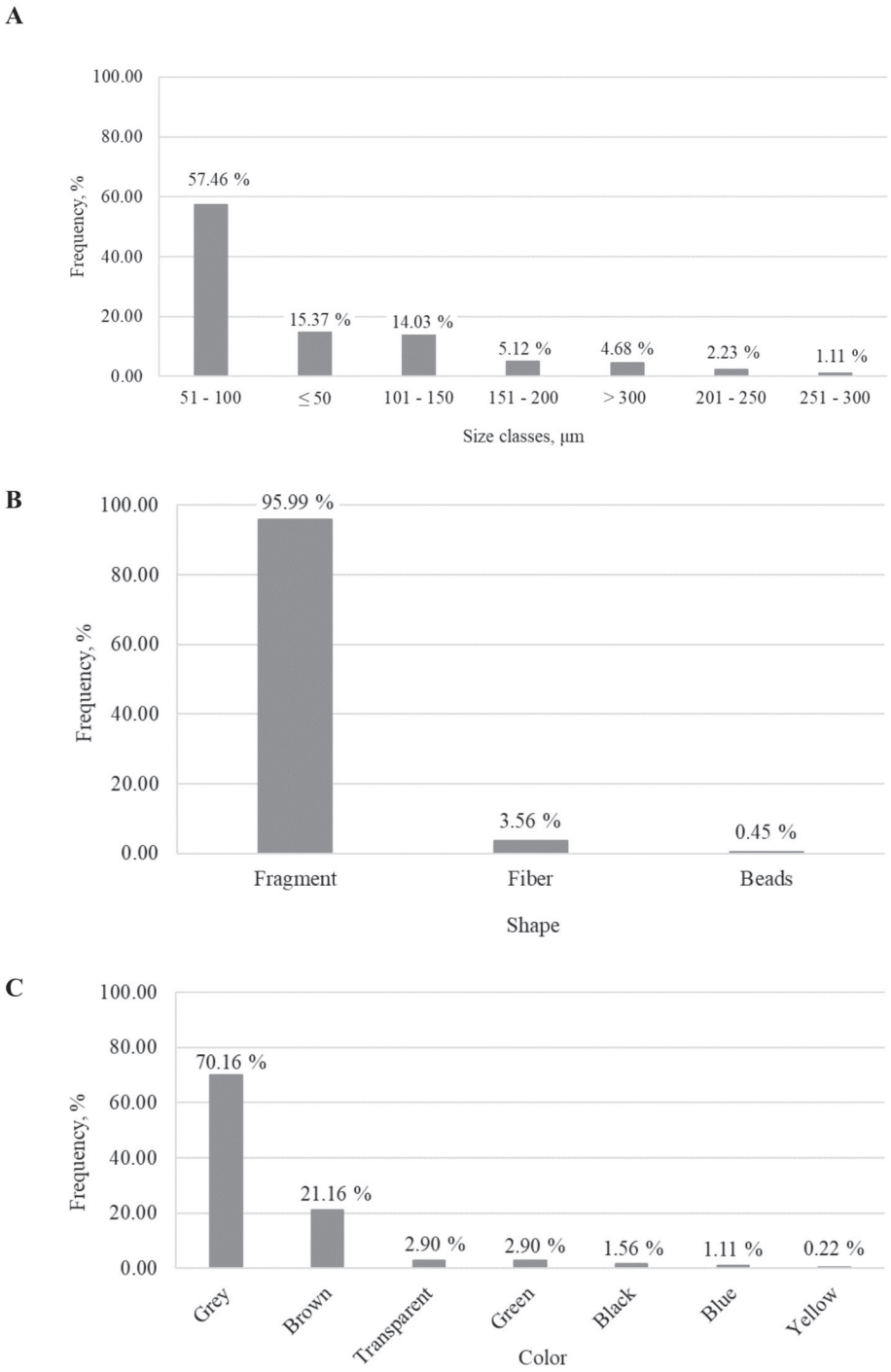

Fig. 2. Size classes (**A**), shape (**B**), and color (**C**) of microplastics identified in beef hamburgers.

MP contaminants detected in blanks were subtracted from the total MP fragments detected in the corresponding sample.

Samples of ultrapure water were spiked with commercial standard of PS (Cospheric LLC, Santa Barbara, CA) to check the potential degradation of PS standard after hot alkaline digestion and to determine the recovery rate of the method. Ten distinct spiking levels, ranging from 22 to 207 MP/kg of water, were tested. Recovery ranged from 66 to 122%, with an average recovery of 84%.

2.6. Statistical analyses

A chi-square test was applied through the PROC FREQ of the SAS 9.4 (SAS Institute Inc., Cary, NC, USA) software. In particular, a first chi-square test was applied on the frequency distribution of contaminated samples, based on MP classes. Similarly, a second chi-square test was applied on the frequency distribution of the overall detected MP particles, again based on MP classes. Significance was set at $P < 0.05$.

3. Results and discussion

3.1. Dimension, morphology, and color of microplastics in beef hamburgers

Dimension, morphology, and color of MP fragments identified in the

Table 1
Summary of sample types, experimental conditions, microplastics (MP) qualitative and quantitative traits in published literature.

References	Sample types		Experimental conditions					MP qualitative traits				MP quantitative traits
	Meat	Processing	Packaging	Country	N	Digestion	Technique[1]	Polymer[2]	Size	Shape	Color	Concentration
This study	Beef	YES	Skin- and flow-packaging	Italy	10	KOH	μ-FTIR-ATR	EVA, Nylon, PA, P(BMA), PC, PES, PE, CPE, PET:PC, PET, PI, POM, PP, PS, PVDC:PMMA, PU, Resin, and Silicone	30.0–3154.0 μm	Beads, fiber, and fragment	Black, blue, brown, green, grey, transparent, and yellow	200.0–38,400.0 MP/kg
Kedzierski et al., 2020	Chicken	NO	Tray and plastic film	France	12	–	μ-FTIR-ATR	PS	300.0–450.0 μm	Generally variable	Black and yellow	1.1–18.7 MP/kg
Habib, Kindi, et al., 2022	Chicken	NO	–	United Arab Emirates and Kuwait	6	KOH	FT-IR spectroscopy	PE	8.2–1454.5 μm	–	Green, white, red, and yellow	0.03–1.2 MP/g
Habib, Poulose, et al., 2022	Goat	YES	–	Middle East and South Asia	5	KOH	FT-IR spectroscopy	PE	15.5–13,089.8 μm	–	White and red	1.2–7.2 MP/g
	Beef	NO			1				14.8–5369.8 μm			6.5 ± 4.4 MP/g
Katsara et al., 2022	Pork	YES	Resealable air-tight PE pouches	Greece	18	–	Raman spectroscopy	PE	–	–	–	–
Van der Veen et al., 2022	Beef	NO	Plastic-packaged	Dutch	2	–	Pyr-GC/MS	PE, PVC	–	–	–	–
	Beef	YES			6			PE, PS, PVC				
	Pork	NO			1			PE, PVC				
	Pork	YES			7			PE, PP				

[1] μ-FTIR-ATR: Fourier-transformed infrared micro-spectroscopy in attenuated total reflectance mode analysis; FT-IR spectroscopy: Fourier-transformed infrared spectroscopy; Pyr-GC/MS: Pyrolysis-gas chromatography/mass spectrometry.

[2] EVA: Ethylene vinyl acetate; PA: Polyacrylate; PBMA: Polybutyl methacrylate; PC: Polycarbonate; PE: Polyethylene; PES: Polyester; CPE: Polyethylene chlorinated; PET:PC: Polyethylene terephthalate-polycarbonate copolymer; PET: Polyethylene terephthalate; PI: polyisoprene; POM: Polyoxymethylene; PP: Polypropylene; PS: Polystyrene; PVC: Polyvinyl chloride; PVDC:PMMA: Polyvinylidene chloride-poly(methyl methacrylate) copolymer; PU: Polyurethane.

Table 2
Morphological and chemical features of the identified microplastics.

Microplastics[1]	*n* of samples	*n* of MP	Size (± SD), μm	Shape			Color							Company
				Beads	Fiber	Fragment	Black	Blue	Brown	Green	Grey	Transparent	Yellow	
1. Ethylene vinyl acetate	3	2	213.00	–	–	1	–	–	1	–	–	–	–	C1
		4	429.00 (± 524.67)	–	–	2	–	–	1	–	–	1	–	C1
		2	30.00	–	–	1	–	–	1	–	–	–	–	C1
2. Nylon	1	4	68.00 (± 33.94)	–	–	2	–	–	2	–	–	–	–	C2
3. Polyacrylate	1	2	96.00	1	–	1	–	1	–	–	–	–	–	C1
4. Polybutyl methacrylate	1	2	160.00	–	–	1	–	–	1	–	–	–	–	C1
5. Polycarbonate	2	222	76.46 (± 34.76)	–	–	111	–	–	13	–	98	–	–	C1
		384	86.00 (± 35.00)	–	–	192	–	–	–	–	192	–	–	C2
6. Polyester	1	2	106.00	–	–	1	–	1	–	–	–	–	–	C2
7. Polyethylene	10	26	72.15 (± 38.24)	–	–	13	–	–	–	–	13	–	–	C1
		4	72.50 (± 12.02)	–	–	2	–	–	2	–	–	–	–	C1
		4	172.00 (± 154.15)	–	–	2	–	–	2	–	–	–	–	C1
		10	342.00 (± 270.78)	–	–	5	–	–	5	–	–	–	–	C1
		60	88.00 (± 63.29)	–	–	30	–	–	30	–	–	–	–	C1
		4	56.00 (± 1.41)	–	–	2	–	–	2	–	–	–	–	C1
		32	109.81 (± 91.67)	–	–	16	–	–	16	–	–	–	–	C1
		10	75.00 (± 27.99)	–	–	5	–	–	–	–	–	5	–	C1
		2	184.00	–	–	1	1	–	–	–	–	–	–	C2
		4	413.00 (± 224.86)	–	–	2	–	–	–	–	2	–	–	C2
8. Polyethylene chlorinated	1	2	133.00	–	1	–	1	–	–	–	–	–	–	C1
9. PET:PC	1	2	83.00	–	–	1	–	–	1	–	–	–	–	C1
10. Polyethylene terephthalate	8	6	1510.00 (± 1431.52)	–	2	–	–	–	–	–	–	2	1	C1
		2	394.00	–	1	–	–	–	–	1	–	–	–	C1
		4	440.00 (± 19.80)	–	2	–	–	–	–	–	–	2	–	C1
		2	1636.00	–	1	–	–	–	–	–	–	1	–	C1
		2	1865.00	–	1	–	1	–	–	–	–	–	–	C1
		2	315.00	–	–	1	–	–	–	–	1	–	–	C1
		2	796.00	–	1	–	–	–	–	–	–	1	–	C2
		4	649.00 (± 502.05)	–	2	–	–	1	–	–	1	–	–	C2
11. Polyisoprene	1	2	42.00	1	–	–	–	–	–	–	1	–	–	C1
11. Polyoxymethylene	3	2	96.00	–	–	1	1	–	–	–	–	–	–	C1
		2	47.00	–	–	1	–	–	1	–	–	–	–	C1
		2	42.00	–	–	1	–	–	1	–	–	–	–	C1
13. Polypropylene	8	26	85.62 (± 54.57)	–	–	13	–	1	–	12	–	–	–	C1
		2	434.00	–	1	–	1	–	–	–	–	–	–	C1
		4	81.00 (± 15.56)	–	–	2	–	–	2	–	–	–	–	C1
		4	61.50 (± 19.09)	–	–	2	–	–	2	–	–	–	–	C1
		8	85.00 (± 55.00)	–	–	4	–	–	4	–	–	–	–	C1
		6	128.67 (± 101.11)	–	1	2	1	–	1	–	1	–	–	C1
		4	72.50 (± 20.51)	–	–	2	–	–	1	–	1	–	–	C1
		6	228.00 (± 185.14)	–	1	2	1	–	–	–	2	–	–	C2
14. Polystyrene	6	2	43.00	–	–	1	–	1	–	–	–	–	–	C1
		2	62.00	–	–	1	–	–	1	–	–	–	–	C1
		2	112.00	–	–	1	–	–	–	–	1	–	–	C1
		2	53.00	–	–	1	–	–	1	–	–	–	–	C1
		2	35.00	–	–	1	–	–	–	–	1	–	–	C1
		2	67.00	–	–	1	–	–	–	–	1	–	–	C2
15. PVDC:PMMA	1	2	1364.00	–	–	1	–	–	–	–	–	1	–	C1

(*continued on next page*)

Table 2 (*continued*)

Microplastics[1]	*n* of samples	*n* of MP	Size (± SD), μm	Shape			Color							Company
				Beads	Fiber	Fragment	Black	Blue	Brown	Green	Grey	Transparent	Yellow	
16. Polyurethane	1	4	357.00 (± 145.66)	–	2	–	–	–	2	–	–	–	–	C2
17. Resin	1	2	119.00	–	–	1	–	–	1	–	–	–	–	C1
18. Silicone	1	2	81.00	–	–	1	–	–	1	–	–	–	–	C1

[1] PET:PC: Polyethylene terephthalate-polycarbonate copolymer; PVDC:PMMA: Polyvinylidene chloride-poly(methyl methacrylate) copolymer.

Table 3
Descriptive statistics[1] of microplastics concentration[2] (MP/kg) in beef hamburgers.

Microplastics	*n* of beef hamburgers	*n* of MP	Mean	SD	CV,%	Minimum	Maximum
Ethylene vinyl acetate	3	8	266.67	115.47	43.30	200.00	400.00
Nylon	1	4	400.00	0.00	0.00	400.00	400.00
Polyacrylate	1	2	200.00	–	–	–	–
Polybutyl methacrylate	1	2	200.00	–	–	–	–
Polycarbonate	2	606	30,300.00	11,455.13	37.81	22,200.00	38,400.00
Polyester	1	2	200.00	–	–	–	–
Polyethylene	10	156	1580.00	1879.60	118.96	200.00	6000.00
Polyethylene chlorinated	1	2	200.00	–	–	–	–
PET:PC	1	2	200.00	–	–	–	–
Polyethylene terephthalate	8	24	300.00	151.19	50.40	200.00	600.00
Polyisoprene	1	2	200.00	–	–	–	–
Polyoxymethylene	3	6	200.00	0.00	0.00	200.00	200.00
Polypropylene	8	60	750.00	769.04	102.54	200.00	2600.00
Polystyrene	6	12	200.00	0.00	0.00	200.00	200.00
PVDC:PMMA	1	2	200.00	–	–	–	–
Polyurethane	1	4	400.00	0.00	0.00	400.00	400.00
Resin	1	2	200.00	–	–	–	–
Silicone	1	2	200.00	–	–	–	–

[1]SD = standard deviation; CV = coefficient of variation. [2]PET:PC: Polyethylene terephthalate-polycarbonate copolymer; PVDC:PMMA: Polyvinylidene chloride-poly (methyl methacrylate) copolymer.

analyzed samples are reported in Fig. 2. The size range of MP observed in the present study was found to be between 30.00 and 3154.00 μm (Table 1, Table 2), with an overall average of 120.00 μm (data not shown). In particular, 15.37% of identified MP were ≤ 50 μm, more than half (57.46%) were in the range of 51–100 μm, 14.03% were in the range of 101–150 μm, 5.12% were in the range of 151–200 μm, 2.23% were in the range 201–250 μm, 1.11% were in the range 251–300 μm, while 4.68% were > 300 μm (Fig. 2A). Larger MP dimensions were reported by Habib, Poulose, et al. (2022), who investigated plastic cutting boards as a source of MP in beef and goat meat samples. The same authors reported that MP in beef meat were in the range between 14.80 and 5369.80 μm, whereas the dimension of MP in goat meat varied from 15.50 to 13,089.80 μm (Table 1). Considering MP from both beef and goat, Habib, Poulose, et al. (2022) reported that 35% were < 500 μm (in the present study 97.77%), 64% of the particles were in the range 500–5000 μm (in the present study 2.23%), whereas 0.7% were > 5000 μm (in the present study 0.00%). Similarly, the average MP size observed in the present study was lower compared to the MP size reported by Kedzierski et al. (2020), who found the majority of plastic particles in chicken meat products comprised in the range between 300 and 450 μm (Table 1). Therefore, it is possible to infer that the technological steps adopted for hamburger manufacturing (pre-cutting, mixing, grinding, and forming) may lead to the fragmentation and miniaturization of MP originally contained in the raw meat.

Most of the MP identified in the present study were characterized by irregular shapes (95.99%). Fibers and beads were observed with considerably lower frequency (3.56% and 0.45%, respectively; Fig. 2B). This agrees with the results reported by Kedzierski et al. (2020), who observed that the morphology of the particles identified was variable (Table 1).

About 97% of the MP identified in the present study were pigmented, with grey, brown, and green being the most prevalent colors (70.16%, 21.16%, and 2.90%, respectively; Fig. 2C). Habib, Kindi, et al. (2022) identified green, white, red, and yellow MP particles to be traced back to the plastic cutting boards used during their trials (Table 1). Instead, Kedzierski et al. (2020) generally detected black and yellow MP particles, associable to the PS trays used in their research (Table 1).

3.2. Quantitative characterization of microplastics

Descriptive statistics of MP concentration in beef hamburgers are reported in Table 3. Microplastics were detected in all analyzed samples. A total of 898 plastic particles belonging to 18 different polymer classes were identified in the analyzed samples (Table 2). Such results suggest a greater variability of polymeric matrices compared to previous studies (Table 1). The most abundant MP in the analyzed samples consisted in PC, PE, and PP, with average concentration of 30,300.00, 1580.00, and 750.00 MP/kg, respectively (Table 3). Among MP fragments characterized in the present study, PA, PBMA, PES, CPE, PET:PC, PI, PVDC: PMMA, resin, and silicone were the least abundant polymers. Indeed, only one particle of these polymers was detected in the analyzed samples (Table 3). Based on MP classes, the chi-square test resulted significant both for the frequency of contaminated samples ($P < 0.001$) and for the frequency of detected MP particles ($P < 0.001$), thus highlighting great contamination variability.

To authors knowledge, scientific data regarding the presence of MP in meat products are limited to only few studies (Table 1). In a recent study conducted in 7 beef cattle farms, Van der Veen et al. (2022) analyzed the presence of MP in different biological substrates, including feed, blood, meat, and milk, confirming that animals can absorb plastic particles they are exposed to in their living environment. The same authors reported that meat processing procedures, including grinding, mixing, adding ingredients, and cooking may represent sources of MP contamination. Habib, Kindi, et al. (2022) and Habib, Poulose, et al. (2022) concluded that plastic cutting boards represented the primary source of PE contamination in commercially sold cut meat. Katsara et al.

(2022) reported that the extent of MP contamination in bacon, salami, and mortadella was positively associated with the storage time, in a way of increased number of PE residues along increased storage time. In particular, the migration of PE from the packaging to the meat started after 9 days of storage and persisted throughout the 28 days of investigation. Finally, Kedzierski et al. (2020) demonstrated that plastic particles from PS trays used in commercial chicken meat adhered to meat surface even after rinsing and would likely be eaten by consumers.

Thus, even if it is worthy to account that the present study does not allow to determine the source of plastic residues contamination, available literature suggests that detected MP are likely and mostly originated from animal body, industrial processing, and packaging. To date, plastic polymers still represent essential components in meat packaging, contributing to maintain product quality, safety, and shelf life. Therefore, also in the field of processed meat products, designing alternative packaging materials becomes crucial to guarantee consumer health further than to enhance environmental sustainability.

4. Conclusions

This is the first study addressing qualitative and quantitative characterization of MP in processed commercial beef hamburgers. Results exhibited a significant variability in polymeric particle classes ($n = 18$). Among identified MP, the most abundant were PC (30,300.00 MP/kg), PE (1580.00 MP/kg), and PP (750.00 MP/kg). It is worthy to report that the analytical method adopted in the present study has several limitations. The amount of the starting sample should be sufficient representative (which means big enough) and easy to process (which means small enough) at the same time, resulting in a difficult-to-achieve compromise. The long time required for sample preparation (about 4 days) and for MP characterization (about 1 day). The need of specialized personnel, costly analytical instruments, dedicated laboratory environments and equipments. Findings of the present study will help to precisely circumscribe the origin of MP contamination, thus allowing to design specific guidelines to limit MP diffusion in processed meat products.

CRediT authorship contribution statement

E. Visentin: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **G. Niero:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Conceptualization. **F. Benetti:** Writing – review & editing, Visualization, Software, Methodology, Data curation. **A. Perini:** Writing – review & editing, Visualization, Software, Methodology. **M. Zanella:** Writing – review & editing, Visualization, Software, Methodology. **M. Pozza:** Writing – review & editing, Software, Methodology. **M. De Marchi:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Data availability

Data will be made available on request.

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GENERAL CONCLUSIONS

This doctoral thesis presents one of the first evaluation of MP contamination in animal-based foods, including dairy products, skim-milk powders, and beef hamburgers. The investigation aimed to identify, characterize, and quantify MP through the application of a rigorous digestion protocol combined with analytical techniques based on Fourier-transform infrared micro-spectroscopy in attenuated total reflectance mode, thus providing novel insights into the occurrence of MP in foods of high dietary relevance.

The results indicate that MP are widespread in animal-based foods, exhibiting substantial variability in abundance, polymer types, size, shape, and color. In dairy products, contamination levels differed among milk, fresh cheese, and ripened cheese, highlighting the importance of considering specific product characteristics when assessing exposure. Skim-milk powders, widely used in industrial cheesemaking across European countries, were also found to contain MP, suggesting that contamination in industrial ingredients may be transferred to final products and ultimately ingested by consumers. Processed meat products, such as commercial beef hamburgers, similarly exhibited considerable MP contamination, illustrating the heterogeneity and pervasiveness of MP in animal-based foods.

The findings emphasize the influence of production processes, packaging, and ingredient sources on contamination levels, thereby providing a basis for identifying potential pathways through which MP may enter the food chain. While this thesis confirms the pervasive occurrence of MP contamination, it also

underscores the necessity of further research to clarify the sources, dynamics, and potential health risks associated with MP in animal-based foods. Advancing such knowledge will be critical for the development of effective mitigation strategies aimed at reducing human exposure and safeguarding food safety.

The findings from the scientific contributions included in this Ph.D. thesis have provided additional benchmarks regarding the presence of MP in processed animal-based products. These results demonstrate that MP are present in such food items, as in many others, and should encourage both the scientific community and policymakers to establish guidelines for large-scale monitoring of MP occurrence in food products, as well as preliminary indications of potential quantitative and qualitative limits for their presence.

OTHER SCIENTIFIC PUBLICATIONS

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2022

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