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Livestock activity biomarkers: Estimating domestication and diet of livestock in ancient samples

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ABSTRACT

Faecal matter is commonly recovered from archaeological sites related to human/animal activity. The identification of its source is essential to understand the domestication process and the relationship between humans and domestic animals in ancient times. Additionally, faecal matter is useful for determining the diet of animals. Therefore, the use of an appropriate biomarker is essential.

The ratios of 5β-Stanols and bile acid biomarkers are most commonly used to identify the biogenic origin of faecal matter. However, other biomarkers such as archaeol can be a good proxy for ruminants.

Conversely, plant-based diet of the animals can be discerned by analysis of faecal matter. *n*-Alkanes are the most common proxies of the animal diet, followed by long-chain fatty acids and long-chain alcohols, and the interest in the analysis of carbon isotopes has recently increased owing to the possibility of distinguishing animal diets.

In this review, we describe the identification of faecal and diet biomarkers in animals. Ratios and proxies used in archaeological fields are also described and discussed to determine the best approach for accurate identification.

1. Introduction

Animal domestication is a sustained, multigenerational, and mutualistic relationship between humans and plants or animals. In this relationship, humans assume a significant level of control over reproduction and plant or animal care to ensure a more predictable supply of a particular resource. Therefore, the plant or animal of interest increases its reproductive success over others which are not controlled. This increment enhances the health of humans and plant or animal (Conolly et al., 2011; Larson et al., 2014; Zeder, 2012a). In terms of archaeology, domestication can be defined as the process whereby the reproduction of a target animal population is appropriated and controlled by human society for material, social or symbolic profit (Vigne, 2011).

The behavioural characteristics of animals make some better candidates than others for domestication. The key factors included in the domestication process are: social structure, sexual behaviour, parent-young interactions, feeding behaviour and habitat choice, and responses to humans and new environments (Hirst et al., 1975; Zeder, 2012a).

Wolves (*Canis lupus*) were the first species domesticated approximately 17,000–15,000 BP or even earlier (Galibert et al., 2011; Savolainen et al., 2002; Vigne, 2011). However, all large mammal domestication took place during the Holocene. This period provided favourable climatic conditions (warm, wet, stable, CO_2-rich environment) and hunter-gatherers started to exploit locally abundant plant resources more efficiently (Blumler and Byrne, 1991; Gupta, 2004; Richerson et al., 2001). These circumstances led to an increase in the population that subsequently resulted in prey depletion and the suppression of resources, which forced humans to focus on extraction strategies never considered before (Alvard and Kuznar, 2001; Zeder, 2006). The earliest herbivore and porcine domestications were detected in the Near East and dated to approximately 11,000 BP. and include the oriental mouflon (*Ovis orientalis*), bezoar goat (*Capra aegagrus aegagrus*), extinct auroch (*Bos primigenius*) and wild boar (*Sus scrofa*) (Arbuckle and Makarewicz, 2009; Chessa et al., 2009; Hongo et al., 2009; Siddiq and Şanlı, 2020; Vigne, 2011), which were predecessors of modern-day sheep (*O. aries*), domestic goats (*Capra hircus*), domestic cattle (*Bos taurus*) and pigs (*Sus domesticus*), respectively. A few millennia later, in

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the 5th millennium BP, horses (*Equus caballus*) and wild asses (ancestors of donkeys) (*Equus africanus*) were also subdued (Hu et al., 2020; Vigne, 2011). The same or similar species were domesticated in other parts of the world at similar periods with apparently no contact between areas (Hristov et al., 2017; Jansen et al., 2002; Larson et al., 2005; Luikart et al., 2001; Pedrosa et al., 2005).

Each animal responds differently to domestication, and therefore three ways have been proposed to explain how animals became integrated into human society: the commensal pathway, the prey pathway, and the directed pathway. The commensal pathway was followed by scavenger animals. These animals, at some point, developed close bonds with their human hosts who began to acquire some tangible benefits from this association. Pigs are possible candidates for this domestication method. The prey pathway was presumably followed for most major livestock species (sheep, goats, and cattle). These animals, in their wild forms, were hunted by humans until their supply was depleted, at which point humans experimented with feeding them to increase their availability. The directed pathway is a domestication process that involves animals with few key behavioural characteristics to comply with domestication and keep their natural instinct for wildlife; therefore, the domestication of these animals required more effort from humans. Horses and donkeys were likely to be domesticated through this pathway (Larson and Fuller, 2014; Zeder, 2012b, 2012a).

The domestication process reflects one of the major changes in the history of humanity. Therefore, it is fundamental to understand the transition from hunting-gathering to agriculture and analyse its impact on biodiversity, evolution of species, and the development of human societies (Yuan and Dong, 2020). This process has been documented through two complementary approaches: archaeological records, and genetic data (Frantz et al., 2020). Archaeological records include the study of skeletal remains (osteological changes, age and sex at death, post mortem marks), isotopic signatures and archaeological context (burial sites, food refuse deposits, corrals, harnesses) (Angelucci et al., 2009; Frantz et al., 2020; Harbers et al., 2020; Makarewicz and Tuross, 2012; Vigne, 2011). The genetic data are based on the study of geographical patterns of diversity, and genetic and phenotypic changes, including developmental, anatomical, physiological and behavioural changes (Frantz et al., 2020; MacHugh et al., 2017; Wright, 2015). The advantages are clear; however, both approaches have disadvantages such as the low rhythm of archaeological analysis, either the loss of most of the biological information or the lack of genetic material, and the high costs of genetic techniques (Schwarze et al., 2018; Vigne, 2011).

Among the archaeological remains, organic residue deposits are an important source of human-land relationship information (Hassan, 1978) and organic geochemical approaches were very useful to obtain this information (Knights et al., 1983; Lin et al., 1978). Organic residue deposits are formed by a human activity depositional process, followed by a natural post-depositional course (Fernández-Eraso and Polo Díaz, 2008). The analysis of these organic residues reliably associated with human settlements, provides significant information to better understand the development of domestication. Organic residue analysis provides insights into the inputs of these remains through the study of specific biomarkers (Evershed, 2008).

Biomarkers are organic compounds that can be related to their biological precursors because of diagenetic processes and cannot be synthesised by any other mechanism. In archaeology, the detailed characterisation of biomarker composition allows for the assessment of the major contributing species in the past (Simoneit, 2002). The association between the organic residue and the original constituent is a valuable aspect of the biomarkers although the alterations suffered through the millennia must be considered. Therefore, knowledge of the chemical and biochemical mechanisms likely to be involved is required. These biomarkers can be retrieved from various sources of archaeological field, such as pottery, soils and sediments, human, animal and plant remains, dyes and pigments, resins and bitumen among others (Bull et al., 1999a; Bull et al., 1999b; Clark et al., 2013; Drieu et al., 2018; Evershed, 2008; Faraco et al., 2016; Fiore et al., 2008; Lucejko et al., 2018; Prost et al., 2017).

The analysis of faecal matter biomarkers in organic residue deposits helps to better understand the development of domestication over time and the past management of animals (Baeten et al., 2012; Harrault et al., 2019a; Prost et al., 2017). Faecal biomarkers are not only able to identify faecal remains but also their biogenic origin which is useful for determining the species of the animals housed and, therefore, herd habits and even community distributions (Mackay et al., 2020; Shillito et al., 2020). Consequently, the analysis of plant biomarkers, once the faecal remains are identified, is useful to determine the diet of the animals and the flora consumed, which allows for the reconstruction of past vegetation (D'Anjou et al., 2012; Wang et al., 2013).

This review compiles the biomarkers used to identify faecal remains as the primary source and their biogenic origin focusing on those related to livestock activity. As a second objective, the aim was to establish the association between the animal source and its plant-derived diet by analysing the plant biomarkers accumulated in the faecal remains. To accomplish both objectives, the previously proposed proxies/ratios are reviewed and discussed.

2. Ancient faecal matter sources

Domestication is necessarily linked to animal husbandry and the requirement of animal housing to breed and protect them from predators. Depending on the weather, humans used different management approaches, setting the animals in open-air areas, rock shelters, or caves (Angelucci et al., 2009; Fernández-Eraso et al., 2015; García-Suárez et al., 2020). Once deposited, animal excrements may be used in different ways, such as the use of dung as a building material or fuel, among other purposes, and the faeces was regularly burned in order to reduce its volume and eliminate parasites (Shahack-Gross, 2011; Vergès et al., 2016c). Their recovery and identification as animal remains are crucial to perform the correct analyses to detect the presence of domestic taxa and reconstruct past vegetation.

Livestock dung is a major occupation signature. Its presence in the settlement context could be indicative of animal husbandry practices and the spatial organisation of these sites (Linseele et al., 2013). Once deposited, it was important to human communities, especially those inhabiting treeless steppes or desert landscapes, providing both building materials and fuel sources (Égüez and Makarewicz, 2018). The accurate identification of dung is not easy because of the different forms in which it was used as a result of the aforementioned management (Portillo et al., 2020). Under certain circumstances, including permanent dry, waterlogged, and frozen conditions, dung can be preserved in its original state owing to the absence of bacterial and fungal activity. It can be found as hardened pellets or in a mineralised state owing to its high content of phosphates and nitrates. Reliable identification of species from the analysis of archaeological dung can be very helpful, especially in the absence of bone remains (Linseele et al., 2013; Shahack-Gross, 2011).

Animal manure (faeces, urine, and straw), such as dung, is also related to human and livestock activity and has been widely studied in the field of archaeology to determine the biogenic origin of the manure (Baeten et al., 2012). Additionally, manure can also be related to agricultural soils because it was widely used as a fertiliser for its high organic matter content and nutritional intake (Birk et al., 2011; Bull et al., 2001). However, the accumulation of plant remains in manure, commonly straw, must be considered when the biogenic origin of the faecal remains is the study objective because the intake of plants changes the primary composition of faeces.

Coprolites are also a source of faecal remains. Direct analysis of this faecal source is especially useful to evaluate the origin of the faecal remains and to obtain evidence of the digestive system and diet (Baeten et al., 2012; García-Suárez et al., 2020; Portillo et al., 2020; Shillito et al., 2011, 2020; Sistiaga et al., 2014a; Sistiaga et al., 2014b)

Fumier deposits are formed by the periodic burning of animal

deposits from the Neolithic to the Bronze age (Angelucci et al., 2009; Fernández-Eraso and Polo Díaz, 2008; Vergès et al., 2016b, Vergès et al., 2016a). They are associated with stabling and stock-keeping of herbivore livestock, with the ultimate purpose of excrement, parasite, and residue removal (Angelucci et al., 2009; Oms et al., 2008). One of the facies of the *fumiers* is not thermically altered; therefore, this is an unquestionable source of livestock dung which is related to farming. Consequently, the study of *fumiers* is especially useful to better understand livestock activity at the beginning of domestication.

3. Biomarkers

As previously defined, biomarkers are organic compounds that can be directly or indirectly related to their precursors and can be used to trace that source (Simoneit, 2002). Additionally, archaeological biomarkers can be defined as organic molecules that are resistant to diagenetic processes, owing to their relative stability or protected in some way by the physical state of the context, and indicative of their original biogenic source (Bull et al., 2002; Evershed, 1993; Linseele et al., 2013).

The usefulness of biomarkers is attached to the possibility of determining their origin, and this requires knowledge of the primary source, that is, reference materials, to make comparisons. The problem in archaeology is the lack of chemical data on the corresponding ancient reference material. However, the use of biomarkers is based on chemotaxonomic and phylogenetic principles, which means that they have some constant and variant characteristics that are the same within the same genus. Moreover, within the same species, there is a close relationship between the composition of the biomarkers (Evershed, 1993). Thus, the development of biomarkers in archaeology has been linked not only to the study of plant and animal remains retrieved from archaeological excavations but also to a recent reference collection of materials with significance to the past (Evershed et al., 1997; Harrault et al., 2019a; Lejay et al., 2016; Prost et al., 2017; Zocatelli et al., 2017).

Nevertheless, the complexity of the samples must be considered prior to drawing conclusions. Organic materials retrieved from archaeological sites are formed by mixtures of compounds, consequently, the progress of the field of biomarker analysis is key to creating the necessary databases (Evershed, 2008, 1993; Simoneit, 2002). However, complexity is increased by abiogenic and biogenic decay (Evershed, 2008, 1993). To assess this, experiments with modern equivalents were conducted. The process of alteration from precursor to products can be accomplished slowly over geological time intervals, or rapidly in hydrothermal systems (Bull et al., 1999a; Bull et al., 1999b; Simoneit, 2002, 1994, 1985).

3.1. Livestock activity biomarkers

Animal signatures on archaeological sites can be detected using the faecal material that they leave behind. Therefore, it is necessary to study the different digestive systems of our target animals to be able to select suitable biomarkers.

Digestion and fermentation occur in separate parts of vertebrate herbivore guts such as ruminants and equids. The stomach is a complex of four different compartments: the rumen, reticulum, omasum, and abomasum (Zhou et al., 2015). They cannot digest certain plant material enzymatically but rely on the gut microbiome in the rumen for this purpose where fermentative digestion takes place (Alexander, 1993; Clauss et al., 2010; Schwarm et al., 2009). The rumen fermentation results in the production of short-chain fatty acids, ammonia, and methane (Zhou et al., 2015). Herbivores can be classified as foregut or hindgut fermenters depending on their microbial digestion. The former has the primary fermentation chamber proximal to the small intestine and the latter distal to the small intestine. Ruminants are often referred to as foregut fermenters and equids to hindgut fermenters (Alexander, 1993; Clauss et al., 2010). Foregut fermentation corresponds to a low-intake, high-efficiency strategy and hindgut fermentation to a high-intake, low-efficiency strategy. Fibrous material requires more time for fermentative digestion and is less energetically efficient than to easily digestible substrates and auto-enzymatic digestion respectively (Clauss et al., 2010; Janis, 1976).

The porcine gastrointestinal tract is markedly different from the gastrointestinal tract of vertebrate herbivores because they are monogastric animals. Their stomach is a single compartment but is divided into four sections: the cardia, fundus, corpus, and pylorus, analogous to stomach of humans (Zhou et al., 2015).

Depending on the different gastrointestinal mechanisms leading to the formation of our study samples, their biomolecular composition varies. This point must be considered when selecting a biomarker, as well as determining the degree of decay of these compounds. Biomarker selection is highly dependent on the physicochemical conditions of the environment, including pH, temperature, light exposure, and redox conditions. Regardless, lipids are expected to be relatively well preserved compared to carbohydrates, proteins and nucleotides (Eglinton and Logan, 1991; Evershed, 2008, 1993). Owing to the nature of bonds and hydrophobic nature of lipids, they are not prone to leaching or microbial degradation in the environment (Evershed, 2008). Consequently, lipids are excellent candidates for use as biomarkers in archaeological investigations.

Mammalian faecal matter is composed of 50% bacteria, and the rest are fatty acids, steroids, and undigested food (Obuseng and Nareetsile, 2013). These lipidic structures, especially faecal sterols such as 5β-stanols and bile acids, have been extensively studied as the best animal and human waste assessment indicators (Bull et al., 2003, 2002; Harrault et al., 2019b; Lin et al., 1978; Obuseng and Nareetsile, 2013; Zocatelli et al., 2017).

3.1.1. 5β-Stanols

Stigmasterol and sitosterol are the common Δ^5-sterols in plant biomass, whereas cholesterol is the dominant Δ^5-sterol in most animal tissues (Prost et al., 2017). 5β-stanols are formed by a microbially mediated reduction of cholesterol, campesterol, sitosterol and stigmasterol in the intestinal tracts of higher mammals. Enteric bacteria saturate the Δ^5 double bond of these lipids, yielding 5β(H) rather than 5α(H) stereoisomers because of anaerobic conditions (Bull et al., 2002; Huang et al., 1981). Soil and gut sterols can be distinguished on this basis, with 5α-products resulting from microbial reduction external to the gut rather than the 5β-products that are produced in the gastrointestinal tract (Fig. 1 (Shillito et al., 2011).

Because of respective diets of animals, the proportions of sterol precursors entering the digestive tract are different (Leeming et al., 1996). Herbivores excrete faeces containing high proportions of 5β-campestanol and 5β-stigmastanol, whereas coprostanol and epicoprostanol are more predominant in omnivorous faeces, although dog faeces do not contain 5β-stanols (Bull et al., 2002, 1999; Harrault et al., 2019b; Leeming et al., 1996; Prost et al., 2017; Tyagi et al., 2009).

Because of the hydrophobic nature of 5β-stanols, they exhibit a preferential association with particulate matter once deposited, and their degradation is extremely limited in this state (Harrault et al., 2019b; Leeming et al., 1996). Several studies have corroborated the

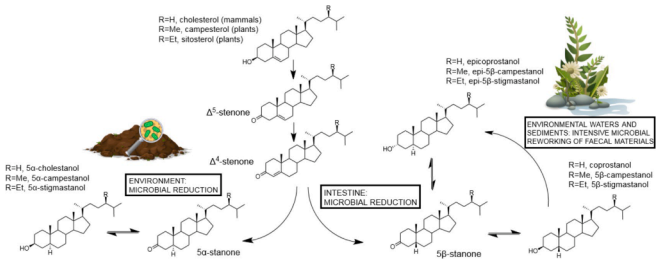

Fig. 1. Formation of 5α- and 5β-stanols from their precursors in the natural environment and in the mammalian gut.

longevity of these compounds, either from humans or animals (Bull et al., 1999a; Bull et al., 1999b; Lin et al., 1978; Prost et al., 2017; Shillito et al., 2011).

However, 5β-stanols are widespread within the soil environment and are also found in soils where no faecal deposition was assessed (Birk et al., 2011; Evershed et al., 1997). The latter can be explained by the formation and deposition of these stanols by plants and soil fauna (i.e., earthworms). Therefore, 5β-stanols alone are often insufficient to prove ancient faecal deposition on ancient soils and have to be combined with other biomarkers (Bethell et al., 1994).

3.1.2. Bile acids

Bile acids are a group of C_{24}, C_{27} and C_{28} steroidal acids (Bull et al., 2002; Reshetnyak, 2013). Physiologically, bile acids are detergents that generate bile flow and facilitate intestinal absorption and transport of lipids, nutrients and vitamins (Chiang, 2009; Reshetnyak, 2013). They are classified into two groups: primary and secondary. The former is produced in the liver as a part of the cholesterol metabolic pathway and is excreted in the intestine through the bile. At this point, these primary bile acids undergo microbial biotransformation, leading to secondary bile acids (Fig. 2 (Elhmmali et al., 2000)). The most important reaction is the dehydroxylation of C_7 (Linseele et al., 2013; MacDonald et al., 1983). Only a small fraction of the secondary bile acids is excreted in the faeces because most bile acids are reabsorbed in the intestine and return to the liver (Elhmmali et al., 2000).

The composition of bile acids in faeces differs among species owing to metabolism, and to a lesser extent, diet (Hagey et al., 2010; Obuseng and Nareetsile, 2013; Prost et al., 2017). In mammals, the dominant primary bile acids are chenodeoxycholic and cholic acids (Obuseng and Nareetsile, 2013), while ruminants mainly produce deoxycholic acid as the predominant secondary bile acid, and omnivores also produce significant amount of lithocholic acid (Bull et al., 2002; Obuseng and Nareetsile, 2013). However, other mammals such as pigs produce substantial amounts of hyocholic and hyodeoxycholic acids (Bull et al., 2002; Elhmmali et al., 1997).

The survival of bile acids has been demonstrated in several archaeological samples (Bull et al., 2003; Knights et al., 1983; Lin et al., 1978; Shillito et al., 2011).

Owing to their stability and correlation with different animal species, 5β-stanols and bile acids have become the best biomarkers to assess animal faecal input in the field archaeology.

3.1.3. Archaeol

Archaeal dialkyl glycerol ether, also known as archaeol (Fig. 3a), is a diether membrane lipid found in the cell membrane of most known

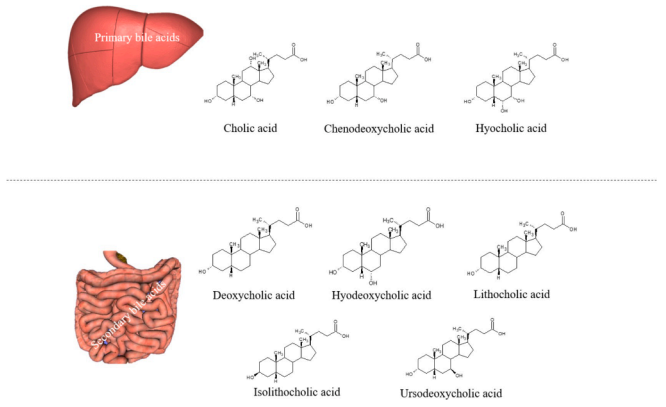

Fig. 2. Primary bile acids are produced in the liver (molecular structures of cholic acid, chenodeoxycholic acid, and hyocholic acid) and secondary bile acids are produced in the intestine (molecular structures of deoxycholic acid, hyodeoxycholic acid, lithocholic acid, isolithocholic acid, and ursodeoxycholic acid).

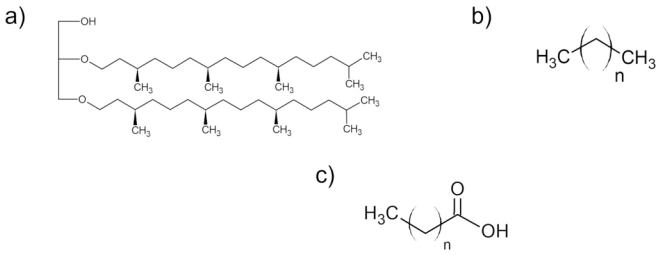

Fig. 3. a) Structure of archaeol, a diether membrane lipid, found in the cell membrane b) Molecular structure of *n*-alkanes, a plant wax component c) Molecular structure of long-chain fatty acids (LCFAs) molecular, a plant wax component.

archaea (Gill et al., 2010). Methanogenic archaea are certain types of microbes found in the rumen that are responsible for methane formation. Livestock emits methane as a part of its natural digestive processes through the use of hydrogen in the stepwise reduction of carbon dioxide (Cheng et al., 2009; Mathison et al., 1998).

Gill et al., (2010) has proposed archaeol as a promising biomarker capable of differentiating foregut fermenters from other animals. Furthermore, its endurance has been confirmed by recovering it from a 2475 ± 30 ^{14}C year ovi-caprid dung. However, some precautions must be taken because of its presence in specific environments, including cold seeps and diverse hypersaline settings (Pancost et al., 2011; Pease et al., 1992; Pancost et al., 2001).

3.2. Animal diet and flora biomarkers

Ancient flora and ancient ways of life were key to domestic animal diet composition in the past. Regarding ancient ways of domestic animal management, different ways of herding have been proposed by archaeologists during early domestication: village-based, seminomadic, transhumant and full-fledged nomadic pastoralism (Abdi, 2003; Makarewicz, 2013). In village-based herding, flocks grazed near the settlement and returned to their pens every evening during warm months. By contrast, during cold months, the herds were fed with collected and stored fodder in their own pens. However, sometimes, the grazing grounds were not enough or were not accessible during the year; therefore, the shepherd had to periodically move the herd access diverse fodder resources (Abdi, 2003). Seminomadic pastoralism involved a combination of herding in the permanent settlement and large movements of the herd through different pastures during most of the year (Abdi, 2003; Kloos et al., 1981). In transhumant pastoralism, during the warmest part of the year the herd grazed on the mountain pastures and returned to lower zones during the coldest seasons (Abdi, 2003; Prag, 1985). Finally, in nomadic pastoralism, animals and shepherds covered long distances between lowlands and highlands looking for the best and most accessible pastures (Abdi, 2003; Miller et al., 2018).

These husbandry strategies changed over time and can be distinguished by diet. Recent studies conducted in the Iberian Peninsula based on stable isotopic analysis of molars from Neolithic cows, sheep, and goats showed dietary differences among species, herds, and animals in the same herds, even between ages and sexes (Sierra et al., 2021, Sierra et al., 2020) which is helpful in understanding the husbandry strategy. Similarly, Villalba-Mouco et al. (2018), used the same analysis for bone collagen to identify the husbandry strategies practised during the Iberian Early Neolithic age.

Therefore, animal faeces containing distinctive compounds related to dietary intake could be useful for the identification of the animal diet and consequently, rebuilding past husbandry strategies (Ali et al., 2005a; Dove and Mayes, 1991). The following are the most well- studied faecal dietary biomarkers.

3.2.1. *n*-alkanes

n-Alkanes are abundant components of plant wax. Owing to their saturated structures and the lack of functional groups, they have high preservation potential and survive in samples for thousands of years (Bush and McInerney, 2013; Eglinton and Logan, 1991; Otto et al., 2005). However, *n*-alkanes can originate from soils, sediments, and paleosols (Handley et al., 2008; Quenea et al., 2004; Sachse et al., 2004; Schefuß et al., 2003; Smith et al., 2007). Odd-carbon-numbered long chain *n*-alkanes, average C_{21}–C_{35}, are typical constituents of higher plant waxes such as of grasses, shrubs or trees (Fig. 3b), and they are a part of the epicuticular leaf wax of terrestrial plants (Ali et al., 2005a; Bush and McInerney, 2013; Laredo et al., 1991; Quenea et al., 2004).

In general, the main origin of long-chain *n*-alkanes are shrubs and angiosperm trees instead of gymnosperm trees. Although plant cultivar, part and age can affect the *n*-alkane concentration, the species of plant is the main contributor (up to 96%) (Dove and Mayes, 1996; Dyson and Herbin, 1968; Eglinton et al., 1962; Laredo et al., 1991). Alternatively, Stránský et al., (1967) characterised herbage by a relatively high occurrence of lower *n*-alkanes (up to C_{23}).

The development of databases including the chemical composition of plant waxes, both antique and modern day, are required to develop the use of these biomarkers as livestock activity biomarkers (Evershed, 1993). Alkane concentrations vary between fresh and dead material within the same species; therefore, adequate sample sizes must be included in the databases (Dove, 1992). The *n*-alkane profile changes among plant species, even between C_3 and C_4 grasses. For instance, the latter accumulates higher concentrations of C_{31}, C_{33}, and C_{35} than the former (Diefendorf and Freimuth, 2017).

The complication with alkanes occurs because of their partial absorption from the animal digestive tract (digestibility). The intake of plants with high digestibility tends to be underestimated (Mayes and Dove, 2000). Recovery from faeces is dependent on the *n*-alkane chain length (Brosh et al., 2003; Oliván et al., 2007). Microbial alkane synthesis in the digestive tract, and the secretion of alkanes into the gut, can affect alkane recovery in faeces. Nevertheless, the rumen microflora of ruminants cannot synthesise alkanes from acetate, the main alkane precursor in plants and bacteria (Dove and Mayes, 1991; Kolattukudy, 1966). Therefore, some corrections must be made before the final interpretation (Dove and Mayes, 2005; Ferreira et al., 2010; Oliván et al., 2007). These corrections vary across studies; Ferreira et al. (2011) used the mean recovery data of the dietary treatment to which the animal belonged and the mean recovery rate across all experimental diets to estimate ruminant diet. Oliván et al. (2007) used six different sets of faecal recovery for goats and four for sheep to correlate the intake of *n*-alkanes and those in the faeces. The objective of these faecal recoveries is to determine the diet of the animal based on the faecal *n*-alkane concentration. To estimate the number of plant species in the diet, they preferably have to be smaller than the number of *n*-alkane markers (Brosh et al., 2003; Oliván et al., 2007). When the number of plants available is higher, or unknown, combining the use of alkanes with other plant wax biomarkers is a useful tool (Ali et al., 2004; Ferreira et al., 2009a; Oliván et al., 2007). Understanding between-species differences in cuticular wax alkane levels is critical for applying *n*-alkanes to palaeoecological interpretations (Bush and McInerney, 2013; Dove, 1992). This method has been used since the 90′s to estimate the species composition of mixtures of herbage in ruminants, equids and pigs (Ferraz de Oliveira et al., 2006; Mayes and Dove, 2000; O'Keefe and McMeniman, 1998). Experiments are mostly conducted on contemporary samples (Ali et al., 2005a; Bush and McInerney, 2013; Dove, 1992), but the possibility of using these methods in ancient specimens is also estimated, although some precautions must be considered, as explained in Section 4.2, and because plant uptake is not accounted.

3.2.2. Long-chain fatty acids

Similar to *n*-alkanes, plant waxes contain long-chain fatty acids (LCFAs) with an average length number of C_{20}–C_{34} (Fig. 3c (Ali et al., 2005a; Dove and Mayes, 1996; Drieu et al., 2018; Mayes et al., 1986).

However, leaf waxes of terrestrial higher plants predominantly contain mixtures of straight-chain, saturated compounds and even-numbered fatty acids (Dove and Mayes, 2005; Schefuß et al., 2003). Within the LCFAs, the shorter ones (C_{22}–C_{26}) represented the highest fraction in the herbage species, while heather had similar proportions of shorter and longer LCFAs (Ferreira et al., 2011).

LCFAs are apparently not absorbed in the digestive tract and are not altered in the digesta; consequently, they have been used as biomarkers because they have high faecal recoveries (Dove and Mayes, 2005; Grace and Body, 1981). However, faecal recovery of LCFAs varies among animal species, especially when comparing ruminants and non-ruminants (Ferreira et al., 2011, 2010). Additionally, within the ruminant species, LCFAs show lower disappearance in cattle than in goats (Ferreira et al., 2011). However, as with *n*-alkanes, LCFA faecal recovery is incomplete and may vary based on chain length between species; therefore, the same precaution must to be taken into account for accuracy (Ali et al., 2004; Dove and Mayes, 2005; Ferreira et al., 2010).

Among plant species, different patterns of these compounds have been demonstrated, confirming them as possible dietary biomarkers (Ali et al., 2005a; Ferreira et al., 2010). In fact, for herbaceous species, LCFA concentrations were found to be greater than the concentration of *n*-alkanes, the latter being unsuitable suitable for this determination because of its reduced concentration (Ali et al., 2005a; Ferreira et al., 2009a). In conclusion, LCFAs provide additional discriminatory information to that provided by *n*-alkanes, and their combination of the two biomarkers is very effective (Ferreira et al., 2011).

An important issue occurs in complex diets, with a large number of dietary components with similar *n*-alkane and/or LCFA profiles. To solve this problem, grouping species with similar profiles has been suggested by several authors (Dove and Mayes, 2005; Ferreira et al., 2007a; Ferreira et al., 2011).

3.2.3. Long chain fatty alcohols

Plant species have a high amount of long-chain fatty alcohols (LCOHs), sometimes even higher than *n*-alkanes (Bugalho et al., 2004; Mayes and Dove, 2000). This allows their use as biomarkers in association with *n*-alkanes and LCFAs, especially in mixtures (Ali et al., 2005a). Several studies have been performed in which plant species were successfully discriminated (Ali et al., 2005a; Bugalho et al., 2004; Kelman et al., 2003; Rao, 2001). One aspect derived from these experiments is that the LCFA with even-chain lengths (1-C_{20}-ol to 1-C_{34}-ol) predominated over the odd-chain ones. Similar to the previous biomarkers, faecal recovery varies with the chain length of LCOHs and corrections have to be done (Ali et al., 2004; Ali et al., 2005a; Kelman et al., 2003).

3.2.4. Carbon isotopes

Approximately, 99% of all carbon on Earth is the ^{12}C isotope while 1% is ^{13}C. The $^{13}C/^{12}C$ ratio ($\delta^{13}C$) varies depending on the material analysed (Minson et al., 1975). Plants can be classified into three groups: C_3, C_4 and crassulean acid metabolism (CAM), depending on the biochemical pathway used in the fixation of atmospheric CO_2 during photosynthesis. C_3 plants use the Calvin cycle with a first reaction intermediate that contains three carbon atoms, and C_4 plants use the Hatch-Stack cycle in which the first reaction intermediate contains four carbon atoms. By contrast, CAM plants can use both carbon fixation pathways (Cerling et al., 1993; Regert, 2011; Schefuß et al., 2003). The C_3 pathway is used by most of the shrubs, cool-season grasses, sedges and almost all the trees, whereas C_4 plants are found in warmer climates of tropical and subtropical regions, conversely, CAM includes succulent plants, such as cacti (Cerling et al., 1993; Gowik and Westhoff, 2011; Schefuß et al., 2003).

The $\delta^{13}C$ value can be used to discriminate plants using either the C_3 or C_4 photosynthetic pathway, owing to their different fractionations of carbon isotopes. Fractionation is the non-linear transfer of isotopes from

the source to the product (DeNiro and Epstein, 1978; Leatherdale, 2013). C_3 plants have lower $\delta^{13}C$ values (-24‰ to -34‰) than C_4 plants (between -6‰ and -19‰), whereas CAM plant values range between those for C_3 and C_4 plants, often similar to those of C_4 (DeNiro and Epstein, 1978; Koch et al., 2017).

Animal forage is very rich in leaves and is also a frequent plant component in biomass; therefore, the literature on carbon isotopic composition of *n*-alkanes is mainly based on leaves (Collister et al., 1994; Diefendorf et al., 2015a, 2015b; Pedentchouk and Zhou, 2018; Rieley et al., 1993). Different plant parts show different fractionations, which may lead to different *n*-alkane concentrations and isotopic composition. However, Rommerskirchen et al. (2006) compared flower, stem, and leaf fractionation and concluded no remarkable differences (Dove et al., 1996). Nevertheless, it is noticeable that leaf wax lipids become more depleted in ^{13}C than the bulk of the plant during biosynthesis, especially by about 6–10‰ in *n*-alkanes; therefore, their $\delta^{13}C$ values can vary between -32‰ and -39‰ in C_3 and between -18‰ and -25‰ in C_4 during leaf wax lipid biosynthesis (Chikaraishi and Naraoka, 2003; Collister et al., 1994; Conte et al., 2003; Rieley et al., 1993; Rommerskirchen et al., 2006). Additionally, dung decomposition in soils alters the natural soil $\delta^{13}C$ value. Égüez and Makarewicz (2018) determined that it was caused by decomposition of the major products of excreted material in dung, lignin and cellulose (already heavily depleted in ^{13}C) , while Schweizer et al. (1999) experiments concluded that this was due to microbial fractionation during decomposition.

Consequently, there is no large isotopic fractionation related to the incorporation of carbon from the diet into an animal, which means that carbon isotopes are advantageous for use as a biomarker (DeNiro and Epstein, 1978; Minson et al., 1975). The isotopic method of dietary analysis can be applied to fossil materials to determine whether the original $\delta^{13}C$ value of some components has been preserved (DeNiro and Epstein, 1978). For instance, *n*-alkanes from dung have been studied to estimate diets using carbon isotopes (Égüez and Makarewicz, 2018).

Additionally, carbon isotopes are useful biomarkers for paleoenvironmental reconstruction. Water-use efficiency and carbon isotope fractionation are related. C_4 plants have an advantage in arid, warm or low pCO_2 climates versus C_3 owing to the CO_2 accumulation mechanism in the pathway of C_4 plants; thus, the fractionation is less. By contrast, in C_3 plants, fractionation is large (Badejo et al., 2014; Lambers et al., 2008). This results in differences of $\delta^{13}C$ values that can be recorded in sediments (Gayantha et al., 2020). Analysing animal plant diet from dung and the different strata beneath may lead to the reconstruction of the paleoenvironmental shifts in history and identify which plants dominated different parts of the world in different eras.

In conclusion, $\delta^{13}C$ values have been used as biomarkers to distinguish between C_3 and C_4 plants and to research climate variations depending on the prevailing plants at each sedimentary level.

4. Interpretation of proxies/ratios

4.1. Domestic animal differentiation proxies/ratios by faecal biomarkers

Table 1 lists the published ratios used to identify domestic animal faecal input and discern different species. They are also described as follows:

4.1.1. Faecal input

Faecal matter can be positively identified by correlating 5β-coprostanol (faecal biomarker) and 5α-cholestanol (reduction in soils) as shown in Ratio 1 (Grimalt et al., 1990).

$$\frac{5\beta - coprostanol}{5\beta - coprostanol + 5\alpha - cholestanol} (\text{Ratio1})$$

This ratio was proposed for faecal input identification in modern water and sediments using three Spanish polluted sites for water (six samples) and one unpolluted site (three samples), two polluted sites for

Table 1
Faecal matter identification ratios.

Information	No.	Ratio	Outcome	Reference
Faecal input	1	$\frac{5\beta - coprostanol}{5\beta - coprostanol + 5\alpha - cholestanol}$	Positive > 0.7 Inconclusive 0.3–0.7 Negative < 0.3	(Grimalt et al., 1990)
	2	$\frac{5\beta - coprostanol}{epi - 5\beta - coprostanol}$	Positive < 1	(McCalley et al., 1981); (Baeten et al., 2012)
	3	$\frac{5\beta - stigmastanol}{epi - 5\beta - stigmastanol}$	Positive < 1	
	4	$\frac{5\beta - coprostanol + epi - 5\beta - coprostanol}{5\beta - coprostanol + epi - 5\beta - coprostanol + 5\alpha - cholestanol}$	Positive > 0.7 Inconclusive 0.3–0.7 Negative < 0.3	(Bull et al., 1999a; Bull et al., 1999b)
	5	$\frac{5\beta - stigmastanol + epi - 5\beta - stigmastanol}{5\alpha - stigmastanol + 5\beta - stigmastanol + epi - 5\beta - stigmastanol}$	Positive > 0.7 Inconclusive 0.3–0.7 Negative < 0.3	(Prost et al., 2017)
Omnivore faeces	6	$\frac{5\beta - coprostanol + epi - 5\beta - coprostanol}{5\beta - stigmastanol + epi - 5\beta - stigmastanol}$	Omnivore > 1 Ruminant < 1	(Bull et al., 1999a; Bull et al., 1999b)
	7	$\frac{5\beta - coprostanol}{5\beta - stigmastanol}$	Human 5.5:1 Ruminant 1:4	(Evershed and Bethell, 1996)
	8	$\frac{5\beta - coprostanol}{5\beta - coprostanol + 5\beta - stigmastanol} x100$	Hervibore < 38% Values between 38% − 73% need a 2.86 factor Human > 73%	(Leeming et al., 1997)
	9	$\frac{epi - 5\beta - coprostanol}{5\alpha - cholestanol + 5\beta - coprostanol}$	Human < 0.01 Cattle, horse, deer > 0.1	(Standley et al., 2000)
	10	$\frac{5\beta - coprostanol + epi - 5\beta - coprostanol}{cholesterol}$	Pig > 3.7 Poultry, cattle < 0.7	(Jardé et al., 2007)
	11	$\frac{campesterol + sitosterol}{cholesterol}$	Pig > 1.5 Human < 1	
Herbivore faeces	12	$\frac{5\beta - coprostanol}{cholesterol}$ or $\frac{5\beta - stigmasterol}{sitosterol}$	Cow, horse ~ 1 Sheep 1–3	(Leeming et al., 1996)
	13	$\frac{epi - \beta - stigmastanol}{\beta - stigmastanol} + \frac{epicoprostanol}{coprostanol}$	Horse 2.1–2.7	(Prost et al., 2017)
	14	$\frac{deoxycholicacid}{chenodeoxycholicacid}$	Horse 0.8–2.1	
	15	$\frac{deoxycholicacid}{lithocholicacid}$	Horse 1.0–3.4	

sediments (Spain and Cuba, nine samples), and two unpolluted areas of Spain (three samples). Grimalt et al. (1990) proposed a Ratio 1 higher than 0.7 as the detection of faeces input. However, in archaeological soil samples, the degradation is greater than that in contemporary samples. In particular, 5β-coprostanol suffers from decay>5α-cholestanol and a comparison with the background of the area is proposed (Bull et al., 2001, 1999)

McCalley et al. (1981) indicated that 5β-stanols can be converted to epi-5β-stanols owing to bacterial reworking in anaerobic environments (Ratios 2 and 3).

$$\frac{5\beta - coprostanol}{epi - 5\beta - coprostanol}(\text{Ratio2})$$

$$\frac{5\beta - stigmastanol}{epi - 5\beta - stigmastanol}(\text{Ratio3})$$

The higher concentration of epi-5β-stanols (>1) indicates that the excrement has been subjected to composting (Baeten et al., 2012; Bull et al., 2001).

Due to the latter, Ratio 1 was modified including epi-5β-coprostanol, a diagenetic transformation product of 5β-coprostanol based on a study carried out in 11 ancient samples removed at different depths (Crete, 4500–3500 yr BP) (Bull et al., 2002; Bull et al., 1999a; Bull et al., 1999b; Simpson et al., 1998):

$$\frac{5\beta - coprostanol + epi - 5\beta - coprostanol}{5\beta - coprostanol + epi - 5\beta - coprostanol + 5\alpha - cholestanol}(\text{Ratio4})$$

Conversely, in ancient samples, the limit of 0.7 would be too high to determine faecal input, although no other limit was proposed ,but a comparison with the background of the area was recommended (Bull et al., 2001; Simpson et al., 1998). Mackay et al. (2020) used this ratio to determine faecal input from the Iron Age in North West Europe.

Finally, Prost et al. (2017) modified the ratio proposed by Bull et al. (1999) (Ratio 4) by changing the target 5β-stanol from the typical human form (5β-coprostanol) to the herbivore (5β-stigmastanol), retaining the same threshold (Ratio 5). This modification was made using modern composite samples from humans and the same herd of cattle, horses, sheep, goats, geese, and pigs.

$$\frac{5\beta - stigmastanol + epi - 5\beta - stigmastanol}{5\alpha - stigmastanol + 5\beta - stigmastanol + epi - 5\beta - stigmastanol}(\text{Ratio5})$$

4.1.2. Omnivore faecal input

Discerning among human, pig, and dog faecal inputs is key to clarifying the context of a sample. In general, omnivorous faeces contain a higher relative abundance of 5β-coprostanol and lithocholic acid than herbivorous faeces (Bull et al., 2002; Harrault et al., 2019b). Bull et al. (2002) introduced 5β-stigmastanol to the ratio to distinguish between ruminants (predominantly 5β-stigmastanol and epi-5β-stigmastanol), modifying a previous one proposed by Evershed and Bethel (1996) and omnivores (predominantly 5β-coprostanol and epi-5β-coprostanol) (Bull et al., 2002; Bull et al., 1999a; Bull et al., 1999b; Shillito et al., 2011).

$$\frac{5\beta - coprostanol + epi - 5\beta - coprostanol}{5\beta - stigmastanol + epi - 5\beta - stigmastanol}(\text{Ratio6})$$

Based on this ratio, omnivores > 1 > ruminants.

4.1.2.1. Humans. Because humans are omnivores, 5β-coprostanol is also the main biomarker of human faeces (Bull et al., 2002; Shillito et al., 2011). A few years before Bull et al. (2002), Evershed and Bethell (1996) proposed a ratio based only on 5β-coprostanol and 5β-stigmastanol, not taking into account epimers as in Ratio 6 using published data (Evershed and Bethell, 1996).

$$\frac{5\beta - coprostanol}{5\beta - stigmastanol}(\text{Ratio7})$$

The relative proportion to assess human input was established at > 1.5:1 (approximately 5.5:1 is the specific value for humans).

Another ratio to discriminate between humans and herbivores, was propose by Leeming et al. (1997) using generally 6 to 7 modern faeces samples from omnivores, herbivores and carnivores and principal component analysis (PCA):

$$\frac{5\beta - coprostanol}{5\beta - coprostanol + 5\beta - stigmastanol}x100(\text{Ratio8})$$

If the result is > 73%, the faecal source would probably be from humans. If the result is < 38%, the input presumably originated from herbivores. However, if the percentage of 5β-coprostanol is between 38% and 73%, a factor of 2.86 can be applied to estimate the proportion of human faecal input (Leeming et al., 1997). This approach with some modifications in the percentages was used by Prost et al. (2017) to classify archaeological samples from the Roman, Bronze and Iron age in Germany and Mackay et al. (2020) to classify archaeological samples from the Iron age in North West.

Additionally, another method based on 5β-stanols to identify human sources among cattle, horses and deer was developed by Standley et al. (2000).

$$\frac{epi - 5\beta - coprostanol}{5\alpha - cholestanol + 5\beta - coprostanol}(\text{Ratio9})$$

Values < 0.01 are related to human contribution, whereas > 0.1 refer to the other animals mentioned.

However, as specified before, 5β-coprostanol is present as the main biomarker in omnivores; therefore, bile acids present in faecal matter are useful for discerning omnivore species. Lithocholic and deoxycholic acids are the main bile acids in humans, and the concentration of lithocholic acid is higher than that of deoxycholic acid (Bull et al., 2002; Evershed and Bethell, 1996).

4.1.2.2. Pigs. Pig faeces can be distinguished by the presence of hyodeoxycholic and hycholic acids, which are not present in any other omnivorous faeces (Bethell et al., 1994; Bull et al., 1999a; Bull et al., 1999b; Evershed and Bethell, 1996).

Additionally, Jardé et al. (2007) thoroughly tested two ratios to distinguish pig manure:

$$\frac{5\beta - coprostanol + epi - 5\beta - coprostanol}{cholesterol}(\text{Ratio10})$$

$$\frac{campesterol + sitosterol}{cholesterol}(\text{Ratio11})$$

Ratio 10, pig slurry corresponds with values > 3.7, while this ratio is < 0.7 in poultry and dairy manures. For Ratio 11, pig faeces are characterised by values > 1.5, while those < 1 identify humans (Jardé et al., 2007). This ratio was proposed using nine samples (five from pig slurry) from the main manure types spread on the soils of Brittany (France).

4.1.2.3. Dogs. According to Leeming et al. (1996) and Shah et al. (2007), dog faeces contain only traces of 5β-stanols. Owing to the lack of microorganisms capable of converting Δ^5-sterols into 5β-stanols, their faeces contain high proportions of cholesterol (Bull et al., 2002). The predominance of cholesterol and the absence of 5β-stanols are characteristic of dog input.

However, a recent investigation by Harrault et al. (2019b), found high amounts of 5β-coprostanol and 5β-stigmastanol in dog faeces. They stated that the differences could be due to the diet of dogs; nonetheless, more studies should be conducted on this species.

4.1.3. Herbivores faecal input

Because of their diet, herbivore excrement contains higher proportions of plant-derived stanols such as 5β-campestanol and 5β-stigmastanol than other faeces (Baeten et al., 2012; Leeming et al., 1996;

Linseele et al., 2013). With this assumption, Leeming et al. (1997) proposed Ratio 8, indicating herbivore input values under 38%.

Regarding bile acids, all species produce a high concentration of deoxycholic acid compared to that of lithocholic acid, although each species has a specific fingerprint (Zocatelli et al., 2017).

4.1.3.1. Ruminants. As a result of their characteristic digestive tract, the predominant biomarkers in ruminant faeces are 5β-stigmastanol and epi-5β-stigmastanol.

As mentioned previously, Ratio 6 based on these characteristic 5β-stanols discriminates between ruminants and omnivores, confirming ruminant faecal input at values < 1 (Shillito et al., 2011).

Additionally, Ratio 7 was developed to distinguish between ruminants (particularly sheep and cows) and humans. In this case, the relative proportion to identify faecal input of sheep and cows was approximately about 1:4 (Evershed and Bethell, 1996).

Notably, sheep may be distinguished from other herbivores based on the presence of higher relative levels of epi-5β-coprostanol, and that their faeces only contain deoxycholic acid as bile acid (Tyagi et al., 2008; Zocatelli et al., 2017).

Regarding cattle, Leeming et al. (1996) proposed a ratio comparing β-stanols and their sterol precursors to distinguish between cows/horses (average close to 1) and sheep (1–3).

$$\frac{5\beta - stanols}{sterolprecursors}(\text{Ratio12})$$

Moreover, cattle could be discerned from humans with Ratio 9, with values > 0.1 an indicating of cattle or horse faecal input (Standley et al., 2000); however the number of samples is not presented.

Cows could finally be characterised because of the total bile acid concentration, which was significantly higher than that of horses (Zocatelli et al., 2017).

Goats could be distinguished by the presence of chenodeoxycholic acid in their faeces, in contrast to other domestic ruminants (Prost et al., 2017).

Finally, archaeol is a recent biomarker used to identify ruminants as a result of the presence of methanogens in their digestive tract, unlike other herbivores (Gill et al., 2010; Prost et al., 2017).

4.1.3.2. Equids. With reference to 5β-stanols, the trends observed in the distribution of faecal stanols from cows and horses are similar (Harrault et al., 2019b). Horses could be identified along with cattle using Ratio 9 and 10, as previously described (Leeming et al., 1996; Standley et al., 2000).

Prost et al. (2017), however, recommended another ratio to identify horse faeces more clearly. Owing to the large amounts of epi-β-stanols, but equally large or smaller contents of 5β-stanols in horse faeces, the following ratio was proposed:

$$\frac{epi-\beta-stigmastanol}{\beta-stigmastanol}+\frac{epicoprostanol}{coprostanol}(\text{Ratio13})$$

Values between 2.1 and 2.7 could be identified as horse input.

Additionally, horse faeces contain chenodeoxycholic and lithocholic acids, characterising them accurately (Prost et al., 2017; Zocatelli et al., 2017).

$$\frac{deoxycholicacid}{chenodeoxycholicacid}=0.8-2.1(\text{Ratio14})$$

$$\frac{deoxycholicacid}{lithocholicacid}=1.0-3.4(\text{Ratio15})$$

Donkey and horse faeces are remarkably similar, despite the lack of chenodeoxycholic acid in donkey faeces, which can identify them. In general, equid faeces are discriminated because of their significantly lower relative abundance of deoxycholic and lithocholic acids than those of cattle and sheep (Prost et al., 2017).

Additionally, unlike ruminants, above, equids cannot produce archaeol; therefore , it can be used as an interesting biomarker (Gill et al., 2010; Prost et al., 2017).

Figs. 4 and 5 represent two ratio/proxies to distinguish between different species of omnivores and herbivores as described by Bull et al. and Prost et al.(Bull et al., 2002; Prost et al., 2017). The former was proposed using published data and the latter from experimental data obtained from composite samples from each species.

In conclusion, a recent study conducted by Harrault et al. (2019b) questioned the validity of ratios based only on four 5β-stanols (5β-coprostanol, epi-5β-coprostanol, 5β-stigmastanol and epi-5β-stigmastanol). Additionally, the 5β-stanols, namely, 5β-lichestanol, 5β-brassicastanol, epi-5β-brassicastanol, 5β-campestanol, epi5β-campestanol, 5β-3β-stigmastanol and epi-5β-3β-stigmastanol were used. They tested 11 5β-stanols in 90 samples from 10 species (from 4 to 23 individuals per sample) and built multivariate statistical models based on PCA and hierarchical clustering on principal components with the relative % concentrations of the analytes. The values were compared with the ratios previously discussed (Table 1). With this method, it was possible to improve the accuracy and clearly distinguish the fingerprints at the species level and to differentiate between winter and summer diets in certain animals. They also highlighted the necessary increase in the number of reference samples with different diets to narrow the faecal fingerprints and continue to improve the methods of differentiation (Harrault et al., 2019b).

All these ratios or proxies have been widely used to classify ancient residues from the Neolithic to Roman ages (Baeten et al., 2012; Ball, 1996; Bull et al., 1998; Gea et al., 2017; Gill et al., 2010; Harrault et al., 2019b; Hjulström and Isaksson, 2009; Lauer et al., 2014; Lin et al., 1978; Linseele et al., 2013; Mackay et al., 2020; Prost et al., 2017; Shillito et al., 2011; Sistiaga et al., 2014a; Sistiaga et al., 2014b). However, some caution must be taken when drawing appropriate conclusions.

The first is the design of the model. Commonly, these models are designed to analyse 20 samples of each target species per scenario to obtain a representative view of the stage (Cuevas-Tena et al., 2017; Devane et al., 2015; Ikeda et al., 2020; Kakiyama et al., 2014). The study by Harrault et. al (2019b) was the only one that built the model using similar criteria and concluded that more biomarkers and reference samples were necessary to improve the model. Additionally, some scenarios were not considered. The diet of animals differs between seasons, and occasionally even within seasons, which could change the proxies or ratios proposed. For instance, in humans, more vegetarian diets enhance excretion of 5β-stigmastanol, which is dominant to coprostanol, and could therefore cause confusion with herbivorous animals (Cuevas-Tena et al., 2017).

Conversely, the validation of the analytical methods used for identification and quantification will enhance the accuracy of the results. The use of signal values as a substitute for concentration is a common approach when the standards of the analytes are not commercially available (Bull et al., 2001, 1998; Elhmmali et al., 1997). Nevertheless, this approach is not recommended because the sensitivity of two different analytes at the same concentration is not necessarily the same (lower concentrations can give higher signals), which can alter the accuracy of the results. Therefore, the use of concentration is recommended. For this purpose, the figures of merit values (accuracy, recovery, apparent recovery, limits of detection and quantification, matrix effect, type of calibration, surrogates, and internal standard used) of the method are necessary. Birk et al. (2012) properly described these values, and subsequent studies adopted this analytical method (Lauer et al., 2014; Prost et al., 2018). Other methods used for the quantification of faecal biomarkers should include all figures of merit to clarify that the results obtained are accurate (Bull et al., 2003, 2001; Derrien et al., 2011). Additionally, some models are built with the methods of Harrault et al. (2019a), and others used methods with modifications, and the latter figures are necessary to understand whether the modifications changed the accuracy of the results (Baeten et al., 2012; Lauer

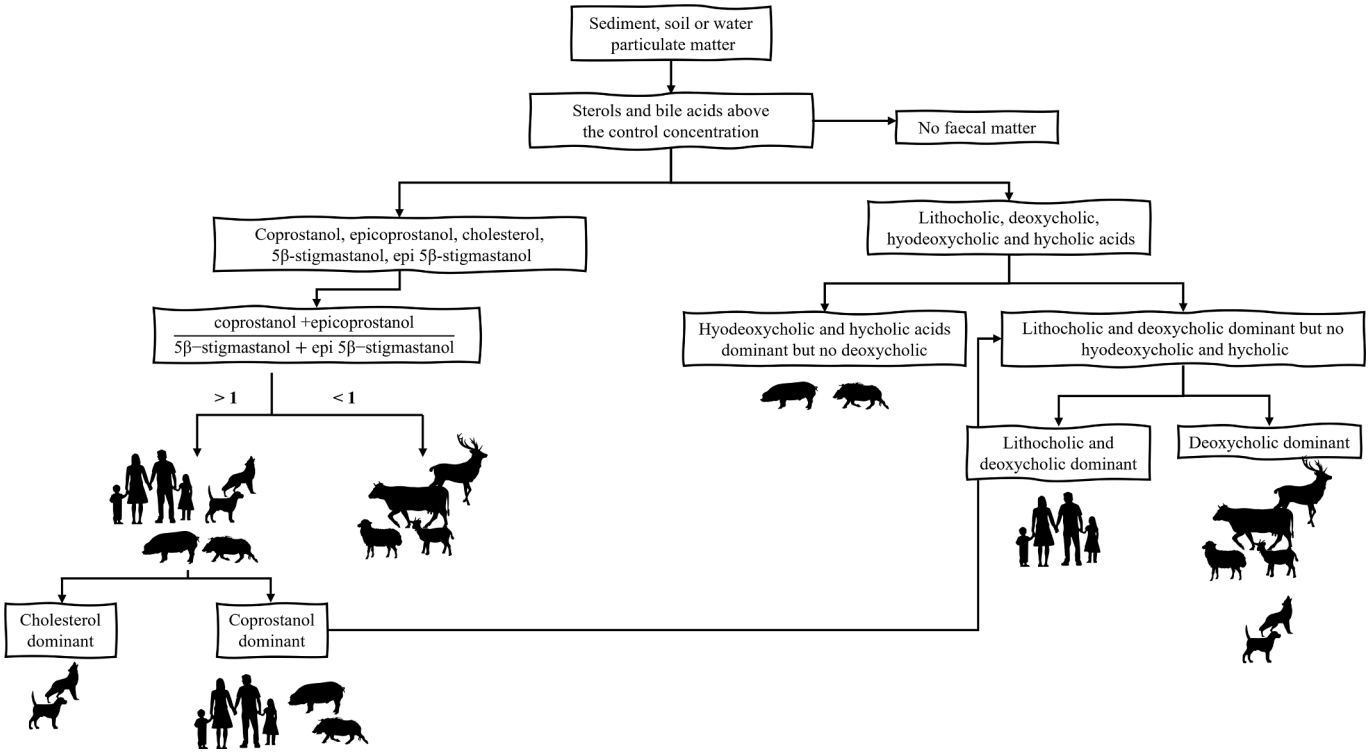

Fig. 4. A summary of the criteria used to determine specific sources of faecal pollution during multiple biomarker analysis, adapted from Bull et al., 2002.

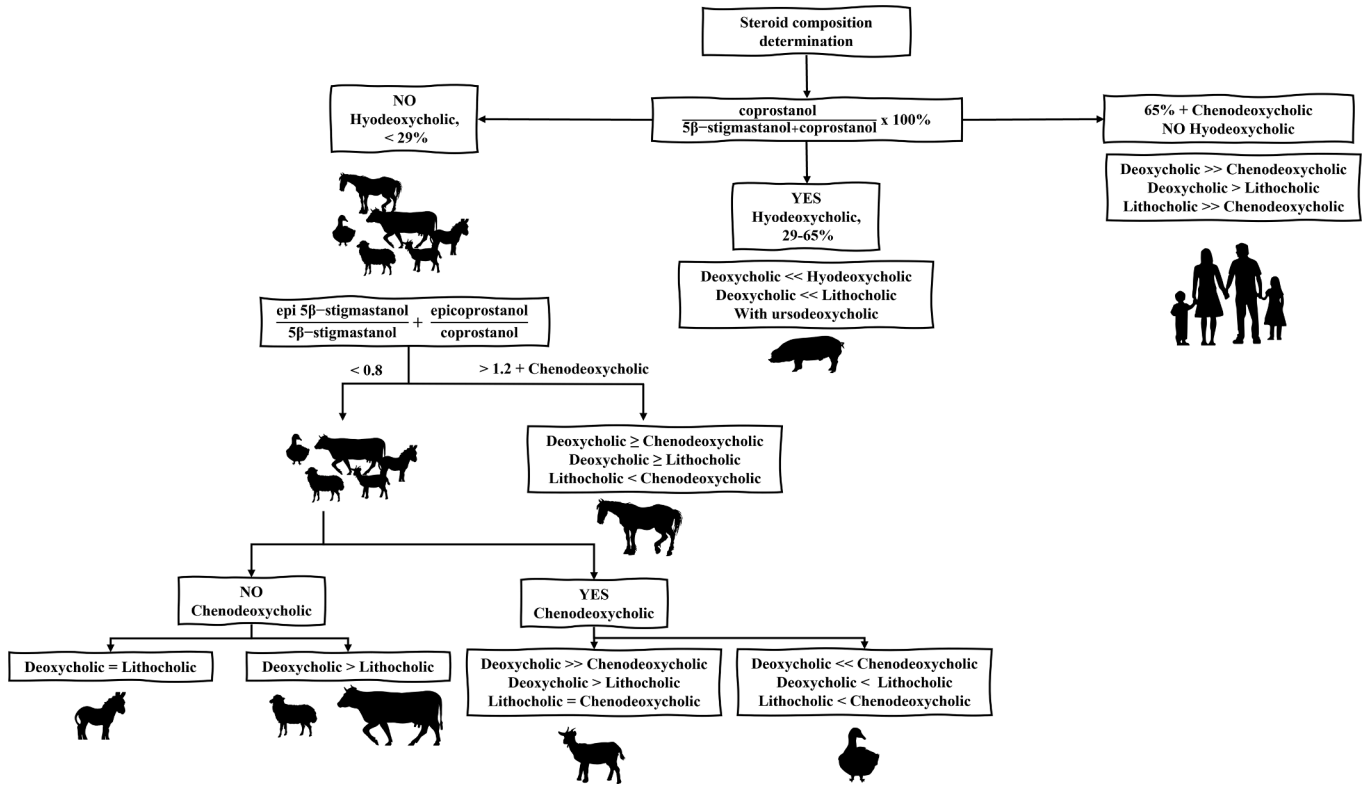

Fig. 5. Proxies and ratios for the identification of faeces using their steroid and bile acid signature, adapted from Prost et al., 2017.

et al., 2014; Mackay et al., 2020). Finally, the use of deuterated analogues as surrogates and internal standards (IS) is recommended instead of compounds which can appear in faeces, such as 5β-pregnan-3α-ol-20-one (a faecal progestagen) (Arora et al., 2020; Oates et al., 2004) or hyocholic acid (common in pig faeces) (Zheng et al., 2021) (Bull et al., 2003; Elhmmali et al., 2000; Lauer et al., 2014; Mackay et al., 2020). Surrogates, commonly incorrectly named IS, are used to correct the losses of target analytes through the method used. The use of compounds

which may be in the sample alters the results obtained at higher or lower concentrations depending on the calibration mode used. The use of deuterated analogues of the target compounds ensures the same behaviour of the analyte and the surrogate which improves the accuracy of the methods. Additionally, deuterated analogues are not naturally occurring, which avoids the over/underestimation of the concentrations.

The last point in this discussion is the approach in which the ratios used to determine faecal materials or classify them are constant over time. Prost et al. (2018) described that the losses of epi-5β-stanols observed in composting manure of cattle and horses were smaller than that of 5β-stanols and concluded that the ratios used by Bull et al. and Baeten et al. are useful to trace faecal matter (Baeten et al., 2012; Bull et al., 2001; Prost et al., 2018). Nevertheless, ratios such as epi-5β-stanols/5β-stanols are not useful for classifying animals from ancient faecal material because the ratios are not constant over time (Prost et al., 2018). This point must be studied further to ensure that the ratios and proxies used from modern faecal matter are appropriately compared with those of ancient ones.

4.2. Diet and flora estimation ratios/proxies

The use of biomarkers to determine the diet composition of grazing animals have been widely studied, see Table 2 (Dove and Mayes, 2005; Ferreira et al., 2005; Laredo et al., 1991; Lin et al., 2009; Oliván et al., 2007), and therefore, the plant components accumulated in animal faeces is known (Ali et al., 2005a,b; Dove and Mayes, 1991; Hamellers and Mayes, 1998b). This is determined using the Kulcyznski index (KSI):

$$KSI = 100 \times \frac{\sum 2C_i}{\sum (A_i + B_i)} \quad (1)$$

where C_i is the lesser percentage of *component i* in the two diets (known vs. estimated), and ($A_i + Bi$) is the sum of the percentages of each plant component in both diets (known vs. estimated). The KSI can vary from 0% to 100%. The former corresponds to a completely different diet and the latter to an identical diet (Ferreira et al., 2011; Ferreira et al., 2009a).

Dietary intake estimation can be conducted using its indigestibility and final faecal recovery (Mayes and Dove, 2000). Especially in ruminants, a correction for incomplete faecal recovery is necessary to avoid underestimating the different proportions of dietary components. (Dove and Mayes, 1996; Ferreira et al., 2011; Ferreira et al., 2007a; Ferreira et al., 2009a; Mayes and Dove, 2000). Because it is not possible to research extinct species (both plants and animals), studies on digestibility conducted on extant, genetically related species could be useful for extrapolating on archaeological samples. However, until now, this has only been hypothesised.

Ancient vegetation and anthropological studies are the main sources of faecal alkane patterns in archaeological samples. Several calculations are available to assess the possible origins of *n*-alkanes.

4.2.1. Based on n-alkanes

4.2.1.1. Average chain length (ACL). ACL, also named as the mean carbon number (MC#) by other authors, is used to evaluate inputs of terrestrial plants versus aquatic algae and phytoplankton in lake sediments (Bush and McInerney, 2013; Cranwell, 1972; Fisher et al., 2003).

$$\frac{\sum (C_n \times n)}{\sum (C_n)} \quad (2)$$

where C_n is being the concentration of each *n*-alkane with *n* carbon atoms. Longer chain lengths (C_{21}-C_{35}) are related to terrestrial plants, whereas most algae show a hydrocarbon distribution in the range C_{17}–C_{21} (Bush and McInerney, 2013; Cranwell, 1972; Han and Calvin, 1969).

Bush and McInerney (2013) proposed C_{31} as grasses representative of grasses and C_{27} and C_{29} as trees and shrubs representative respectively. C_4 grasses tend to accumulate higher amounts of long chain *n*-alkanes (C_{31}, C_{33}, C_{35}) than those accumulated in C_3 grasses and woody angiosperms (Diefendorf and Freimuth, 2017; Rommerskirchen et al., 2006). Alternatively, Garcin et al. (2014) proposed that C_3 plants accumulate more C_{29} than C_4 plants. Therefore, C_{29} alkanes can be used as C_3 plant biomarkers and, C_{33} and C_{35} as C_4 plant biomarkers (Diefendorf and Freimuth, 2017).

4.2.1.2. Carbon preference index (CPI). The CPI measures the relative abundance of odd over even carbon chain length (Bush and McInerney, 2013; Weber and Schwark, 2020).

$$\frac{\sum_{odd}(C_{21-33}) + \sum_{odd}(C_{23-35})}{2\sum_{even}C_{22-34}} \quad (3)$$

Values > 1 indicate a predominance of odd numbers of carbon atoms, typical of terrestrial higher plants (grasses, shrubs, and trees) (Diefendorf et al., 2011; Égüez et al., 2018; Mayes and Dove, 2000; Weber and Schwark, 2020).

4.2.1.3. Terrigenous/aquatic ratio (TAR_{HC}). Silliman et al. (1996) developed this ratio to asses land plant waxes (values > 4) versus algae input (values < 1) (Ortiz et al., 2013):

$$TAR_{HC} = \frac{C_{27} + C_{29} + C_{31}}{C_{15} + C_{17} + C_{19}} \quad (4)$$

This approach was used to study sediments from the Rochester Basin of Lake Ontario (x-1280 BP) and the sediment from a volcanic lake in Spain (middle and upper Pleistocene) (Ortiz et al., 2013; Silliman et al., 1996).

4.2.1.4. Proxy aquatic (P_{aq}). The ratio proposed by Ficken et al. (2000) to distinguish terrestrial and submerged/floating plants:

$$P_{aq} = \frac{C_{23} + C_{25}}{C_{23} + C_{25} + C_{29} + C_{31}} \quad (5)$$

where $P_{aq} < 0.1$ corresponds to terrestrial plants, 0.1–0.4 to emergent plants and 0.4–1 to submerged/floating plants. This proxy was used in aquatic plants from lakes in Kenya (1995–1996 CE). In total, 23 samples were collected, and as described by Ficken et al. (2000), more analysis from modern plants and sediments should be performed to validate the proxy. Sikes et al. (2009) also used this proxy in modern samples from New Zealand.

4.2.1.5. C_{27}/C_{31} ratio. The C_{27}/C_{31} ratio is adequate for determining the biogenic origin of *n*-alkanes (1 ≥ value < 2) (Wang et al., 2013):

$$\frac{C_{27}}{C_{31}} \quad (6)$$

Wang et al. (2013) used this ratio in lacustrine lake in China (1852–2010-year AD) to determine that the organic matter was derived from biogenic hydrocarbon.

4.2.1.6. Least-squares procedure. When the number of plant species in the diet exceeds the number of *n*-alkane markers, the estimation using *n*-alkanes is not accurate. Using a least-squares procedure allows the use of more number of alkanes than that of the components in the mixture (Mayes and Dove, 2000). This could be helpful when the diet is unknown, as it searches for the best fit rather than a unique solution.

Three least-squares procedures have been used by different authors: "Microsoft Excel Solve" (an iterative routine) by Mayes et al. (1994), "Genstat 5" (simple linear mathematics) by Newman et al. (1995) and "Eatwhat" (known as non-negative least squares) by Dove and Moore (1995). The procedure minimizes the discrepancies between the actual

Table 2
Plant species characterized by biomarker detection from domestic animal faeces.

Authors	Biomarkers	Plant species	Authors	Biomarkers	Plant species	Authors	Biomarkers	Plant species
(Bugalho et al., 2004)	*n*-alkanes LCOH	*Austrodanthonia pilosa, Austrodanthonia racemosa Austrodanthonia richardsonii Axonopus fissifolius Bothriochloa macra Bromus catharticus Chloris gayana Cynodon dactylon Digitaria didactyla Elymus scaber Festuca arundinacea Imperata cylindrica Lotus corniculatus* "Prostrate" *Lotus corniculatus* cv. Goldie *Lotus pedunculatus* cv. Sharnae *Lotus pedunculatus* cv. Maku *Microlaena stipoides Paspalum dilatatum Paspalum notatum Phalaris aquatica Pennisetum clandestinum Setaria anceps Sporobolus indicus* cv. major *Themeda australis Trifolium glomeratum Trifolium repens Trifolium striatum Vulpia myuros*	(Dove and Mayes, 1991)	*n*-alkanes	*Acacia aneura Aristida jerichoensis Bassia diacantha Brachiaria decumbens Chloris gayana Dactylis glomerata Digitaria decumbens Dodonea attenuata Duboisia hopwoodii Eragrostis eriopoda Holcus lanatus Leucaena leucocephala Lolium perenne Lolium multiflorum Lolium rigidum Medicago sativa* cv. Siriver *Monachather paradoxa Ornithopus compressus Paspalum dilatatum Pennisetum glaucum Phalaris aquatica Phleum pratense Setaria sphacelata Stylosanthes hamata* cv. Verano *Stylosanthes scabra Trifolium balansae Trifolium pratense Trifolium repens* cv. Haifa *Trifolium subterraneum* cv. Larisa *Trifolium subterraneum* cv. Dinninup *Trifolium subterraneum* cv. Mt Barker *Vulpia* sp.	(Dove and Mayes, 2005)	*n*-alkanes LCOH	*Austrodanthonia racemosa Austrodanthonia richardsonii Bothriochloa macra Brassica oleracea Bromus catharticus Calluna vulgaris Chloris gayana Cynodon dactylon Digitaria dactyla Fagus sylvatica Festuca arundinacea Lolium perenne Lotus corniculatus* cv. Goldie *Lotus pedunculatus* cv. Maku *Microlaena stipoides Paspalum dilatatum Paspalum notatum Pennisetum clandestinum Phalaris aquatica Picea sitchensis Pinus sylvestris Setaria anceps Themeda triandra Trifolium glomeratum Trifolium repens Trifolium subterraneum Trifolium striatum Vulpia myuros*
(Ferreira et al., 2009b)	*n*-alkanes	*Agrostis* spp. *Calluna vulgaris Erica arborea Erica australis Erica cinérea Erica umbellata Lolium perenne Poa* spp. *Trifolium repens Ulex gallii*	(Ali et al., 2005b)	*n*-alkanes LCOH LCFA	*Acer pseudoplatanus Brassica oleracea Calluna vulgaris Chamaenerion angustifolium Fagus sylvatica Juncus effusu Lolium perenne Luzula sylvatica Picea sitchensis Pinus sylvestris Pseudotsuga menziesii Vaccinium myrtillus*	(Mayes et al., 1994)	*n*-alkanes	*Betula nana Betula pubescens Carex* spp. *Deschampsia cespitosa Deschampia flexuosa Eriphorum* spp. *Junniperus comunis Melampyrum sylvaticum Salix* spp. *Tricophorum cespitosum*
(Kelman et al., 2003)	*n*-alkanes LCOH	*Austrodanthonia richardsonii Lotus corniculatus* cv. Goldie *Lotus pedunculatus* cv. Maku *Lotus pedunculatus* cv. Sharnae *Phalaris aquatica Trifolium glomeratum Trifolium repens Trifolium striatum Vulpia myuros*	(Ferreira et al., 2011)	*n*-alkanes LCFA	*Agrostis capilaris Calluna vulgaris Erica arborea Erica australis Erica cinérea Erica umbellata Lolium perenne Poa annua Trifolium repens*	(Fraser et al., 2006)	*n*-alkanes LCOH	*Calluna vulgaris Carex* spp. *Erica tetralix Festuca* spp. *Juncus effusus Molinia caerulea Vaccinium myrtillus*
(Dove and Charmley, 2008)	*n*-alkanes LCOH	*Lolium rigidum Phalaris aquatica Trifolium subterraneum Triticum aestivum*	(Ali et al., 2004)	LCOH LCFA	*Agrostis capillaris Betula pendula Calluna vulgaris Vaccinium myrtillus*	(Hameleers and Mayes, 1998a)	*n*-alkanes	*Lolium perenne* cv. Merlinda *Lolium perenne* cv. Morgana

(*continued on next page*)

Table 2 (*continued*)

Authors	Biomarkers	Plant species	Authors	Biomarkers	Plant species	Authors	Biomarkers	Plant species
								Trifolium repens cv. Menna *Trifolium repens* cv. Milkanova
(Brosh et al., 2003)	*n*-alkanes	*Ceratonia siliqua* *Quercus calliprinos* *Triticum aestivum* *Triticum Ariel* *Vicia atropurpurea* *Vicia sadot*						

faecal alkane ratios (corrected or not) and the estimated ratios (different combinations of diet), that is:

$$\sum [actual - estimated]^2_{marker:1\cdots n} \text{ or,}$$

$$\sum [F_i - (xA_i + yB_i + zC_i)]^2_{marker:1\cdots n} \quad (7)$$

where F_i corresponds to the actual alkane faecal concentration *i*; x, y and z are the respective amounts of dietary components A, B, and C, and A_i, B_i and C_i correspond to the respective concentrations of *i* in A, B and C (Dove and Mayes, 2005; L. M.M. Ferreira et al., 2009a; Salt et al., 1994).

A comparative study by Hameleers and Mayes (1998a) in dairy cows concluded that the authors gave almost identical estimates of diet composition.

4.2.1.7. Leave/grass ratio. ĎAnjou et al. (2012) proposed Equation 8. C_{27}–C_{29} *n*-alkane sources are leafy vegetation, and C_{31} grasses and small shrubs. This ratio was used in studies on sediment from a lake in Norway (0–7000 BP) for the identification of grassland or forest-origin plants.

$$\frac{C_{25} + C_{27} + C_{29}}{C_{29} + C_{31}} \quad (8)$$

The high values correspond to forest origin plants and low values to grass and small shrub plants; however, a specific value is not proposed to distinguish both sources.

The main disadvantage of these equations for application to ancient soils is their stability over time and the impossibility of correcting them properly. Many of the studies carried out are based on modern faeces, although several authors have tried to compare their results with ancient soils in order to determine the flora (D'Anjou et al., 2012; Diefendorf et al., 2011; Ficken et al., 2000; Ortiz et al., 2013; Silliman et al., 1996). However, none of these researchers used only one equation or *n*-alkanes as a proxy. They used them as complementary information.

As Jansen and Wiesenberg (2017) described, when molecular proxies are used, several factors must be considered: the content and composition of the proxy considering the life stage, plant part, external factors, possible microbial inputs, roots, vegetation, and the transformation and degradation of the proxies and the soil.

In this particular case, *n*-alkane composition changes depending on the plant, life stage, and external factors (Diefendorf et al., 2011). Although this composition could be determined, the degradation of the faecal or organic matter can produce *n*-alkanes and alter the equation values, and roots and vegetation near the sample can contribute to the modification of the equation (Jansen et al., 2019; Li et al., 2018). Therefore, *n*-alkane-based proxies must be used as complementary data to determine the diet/flora in ancient faecal matter.

4.2.2. Based on long-chain fatty acids

Parallel to Equation 4, fatty acids can also assess terrestrial (C_{24}, C_{26}, and C_{28}) versus aquatic plant (C_{12}, C_{14}, and C_{16}) origin (Silliman et al., 1996).

$$TAR_{FA} = \frac{C_{24} + C_{26} + C_{28}}{C_{12} + C_{14} + C_{16}} \quad (9)$$

Since Grace and Body (1981) suggested the use of LCFAs to estimate the digestibility of free-ranging herbivores for the first time, few studies have used them to infer diet. Despite their presentation as a favourable biomarker that suffers no alteration in the digestive tract, the analytical method for their quantification is not as robust as required (Ali et al., 2005a; Dove and Mayes, 2005). Regarding the latter, studies including LFCAs to estimate diet noticed high but incomplete faecal recoveries (Ali et al., 2004; Dove and Mayes, 2005). Consequently, as with *n*-alkanes, the incomplete faecal recovery forces a correction to be made to avoid underestimations. For this purpose, the mean recovery data for the dietary treatment to which the animal belonged, and the mean recovery rate across all experimental diets must be used (Ferreira et al., 2011).

Within the LCFAs, the shorter ones (C_{22}–C_{26}) are predominantly found in herbage species, whereas heather has similar proportions of shorter and longer LFCAs.

Ferreira et al. (2011) compared LCFAs, alkanes and their combination for diet estimation using the Genestat least-squares procedure. Overall, the combination of biomarkers was the most accurate (Eq 1), whereas LCFAs alone was more accurate than alkanes alone. Similar to *n*-alkanes, LCFAs show a high disadvantage for use as a unique dietary biomarker from ancient faecal matter (Derrien et al., 2017). The cross contamination of the sample owing to its degradation, and the degradation of roots and vegetables near the sample, considerably alter the equations based on LCFAs (Wiesenberg et al., 2010). Additionally, these compounds are labile and quite easy to oxidise and degrade (Whelton et al., 2021).

Conversely, the sources of cross-contamination of LCFAs during the analytical method are countless. In the sampling and during the analysis, the faecal matter and the extracts must not come into contact with any kind of plastic because of their high C_{16} and C_{18} contents. Therefore, it is recommended that glass sampling containers are used and that the sample should be covered in aluminium foil to avoid this contamination (Wang et al., 2009; Whelton et al., 2021). Additionally, the glass material must be thoroughly cleaned with chloroform or methanol.

4.2.3. Based on Long-chain fatty alcohols

Several authors have used LCOHs to estimate diet, especially in addition to the previous biomarkers, with satisfactory results (Ali et al., 2004;Ali et al., 2005a; Bugalho et al., 2004; Dove and Charmley, 2008; Fraser et al., 2006; Kelman et al., 2003). As with *n*-alkanes and LCFAs, LCOHs are limited by incomplete faecal recovery and correction factors have to be applied, either using recovery data for individual animals, mean recovery data for grouped animals, or grand mean values for faecal marker recovery across all diet possibilities (Dove and Charmley, 2008).

Various computer software programs have been used to estimate diet composition regarding LCOH alone and combined with *n*-alkanes:

- EatWhat: This package is based on a non-negative, least-squares procedure and estimates the diet component by combining the recovery corrected data of alkanes or LCOHs. The discrimination between the group components is made by PCA. Additionally, to determine whether LCOH provides additional information about

plant species, orthogonal procrustes rotation is used (Bugalho et al., 2004; Dove and Charmley, 2008; Dove and Moore, 1995).

- MATLAB (MathWorks 2004): This software is used as a programming and numeric computing platform and the "fgoalattain" function uses a sequential quadratic programming multi-objective to optimise the method. Similarities between the presumed diet and faecal content are estimated by considering the proportions of the components in the diet (Fraser et al., 2006).

In conclusion, none of the biomarkers appeared to be ideal because of their incomplete faecal recovery. However, the combined analysis and development of software that can afford the complexity of a free ranging animal are promising. Nevertheless, this approach must be revised when ancient samples are studied because these compounds are not useful for determining past vegetation and other biomarkers, and analyses, such as pollen analysis, are necessary (van Mourik and Jansen, 2013).

5. Conclusion

The aim of this work was to provide as much information as possible about the interpretation of faecal analysis in the archaeological field. This information includes biomarkers to assess faecal inputs and presumably identify the animal source. Additionally, faecal matter is also a source of dietary information; therefore, it could be possible to identify the related animal and its diet from the same sample.

The analysis of the biomarkers cited in this work is interesting and useful for archaeological samples. However, the identifications were based on analogies between the faeces and reference specimen composition. This requires the development of databases to assign similarities. A complication in the archaeological field is the fact that the animal species linked to the sample are usually extinct or have evolved into a different species. Additionally, owing to climatic changes, the present-day world flora is not dispersed as it was in the past. Therefore, collaboration with both, zooarchaeologists and paleo-botanists is essential to improve the interpretations of these analyses.

However, as Harrault et al (2019a) mentioned before, classic biomarkers are useful, yet insufficient to enhance robust identification. Their study increased the biomarkers from four 5β-stanols to 11, improving their results. This improved the study of these biomarkers and research, not only of 5β-stanols, but of other chemical compounds too. Additionally, advances in chemical and computer technologies have facilitated the development of this promising field in archaeology and history.

The lack of biomarker pattern databases of plants is problematic because, without them, plant identification from archaeological samples would be unattainable. Alternatively, despite the insufficient quantity of archaeological plant remains, more efforts should be made to increase their chemical analysis. Therefore, better databases can be assembled.

Few attempts have been made to improve animal identification and research on new biomarkers. The development and improvement of analytical techniques and the search for new biomarkers are necessary.

In conclusion, the analysis of faecal matter in archaeological sites, underrated at the beginning, provides plenty of information. In fact, this method is convenient when combined with other proxies, and if used alone, it becomes essential. However, to increase reliability, a thorough background review must be done by means of reference sample analysis. The research in progress and because of its significance, we believe that better results will be obtained in the near future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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