Distinguishing between sheep and goat in archaeological *fumiers* through faecal lipid biomarkers: The case of Belmaco Cave (Canary Islands, Spain)

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ABSTRACT

Lipid biomarker analysis focused on steroids has shown to have great potential for discriminating between animal faecal inputs in archaeology. This is particularly interesting when applied to stabling deposits to assess herding strategies and livestock composition. Here we present the results of a sedimentary faecal biomarker study conducted at Belmaco Cave, a pre-European archaeological site in La Palma (Canary Islands). The site was occupied by indigenous pastoralist groups over the course of five centuries prior to the first arrival of the Europeans in the 15th century. Previous soil micromorphological data indicates recurrent sheep and/or goat penning and periodic burning of the pen in the central area of the cave. We selected bulk sediment samples from unburned, charred and ashed dung layers and carried out sterol, stanol and bile acid analysis. Our results suggest that the main source of faecal matter is sheep. We also found that even though charred and dung ash layers contained lower concentrations of the biomarker compounds, these were still sufficient to provide information on the origin of the faecal remains. These data add to our current knowledge of the aboriginal Canary Island pastoralist economy.

1. Introduction

La Palma, an island around 400 km off the African coast and part of the Canary Islands archipelago, was first settled by Imazighen groups from Northwest Africa (Fregel et al., 2009) during the first centuries of the CE (Morales et al., 2013; Velasco Vázquez et al., 2019; Morales et al., 2023), and later by Europeans at the end of the 15th century. Traditionally, the indigenous population that lived in La Palma prior to the European arrival are known as Auaritas. Our current knowledge of the Auarita economy relies heavily on ethnohistorical reports. European chronicles report that the aboriginal inhabitants of La Palma subsisted merely on animal husbandry and plant gathering (Abréu Galindo, 1977; Cioranescu, 2004), in contrast to other islands of the archipelago where agriculture was practised. According to these chronicles, the herds mainly comprised goats (*Capra hircus*), sheep (*Ovis aries*), and pigs (*Sus domesticus*) (Abréu Galindo, 1977; Cioranescu, 2004; Frutuoso, 1964; Marín De Cubas, 1986). This information is supported by zooarchaeological evidence gathered from the archaeological cave sites of El Tendal and El Rincón (Pais Pais, 1996; Pérez González, 2007), which also points to the presence of higher proportions of sheep and goats than pigs. According to the chronicles, the Europeans were responsible for the introduction of cattle after the conquest of the island (Abréu Galindo, 1977).

Some researchers have proposed that, like nowadays, indigenous pastoralist groups in La Palma practised short-range transhumance from the coastal shrublands to the upper pine forest and summit scrub, and

that pasture distribution was a determining factor for territorial control and settlement patterns across the island (Martín Rodríguez, 1986, 1992; Pais Pais, 1996, 2008). However, this hypothesis possibly applies only to goats. In La Palma, fodder present in the pine forest and the summit are found at ca. 1200 m a.s.l. in the north and at 1000 m a.s.l. in the south. At these altitudes, the terrain is rugged and difficult for sheep, which tend to remain within the midlands and lowlands (Martín Rodríguez, 1992; Pais Pais, 1996). It has therefore been hypothesised that sheep herding among the aboriginal Auarita population is constrained to the midlands and lowlands, an issue which this study addresses.

Distinguishing sheep from goat bone remains in archaeological contexts is problematic (Balasse and Ambrose, 2005). This problem has been approached through zooarchaeological morphological and metric studies (Balasse and Ambrose, 2005; Grine et al., 1986; Gron et al., 2020; Halstead et al., 2002; Mallet et al., 2019; Pilaar et al., 2019; Salvagno and Albarella, 2017; Zeder and Lapham, 2010; Zeder and Pilaar, 2010), and more recently geometric morphometrics (GMM) (Haruda, 2017; Jeanjean et al., 2022), archaeogenetics (Bar-Gal et al., 2003; Loreille et al., 1997; Newman et al., 2002; Pilaar et al., 2019), mass spectrometry (ZooMS) (Buckley et al., 2010; Buckley and Kansa, 2011; Pilaar et al., 2019; Prendergast et al., 2019), stable isotope analysis (Balasse and Ambrose, 2005; Pilaar et al., 2019; Prendergast et al., 2019), and faecal lipid biomarkers (Prost et al., 2017). Regarding the latter, faecal steroid analysis has been applied in various contexts to differentiate among mammal faecal sources based on the presence/absence and relative abundance of specific 5β-stanols and bile acids (Bull et al., 2002; Shillito et al., 2011; Zocatelli et al., 2017; Prost et al., 2017; Harrault et al., 2019; Reggio et al., 2023; Taylor et al., 2023; see also Vázquez et al., 2021 and references therein). However, the current method does not discriminate between sheep and cattle, which are similar in terms of 5β-stanol and bile acid composition. Since we do not expect presence of cattle in La Palma pre-European archaeological sites (Abréu Galindo, 1977; Cioranescu, 2004; Pais Pais, 1996), the potential to use this method to distinguish sheep and goat is high, as chenodeoxycholic acid (CDCA) is present in goat faeces, while seems to be absent in sheep dung

(Prost et al., 2017; Zocatelli et al., 2017).

In this study, we apply sedimentary faecal lipid biomarker analysis to distinguish between sheep and goat presence in a *fumier* deposit in La Palma found in Belmaco Cave, an archaeological site in the southeastern midlands of the island spanning from the end of the 9th to the beginning of the 15th century AD (Marrero Salas et al., 2016). *Fumiers* are sedimentary deposits found in cave entrances or rockshelters, formed from recurrent animal stabling and periodic burning of the animal dung (Angelucci et al., 2009; Brochier, 1996; Brochier et al., 1992). Based on their microscopic components, and on the presence of goat/sheep bone remains and rock polish on the cave walls, *fumiers* are commonly associated with sheep and goat (Brochier et al., 1992; Vergès and Morales, 2016). *Fumier* deposits are recognized on a stratigraphic profile by their visible successions of thin, brown, black and white subhorizontal, undulating layers (sometimes referred to as "layer cake" facies) (see Supplementary Information (SI) Fig. S1) and are also characterised by their relative scarcity in archaeological materials (Angelucci et al., 2009; Bergadà and Oms, 2021; Brochier et al., 1992).

Soil micromorphological studies have interpreted the different layers as (i) brown: unburned or mildly burned sheep/goat dung layers, with abundant faecal spherulites, and phytoliths; (ii) black: concentrations of microscopic charred plant residues; (iii) white/grey: wood and/or sheep/goat dung ash (spherulite-rich), sometimes intermixed (Angelucci et al., 2009; Bergadà and Oms, 2021; Égüez et al., 2016; Polo-Díaz et al., 2014, 2016). Sedimentary faecal lipid biomarker studies of *fumier* deposits are scarce (Gea et al., 2017; Vallejo et al., 2022a, 2022b). The results of these studies suggest that unburned dung sediments preserve biomarkers of dung sources, while burned sediments show problematic data, particularly ashy sediment (Gea et al., 2017; Vallejo et al., 2022a, 2022b). Here, to establish the animal source of the Belmaco Cave fumier and explore the potential of steroid analysis on burned sediments, we carried sedimentary faecal lipid biomarker analysis on differently thermally altered facies (brown, black and white) of the Belmaco Cave *fumier*, which has been previously investigated through different microstratigraphic techniques and shows a well preserved, *n*-alkane-rich sequence, including the white facies (Fernández-Palacios et al., 2023).
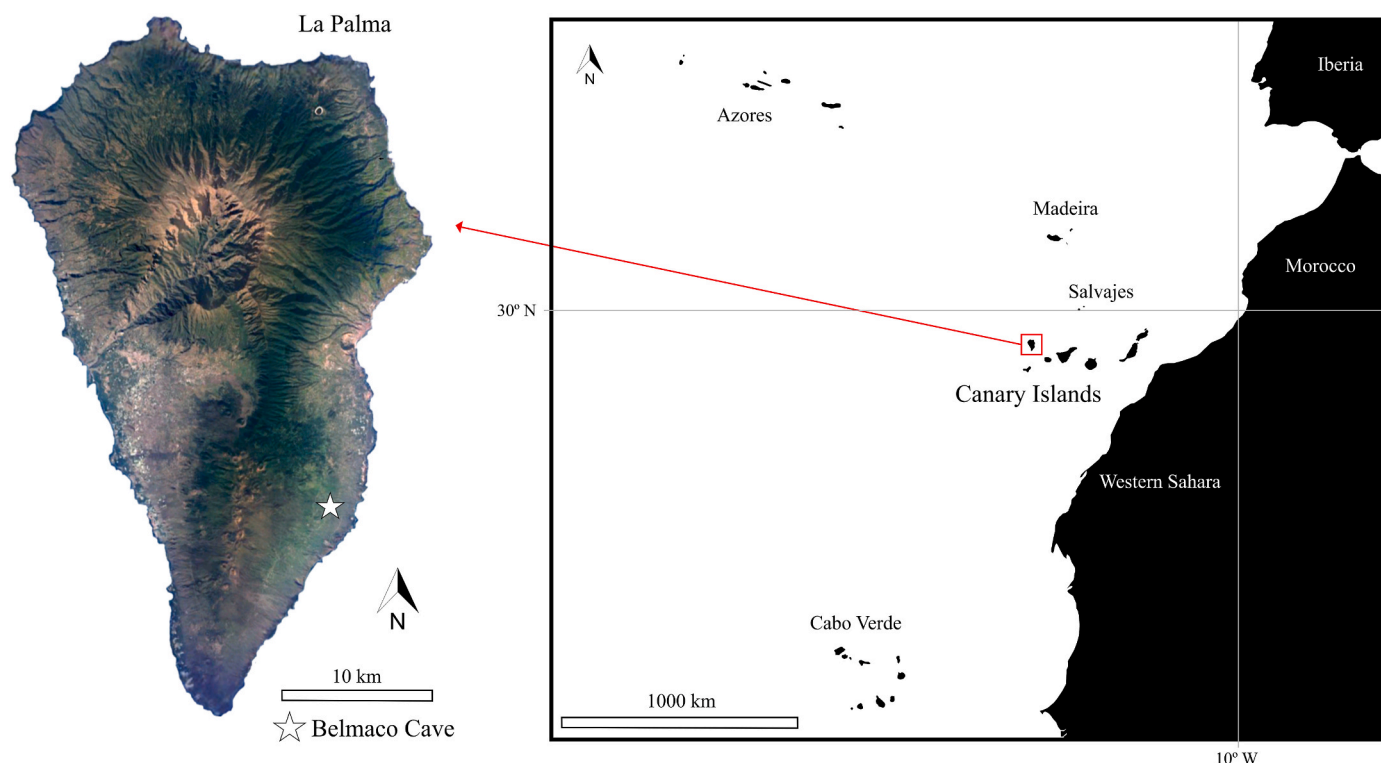

Fig. 1. Map showing location of La Palma and Belmaco Cave archaeological site.

Fig. 2. a) General view of Belmaco Cave and the stratigraphic profiles under geoarchaeological investigation. The red circle shows the location of the *fumier* deposit in the central part of the rockshelter. b) View of the perpendicular Profiles A and B, and adjacent Profile C.

We also conducted zooarchaeological analysis on an available sample of the site's faunal assemblage.

2. Materials and methods

2.1. Study site and field sampling

Belmaco Cave is located in the southeastern midland of La Palma island (Canary Islands) at an altitude of 362 m a.s.l. (28° 34′ 40′′ N; 17° 46′ 36′′ W) (Fig. 1). Archaeological investigations have suggested that the cave, at least its central area, was used by the indigenous people of La Palma as an animal pen from 882 to 1015 cal. AD (Profile B, Unit XXI; Beta382867 (2σ)) to 1305–1419 cal. AD (Profile A, Unit III; Beta382865 (2σ)) according to radiocarbon dating performed on ovicaprid bone remains (Figs. 2 and 3) (Marrero Salas et al., 2016). This evidence points towards a pastoral use that lasted for several centuries almost coinciding with the arrival of European explorers and conquerors that ultimately led to the disappearance of the aboriginal inhabitants.

Sampling for lipid analysis was carried out in the central part of the cave on two stratigraphic profiles: Profile A, exposed facing East, and Profile B, which is perpendicular to A and faces South (see Figs. 2 and 3). Field observations in 2013 had already noted a thin-layered and well-bedded deposit in Profile A (Marrero Salas et al., 2016). Subsequent micromorphological analysis of this sequence identified a recurrent superposition of unburned dung layers (described as microfacies type 1
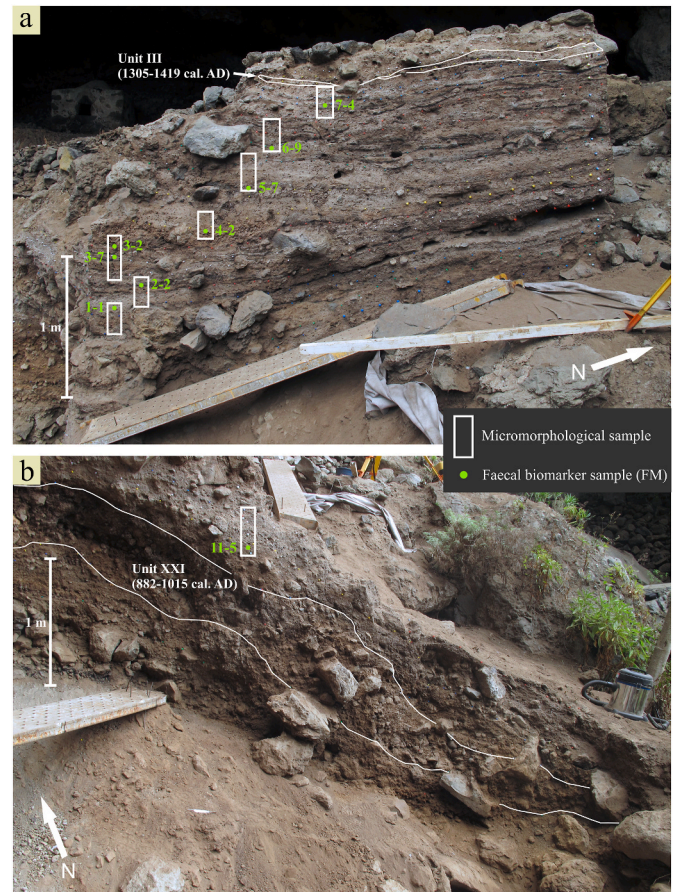
Fig. 3. Faecal biomarker samples collected from areas previously analysed through soil micromorphology in a) Profile A, and b) Profile B.

or MFT 1), overlaid by carbonized black layers (MFT 2), overlaid by dung ash layers (MFT 3) (Fernández-Palacios et al., 2023) . Faecal biomarker analysis targeted each of these studied microfacies types. In order to ensure lipid signal and avoid degradation, samples within these MFTs were selected based on highest TOC (Total Organic Carbon) values and highest total *n*-alkane concentration (Fernández-Palacios et al., 2023). A total of 9 bulk sediment samples (reported in this study as faecal biomarker sample or FM) (see Fig. 3), from each MFT, were collected with sterilized metal tools and nitrile gloves from the negatives of the micromorphological blocks in the field. Around 2 cm of the

Table 1
List of standards and key ions (m/z) used in this study.

Analyte	Purity	Supplier	Key ions (m/z)
Stanols/sterols			
5α-Stigmastanol	–	Avanti Polar Lipids Inc.	215, 383, 473
Cholestanol	–	Avanti Polar Lipids Inc.	445
Stigmasterol	–	Cayman Chemical Company	129, 394, 484
Cholesterol	≥99%	Sigma-Aldrich	329, 368, 458
Coprostanol	≥98%	Sigma-Aldrich	215, 257, 355, 370
β-Sitosterol	–	Sigma-Aldrich	129, 396, 486
Bile acids			
Chenodeoxycholic acid	≥96%	Sigma-Aldrich	255, 355, 370
Ursodeoxycholic acid	≥99%	Sigma-Aldrich	255, 370, 460
Deoxycholic acid	≥98%	Sigma-Aldrich	255, 345, 370
Lithocholic acid	≥95%	Sigma-Aldrich	215, 257, 372
Hyodeoxycholic acid	≥98%	Sigma-Aldrich	255, 355, 370

-: not provided.

Table 2
Calculated and estimated concentrations per faecal biomarker sample (FM) and microfacies type (MFT) (* = estimated concentration based on its area relative to the area of the IS). Steroid abbreviations: Cop = Coprostanol; Epi-cop = Epicoprostanol; Chol = Cholesterol; 5α-Chol = 5α-Cholestanol; 5β-Stig = 5β-Stigmastanol; Epi-Stig = Epi-5β-Stigmastanol; Stigste = Stigmasterol; β-Sit = β-Sitosterol; 5α-Stig = 5α-Stigmastanol; LCA = Lithocholic acid; DCA = Deoxycholic acid.

FM	Depth (cm)	MFT	Condition	Cop	Epicop	Chol	5α-Chol	5β-Stig
				(µg/g dry sample)				
7–4	48.5	1	Unburned	$0.65 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.99 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.69 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	$3.11 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$
6–9	80	3	Burned	$0.46 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.64 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.53 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	$1.98 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$
5–7	112.5	2	Burned	$0.21 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.20 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.60 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.39 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.87 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$
4–2	145	3	Burned	$0.39 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.44 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.82 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.59 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$1.54 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$
3–2	154	2	Burned	$0.31 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.33 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.39 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	$1.19 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$
3–7	160	1	Unburned	$1.23 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$1.07 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$1.11 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	$6.12 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$
2–2	182.5	2	Burned	n.d.	n.d.	$0.32 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	$0.75 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$
1–1	202.5	3	Burned	n.d.	n.d.	$0.17 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	$0.77 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$
11–5	298	1	Unburned	$0.43 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.6 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.59 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	$1.94 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$
Control	–	–	–	n.d.	n.d.	n.d.	n.d.	n.d.

sections' external surface was carefully removed prior to sample collection to avoid modern contaminants that could have accumulated between the time when the blocks were collected and the sampling for steroid analysis. Samples were collected from each of these negative sections (8 samples from Profile A and 1 sample from B) to address the temporal variability of the sequence and to coincide with the areas analysed through micromorphology. An additional control sample was collected from a natural sequence outside of the *fumier* deposit, around 20 m away. The sediment of the control sample shows a high detrital component and an orange colour indicative of its geogenic nature. Its stratigraphic position with respect to the *fumier* (around 1.5 m below) shows that it is not contemporary. Samples for lipid analysis were packed in aluminum foil to avoid cross contamination and stored at −20 °C to avoid bacterial degradation (Brittingham et al., 2017) at the Archaeological Micromorphology and Biomarkers Laboratory (AMBI LAB, University of La Laguna).

The faunal assemblage recovered during the 2013 excavation season was analysed. It comprises 167 faunal remains (NISP) from Profiles A, B and C (see Fig. 2b) (Navarro Mederos et al., 2013; Marrero Salas et al., 2016). The zooarchaeological data is presented in the Supplementary Information (see SI Table S1 & Text S1).

2.2. Lipid extraction and saponification

Processing started with oven drying samples for 48 h (constant mass) at 60 °C. Once dried, samples were homogenized by means of an agate mortar and pestle and consecutively subsampled by collecting 5.000 ± 0.065 g of sediment per sample. In order to obtain the total lipid extract (TLE), sediment was mixed with 40 mL of dichloromethane:methanol (DCM:MeOH 2:1 v/v). Solvents were then sonicated for 30 min (T < 30 °C), centrifuged for 10 min (4700 rpm), and filtered through glass fibber paper and sterilized glass wool. This process was repeated three times and, thereafter, samples were evaporated with a constant flow N_2 at 40 °C. The TLE extraction process follows Herrera-Herrera and Mallol (2018) and Jambrina-Enríquez et al. (2018) optimized for sterols, stanols and bile acids extraction (Elhmmali et al., 1997; Pescini et al., 2023).

Next step was saponification, which was conducted according to the procedure proposed by Elhmmali et al. (1997), with minimum modifications (Pescini et al., 2023). For this purpose, 5 mL of potassium hydroxide (KOH) solution (5M) in MeOH 90% v/v was added to the sample TLE for alkalization. Hyocholic acid (Avanti Polar Lipids Inc.) was also added as Internal Standard (IS) (25 mg/L). Samples were heated at 100 °C for 60 min. After heating, 20 mL distilled Milli-Q water was added and samples were acidified using a HCl (6 M) solution until reaching pH 3–4. Lipids were then extracted by adding 10 mL DCM and mixing with vortex (1 min), a step that was repeated three times (total of 30 mL DCM per sample). Then, solid phase extraction (SPE) was carried

out for each sample using polymerically bonded aminopropyl phase columns (500 mg sorbent, Supelco), which were preconditioned and activated with 6 mL *n*-hexane. Evaporated samples were reconstituted with 1 mL *n*-hexane and transferred into the columns. Sterols/stanols were eluted with 6 mL of DCM/2-propanol (2:1 v/v), while carboxylic acids were eluted with 12 mL of 3% v/v acetic acid in diethyl ether.

Carboxylic acids were consecutively derivatized by methylation with 5 mL MeOH and 400 µl sulfuric acid (H_2SO_4). Methyl nonodecanoate (C19:0, purity ≥98%, Sigma-Aldrich) was used as IS (8 mg/L). Methyl esters were obtained by heating at 70 °C for 240 min. After this time, the sample was neutralised with 10 mL of a saturated bicarbonate solution (2.125 g in 25 mL of Milli-Q water). Derivatized lipids were extracted three times with 3 mL hexane (total of 9 mL hexane per sample) and dried under a gentle N_2 flow in the Organomation evaporator. Methylated carboxylic acids were again fractionated by silica gel column chromatography (600 mg sorbent). These samples were reconstituted using 1 mL 2:1 v/v DCM/hexane and transferred into the columns. Elution with 5 mL 2:1 v/v DCM/hexane allowed us to retrieve monocarboxylic fatty acids methyl esters, and consequent elution with 5 mL 2:1 v/v DCM/MeOH for hydroxy carboxylic acid methyl esters (including bile acids). Again, the eluted compounds were evaporated in the Organomation.

Finally, all fractions of every sample (sterols/stanols, monocarboxylic, and hydroxycarboxylic fatty acids) were silylated and injected in less than 24 h (Knapp, 1979). All 30 fractions were dissolved with DCM and mixed with 100 µL of N,O-Bis(tri-methylsilyl) trifluoroacetamide (BSTFA) + trimethylchlorosilane (TCMS) 99:1 v/v to obtain trimethylsilyl (TMS) ethers. 1 µL of 5α-androstan-3-ol (400 mg/L) as IS was also added. Derivatization lasted for 60 min at 80 °C. Reagents were then dried and redissolved with 50 µL DCM prior to injection.

Method validation was carried out by conducting calibration, sensitivity (limits of detection and quantification -LOQs), and recovery studies. For calibration study, six different concentration levels (n = 6) were injected in triplicate (from 0.002 to 0.200 µg/g for sterols/stanols and from 0.053 to 0.750 µg/g for bile acids). The curves were established based on the area ratio between each analyte and the IS. R^2 equal to or greater than 0.990 were obtained in all cases. The LOQ was defined as the lowest concentration that provides a signal-to-noise ratio higher than 10 for the quantification ions, considering the recovery of the method and the preconcentration and/or dilution factors. The LODs was analogously defined but using a signal-to-noise ratio of 3. LOQs in the range 2.2 ng/g-0.1 µg/g dry sediment were obtained. In order to evaluate the effectiveness and reproducibility of the extraction procedure, a recovery study was conducted at different concentration levels (0.050–0.100 µg/g dry sediment for sterols and 0.375 µg/g dry sediment for bile acids). Three samples were spiked and extracted for this purpose (n = 3). The study involved comparing the peak areas of analytes in

Epi-Stig	Stigste	β-Sit	5α-Stig	LCA*	DCA*
(μg/g dry sample)					
$6.68 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.78 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$3.23 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$1.44 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.02 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$1.89 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$
$3.81 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	$1.16 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.56 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$1.80 \cdot 10^{-4} \pm 0.89 \cdot 10^{-4}$	$6.53 \cdot 10^{-4} \pm 1.39 \cdot 10^{-4}$
$1.39 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	$1.15 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.02 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$3.77 \cdot 10^{-4} \pm 0.00 \cdot 10^{-4}$	$0.11 \cdot 10^{-1} \pm 0.05 \cdot 10^{-1}$
$2.07 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	$1.64 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.13 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	$3.90 \cdot 10^{-4} \pm 0.75 \cdot 10^{-4}$
$1.72 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	$0.75 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.08 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	$0.01 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$
$7.44 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	$5.36 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$2.99 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.06 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$4.26 \cdot 10^{-1} \pm 0.22 \cdot 10^{-1}$
$0.84 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	< LOQ	n.d.	n.d.	$1.73 \cdot 10^{-4} \pm 0.00 \cdot 10^{-4}$
$0.95 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	< LOQ	n.d.	n.d.	$0.73 \cdot 10^{-4} \pm 0.20 \cdot 10^{-4}$
$3.96 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	n.d.	$2.29 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$0.4 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$	$5.71 \cdot 10^{-4} \pm 0.20 \cdot 10^{-4}$	$0.2 \cdot 10^{-1} \pm 0.00 \cdot 10^{-1}$
n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

samples that were spiked at the beginning and end of the extraction process. We obtained valid lipid extract recovery values ranging between 63 and 119% with RSDs ≤ 14%.

2.3. Instrumental analysis

Sterols and carboxylic acids were analysed using Gas Chromatography-Mass Spectrometry (GC-MS). A GC-Agilent 7890B chromatograph coupled with a MSD Agilent 5977A single quadrupole (Q) mass spectrometer with an electron impact interface (equipped with multimode injector (MMI), automatic autosampler, and bonded fused silica HP-5ms capillary column: (5%phenyl)-methylpolysiloxane, 30 m, ID: 250 μm, film thickness 0.25 μm; Agilent Technologies), was used to determine and quantify the steroids. Data collection and processing was performed using MassHunter Workstation software (Agilent Technologies). The GC oven temperature was initially programmed at 70 °C (held for 2 min), then increased to 140 °C (rate: 12 °C/min), and finally to 320 °C (heating rate 6 °C/min, held for 16 min). The carrier gas flow (helium) was set at 1 mL/min while the multimode injector (split ratio 5:1), was programmed at a temperature of 70 °C (0.85 min) and heated to 300 °C (rate: 720 °C/min). Regarding MS conditions, the Q was operated in a full-scan mode (m/z range from 40 to 580). Electron ionization energy was established at −70 eV and temperatures for the ion source were set at 230 °C and 150 °C, respectively. Solvent delay was programmed to 7 min to avoid damage to the mass source filament.

Compound identification was achieved through elution order comparison, observation of peak signals corresponding to specific ion fragments (Prost et al., 2017), reference spectra obtained from the NIST mass spectra library, as well as pure derivatized internal standard injections (Table 1). Sterols/stanols were quantified using calibration curves (A_{peak}/A_{IS} vs concentration, $R^2 \geq 0.9900$). For bile acids, since the detected quantities were below the LODs, concentrations were estimated using the internal standard area and considering the sensitivity obtained by injecting the corresponding derivatized standards.

2.4. Ratios

The ratios of steroid compounds utilized to confirm the presence of excrements and identify its animal source are the following:

● Ratio I: Indication of presence or absence of faecal matter; modified by Prost et al. (2017), using known herbivore stanols, from Bull et al. (1999). A value above 0.7 tends to indicate its presence; values between 0.3 and 0.7 do not allow to determine presence or absence; values below 0.3 confirm absence of faecal matter.

(5β-stigmastanol + epi-5β-stigmastanol) /(5α-stigmastanol + 5β-stigmastanol + epi-5β-stigmastanol)

● Ratio II: Indication of herbivore (<29%), pig (29%–65%), or human (>65%) derived faecal matter (Leeming et al., 1997; Prost et al., 2017). We applied the modified threshold values, and considered bile acids, as indicated by Prost et al. (2017).

coprostanol / (coprostanol + 5β-stigmastanol) x 100%

● Ratio III: Indication of ruminant (<1) or pig/human (>1) faeces (Shillito et al., 2011).

(coprostanol + epicoprostanol) / (5β-stigmastanol + epi-5β-stigmastanol)

● Ratio IV: Indication of horse (1.0–3.4) or ruminant (>10) (Prost et al., 2017; Gea et al., 2017).

deoxycholic acid / lithocholic acid

2.5. Ordination analysis

Principal components analysis (PCA) was performed to better visualize differences between burned and unburned samples. The Open-Source software R (version 4.1.1) (https://www.r-project.org/) and the packages "factoextra" and "FactMineR" were used for this purpose.

3. Results and discussion

3.1. Faecal matter identification

Faecal compounds were identified in all archaeological samples. Table 2 and Fig. 4a show the calculated concentration of sterols and stanols and estimated concentration of bile acids. Total concentrations range between $7.44 \cdot 10^{-1}$ μg/g of dry sample and < LOQ. No steroids were observed in the control sample as expected for a natural sequence outside the *fumier* deposit and it further confirms the anthropogenic nature of the faecal matter preserved in the *fumier* sequence. Values obtained from the calculated ratios are presented in Table 3.

The proportions of sterols and stanols are reflected in Ratios I-III. Ratio I corroborates the presence of faecal matter in all of the unburned and burned *fumier* sediment samples (see Table 3) as a consequence of higher 5β-stanols concentration compared to 5α-stanols. It has been reported that 5β-stanols can also be produced naturally in sedimentary deposits without faecal input, although in smaller amounts than 5α-stanols (Birk et al., 2011). However, the micromorphological characterisation of the *fumier* further suggests that 5β-stanols preserved at
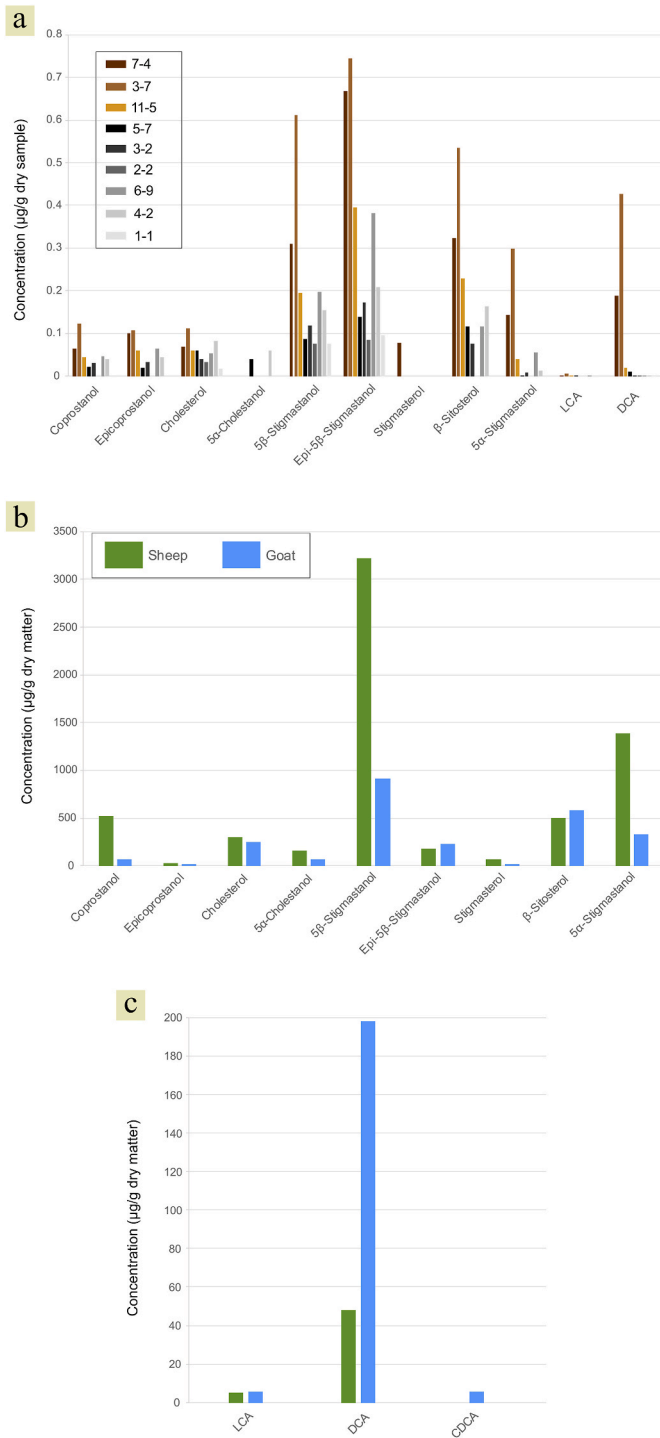
Fig. 4. a) Steroid concentrations per FM sample from Belmaco Cave *fumier*. Colours indicate MFTs: browns refer to MFT 1, blacks to MFT 2, and greys to MFT 3. b) Sterol and stanol concentrations from reference sheep and goat dung obtained by Prost et al. (2017) (see Table 2 in the referenced article). Note the difference in concentration of Epi-5β-Stigmastanol between archaeological samples from Belmaco and modern dung. c) Bile acid concentrations from reference sheep and goat dung obtained by Prost et al. (2017) (see Table 3 in the referenced article). Note higher concentrations of DCA compared to LCA, and presence of CDCA in goat.

Belmaco Cave should mainly derive from animal faecal matter (Fernández-Palacios et al., 2023). Ratios II and III also yielded informative values in all the samples regardless of thermal alteration. Ratio II indicates a clear dominance of herbivore dung in all MFTs and allows us to

discard omnivore sources. Likewise, Ratio III corroborates a ruminant source for all the samples, in accordance with the nature of the *fumier* deposit (Fernández-Palacios et al., 2023) and with Caprinae identification in the zooarchaeological record (SI Table S1). Thus, burning does not prevent us from using Ratios I – III. It is true, however, that FM 2-2 and FM 1-1 show notably different values of Ratios I – III compared to the rest of the samples due to the absence of 5α-stigmastanol and (epi) coprostanol, probably related to a higher thermal degradation degree.

To further clarify the origin of the faecal remains deposited at Belmaco Cave, we turn to the bile acids (Prost et al., 2017). The unburned dung layers (FM 7-4, FM 3–7, and FM 11-5) show informative values for Ratio IV, and these suggest presence of ruminant faecal matter. Ratio IV values from burned layers are variable: FM 5–7 (MFT 2) shows a value > 10, while FM 6–9 (MFT 3) displays a value < 10. The rest of the samples do not indicate any value due to the absence of lithocholic acid (see Table 2). Samples in which both deoxycholic acid (DCA) and lithocholic acid (LCA) have been detected, show DCA proportions ten times higher than those of LCA, which combined with our sterol and stanol analysis also supports the identification of a ruminant producer, and discards humans as potential pollutants. The only exception is FM 6–9, where DCA is only slightly higher than LCA, and cannot guarantee ruminant source based only on this ratio.

Chenodeoxycholic acid (CDCA), hyodeoxycholic acid (HDCA), and ursodeoxycholic (UDCA) were not detected in any of the samples, including the unburned sediment samples, allowing us to also rule out pig, horse, goose, and, most importantly, goat sources according to the composition of referential faecal samples analysed by Prost et al. (2017). The zooarchaeological data indicates presence of pig, but only in a low percentage from the total assemblage (see SI Table S1 & Text S1). The presence of pig bones could be related to human consumption and not pig keeping in the rockshelter. It has been suggested that prehispanic pigs might have roamed free in the laurel forest of some islands (García Morales, 1989). If this was the case also in La Palma, Belmaco Cave is located relatively far away from the laurel forests in the north and northeast of the island, hence we would not expect free roaming pigs in the vicinity of the site. Stand-alone LCA and DCA are associated with donkey, cattle and sheep (Prost et al., 2017) (see Fig. 4b and c for referential data obtained by Prost et al. for goat and sheep). Zocatelli et al. (2017) also provide referential data that shows an absence of CDCA in sheep and cattle excrements. Donkey input can also be discarded as the estimated DCA concentration is much higher than the LCA concentration (Prost et al., 2017). Finally, cattle can also be discarded due to their absence in La Palma during prehispanic times. Thus, the faecal input at Belmaco Cave appears to be indicative of sheep from a molecular perspective. We expect a good bile acid preservation at a centennial timescale (Zocatelli et al., 2017). However, caution must be given to the burned facies as their original faecal signal is likely degraded.

On the other hand, the zooarchaeological analysis based on comparative anatomy is not able to differentiate among Caprinae species, and thus, not able to rule out the presence of goat at Belmaco due to high bone fragmentation (SI Text S1). Future ZooMS analysis on the Caprinae faunal remains might prove useful to corroborate our current hypothesis.

3.2. Steroid preservation in fumiers and analytical viability

A PCA analysis was performed to explore the observed differences among MFTs at the Belmaco Cave *fumier* deposit in greater depth (Fig. 5). The explained and accumulated variance, loadings and co-ordinates are given in the Supplementary Information (SI Tables S2–5; Fig. S2). 87% of the total system variance is explained by the first two dimensions. Dimension 1 shows a thermoalteration pattern by discriminating between the unburned dung layers (MFT 1), which are characterized by the higher concentrations of steroid compounds (arrows of different colours) to its negative scores, and the carbonized (MFT 2) and dung ash (MFT 3) samples, which, together with the control

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Table 3

Values obtained from calculated Ratios I – IV per faecal biomarker sample (FM) and microfacies type (MFT). The control sample is not included because no steroids were detected.

FM	MFT	Condition	Ratio			
			Nº I	Nº II	Nº III	Nº IV
			(5β-stigmastanol + epi-5β-stigmastanol)/(5α-stigmastanol + 5β-stigmastanol + epi-5β-stigmastanol)	coprostanol/(coprostanol + 5β-stigmastanol) x 100(%)	(coprostanol + epicoprostanol)/(5β-stigmastanol + epi-5β-stigmastanol)	deoxycholic acid/ lithocholic acid
7–4	1	Unburned	0.87	17.31	0.17	110.18
6–9	3	Burned	0.91	18.84	0.19	3.62
5–7	2	Burned	0.99	19.25	0.18	28.43
4–2	3	Burned	0.96	20.24	0.23	–
3–2	2	Burned	0.97	20.8	0.22	–
3–7	1	Unburned	0.82	16.78	0.17	79.41
2–2	2	Burned	1	0	0	–
1–1	3	Burned	1	0	0	–
11–5	1	Unburned	0.94	18.25	0.17	34.72

Ratio I > 0.7 = presence of faecal matter.
0.3–0.7 = unable to determine presence or absence of faecal matter.
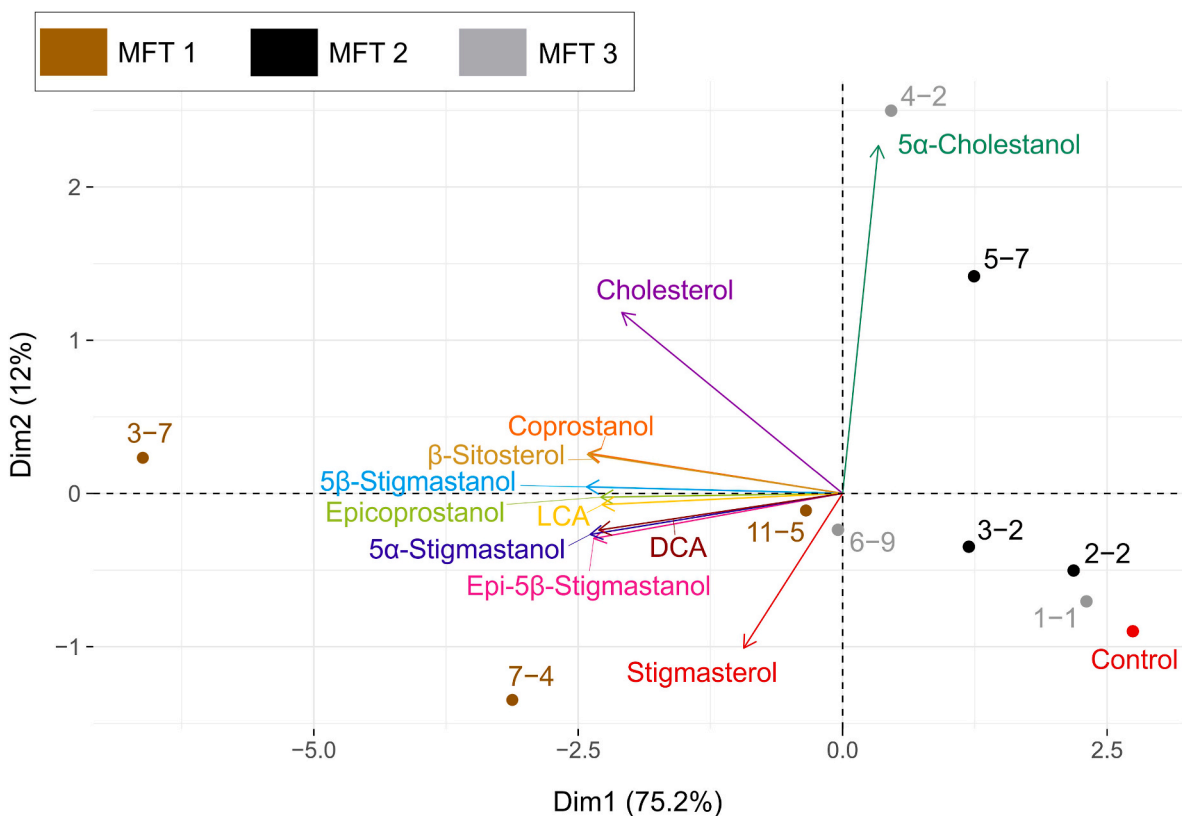< 0.3 = absence of faecal matter.
Ratio II < 29% = herbivore).
29%–65% = pig >65% = human.
Ratio III < 1 = ruminant >1 = pig/human.
Ratio IV > 10 = ruminant.
1.0–3.4 = horse.

Fig. 5. Principal Component Analysis of faecal biomarker samples from Belmaco Cave *fumier*. FM samples are coloured by MFTs. Each steroid compound also received a different colour.

sample (red), are characterized by lower concentrations or a complete absence of steroid compounds, to its positive values. Dimension 2 shows a gradient between 5a-Cholestanol (only present in FM 4-2 and FM 5–7) and Stigmasterol (only existing in FM 7-4).

We observe a general decrease in steroid concentrations affecting both the carbonized and the dung ash layers in comparison with the unburned dung layers. However, in this case, the dung ash layers

showed a total faecal biomarker concentration of nearly 2 µg/g of dry sample, while carbonized layers barely surpass 1 µg/g of dry sample (Fig. 6). A previous lipid biomarker study of Belmaco Cave focusing on the *n*-alkanes, showed a similar pattern. The dung ash layer samples yielded distinct odd-over-even distributions indicative of good lipid compound preservation (Fernández-Palacios et al., 2023). Gea et al. (2017) suggest that burned layers might absorb leaching compounds
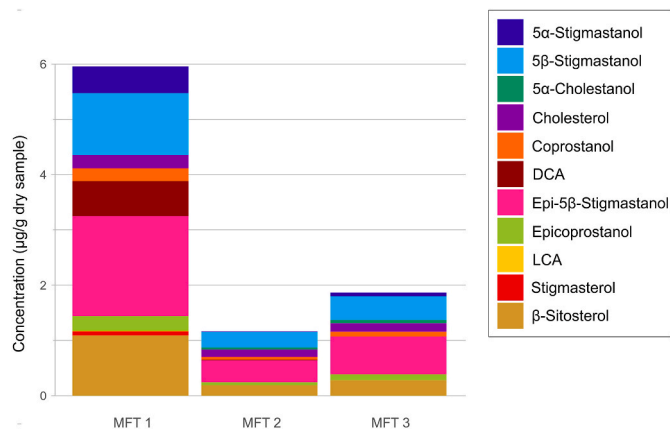
Fig. 6. Summed steroid concentration per MFT (microfacies types). MFT 1 = unburned dung layers; MFT 2 = carbonized black layers; MFT 3 = dung ash layers. Colours of steroids correspond to the colours of arrows in Fig. 5.

from unaltered or partially burned layers higher up the profile. Alternatively, the compounds could be linked to the presence of isolated fresh or charred particles within the ash layers (Jambrina-Enríquez et al., 2022), which has been documented by previous micromorphological analysis (Fernández-Palacios et al., 2023). The latter scenario could explain why samples FM 6–9 and FM 4-2 show a higher steroid summed concentration than samples belonging to MFT 2 and a similar summed concentration to FM 11-5 in MFT 1.

Steroid preservation at Belmaco Cave could be compared to the other case studies on *fumiers* to assess the effect of thermal alteration on the faecal biomolecular signal in carbonized and ashy layers and explore analytical viability for source determination. In the San Cristóbal rockshelter (Rioja Alavesa, Spain) Neolithic and Chalcolithic *fumier* deposits, black (unburned organic matter) and brown (partially burned) layers preserve sufficient traces of sterols and stanols as to identify their source as ruminant. However, most of the white (ash) layer samples did not yield any identifiable residue, due to the low concentrations of faecal biomarkers present, particularly coprostanol, epicoprostanol, and stigmastanol. DCA showed a similar pattern, appearing most depleted in the white layers. LCA appears to be more stable across the different burned facies, but consistently showed lower concentrations than DCA (Gea et al., 2017).

At El Mirador Cave (Burgos, Spain) Neolithic - Bronze Age *fumier* deposit, bile acids were well-preserved, confirming the presence of ruminants (DCA > LCA) and pigs (HDCA) across the sequence, despite the degree of burning. Unburned layers (v/vl & a) show the highest bile acid concentrations. As expected, partially burned facies (b) and charcoal-rich facies (c) also showed higher concentrations than burned white (w), grey (g) and "tutti-frutti" (tf) layers, in which these faecal compounds were most depleted (Vallejo et al., 2022a). Sterols and stanols at El Mirador also indicated a predominant ruminant source and displayed a similar pattern of bile acid degradation (Vallejo et al., 2022b). However, they also documented two exceptions: a fully burned grey facies (g) and a charred facies (c) showing β-sterol concentration similar to the unburned facies (Vallejo et al., 2022b).

3.3. Archaeological implications

The presence of sheep dung as the main source of faecal matter at Belmaco Cave is consistent with previous *n*-alkane data (Fernández-Palacios et al., 2023), which suggests a predominant herbaceous plant input. Sheep, predominantly feed on herbs and grasses (i.e. grazers), in contrast to goats, which given the choice often show a browsing behaviour (Balasse and Ambrose, 2005; Bosma and Bicaba, 1997; Negi et al., 1993; Sanon et al., 2007). Also, Belmaco Cave is an easily accessible shelter located in the southern midlands of La Palma. According to

ethnographic reports, herds dominated by sheep tend to remain in the low- and midland areas, close to the habitational spaces, due to the orographic complexity of La Palma highlands, easily traversed by more agile goats (Martín Rodríguez, 1992; Pais Pais, 1996).

On the other hand, we have documented pine needles embedded in the *fumier* carbonized and dung ash layers (Fernández-Palacios et al., 2023), which is an indication that indigenous herders occupying Belmaco visited the pine forest for litter gathering. We do not know if this mobility included the herd. However, the pine forest that dominates the Cumbre Vieja highlands (southern volcanic rift zone) at ca. 1000 m a.s. l., is more accessible than the pine forest in the northern Caldera de Taburiente, which is found at ca. 1200 m a.s.l. and displays a more abrupt terrain (Ceballos and Ortuño, 1976), making a short-range sheep transhumance more plausible in the south than in the north.

Keeping separate herds is a livestock management strategy still used today as a way of focusing on the production of particular resources, although it is unknown if this was the primary objective of indigenous herders at Belmaco.

4. Conclusions

This study has shown the potential of studying sterols/stanols and bile acids to identify possible sources of faecal residues in archaeological sediments. The faecal biomarker analysis carried out across the Belmaco Cave *fumier* deposit coupled with data from previous geoarchaeological investigations suggests that its formation was related to recurrent sheep stabling. This management strategy of separating sheep from goat herds could have been associated with the orographic complexities of La Palma highlands, which might have constrained indigenous sheep herders to stay in the low- and midlands of the island.

Concerning the preservation potential of biomolecular faecal compound in sediments affected by heat, our study indicates analytical viability in charred and ashy archaeological sediment. However, we have observed that bile acids are often not preserved in burned layers. This implies a lower resolution of faecal source identification in burned samples.

Author contributions

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.quaint.2023.08.012.

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