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RESEARCH ARTICLE

Combing through museum collections. A "museomic" application of ZooMS

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Abstract

This article presents the application of Zooarchaeology by Mass Spectrometry (ZooMS) to osseous Longobard artifacts from the collection of the "Musei Reali di Torino" (MRT; Torino, Italy). Like most archaeological items made of worked bone/antler in museum collections, the raw material of such specimens is usually attributed to deer, often without accurate taxonomic attribution. Therefore, the main aim of the present investigation was to shed light on taxonomical aspects using biomolecular approaches.

We first examined the collagen preservation of the artifacts, then we compared three sampling methods (invasive, eraser-based, and bag-based), and we evaluated the quality of the collagen fingerprint obtained. Overall, we found a good, albeit not optimal, biomolecular preservation status, even in heavily restored objects coming from the 19th-century collections.

Out of 37 specimens analyzed through matrix-assisted laser desorption ionization–time of flight–mass spectrometry (MALDI-TOF-MS) and nanoHPLC-tandem MS, 31 yielded usable data. The results confirmed the widespread use of cervid as the osseous raw material for comb-making in Longobard times in Piedmont, but we also found that bovine bones (*Bos* but also other taxa belonging to family Bovidae, such as caprines) and equid bones were exploited—demonstrating opportunistic use of animal resources. As far as the method is concerned, the ZooMS peptide markers useful to distinguish between bovids and cervids (*m/z* 1580 vs*m/z* 1550) (Buckley et al., 2009) are more frequently detected when analyzing bone chips, that is, with the invasive sampling method, rather than collagen extracts obtained using non-invasive techniques. Nonetheless, the eraser method (eZooMS) seems to be a good trade-off between invasivity and quality of the information obtained: eZooMS sampling does not leave visible marks on the object and therefore can contribute to facilitating the routine application of biomolecular methods in the daily practice of museum conservation laboratories. Indeed, an important outcome of the present study has been the establishment of a close collaboration between museum and biomolecular specialists.

Taken together, our results suggest that the Longobards had a preference towards locally available resources, although this work did not highlight a clear association between raw material (deer, cattle, and other bovids) and object typology (in the case of combs) or function, except for buttons. The overall information obtained by this study confirms the potential of biomolecular approaches for reconstructing the biography of museum objects with a long and complex life and demonstrates the value of zooarchaeological study of museum collections.

KEYWORDS
bone artifacts, combs, Longobards, nanoHPLC-tandem MS, museum collections, non-invasive collagen extraction, object biography, ZooMS

1 | INTRODUCTION

Archaeological finds made of worked osseous materials can testify to the relationships between human populations and their environment, often revealing a nuanced boundary between pragmatic and cultural choices made by an object's past crafters and users (De Marchi, 2014a, 2014b; de Vingo, 2015). Analysis of such finds often occurs several years after excavation, in which case we have to consider the effects of storage on the artifacts. We employ the framework of object biographies (Gosden & Marshall, 1999; Hoskins, 1998; Humphries & Smith, 2014) to integrate both the archaeological life of the analyzed objects and their later (and still ongoing) museum life.

In this study we focus on Longobard funerary objects made of osseous materials, which are currently stored in the Musei Reali of Torino (MRT). Longobard communities lived in Piedmont (northwestern Italy) around 575–774 CE, a period that partially overlaps with the Late Antique Little Ice Age (536–660 CE), and has been famously associated with a wide range of societal transformations at the global scale (Büntgen et al., 2016; Degroot et al., 2021). Agricultural practices, including animal management strategies, changed towards a controlled local production system (Brogiolo & Chavarría, 2020), and craft productions may have relied increasingly on local resources compared to previous late roman periods (Rottoli, 2014). Longobard crafters used bones and antlers to produce utilitarian objects, which could embody specific values for the owner, the donor, or the crafter. Items such as combs were used in everyday hygiene practices, also included in burial rites, and could therefore express social, cultural, and ideological values to past communities (Ashby, 2016; De Marchi, 2014a; Giostra, 2011, 2017). The types of animals used to create these objects, then, can have implications for understanding the value and choices of people in the Longobard communities, as well as the impact of environmental changes.

The taxonomic identification of the objects is hampered by the fact that crafting activities typically obliterate any diagnostic features that distinguish among animals. However, biomolecular methods, such as Zooarchaeology by Mass Spectrometry (ZooMS) (Buckley et al., 2009; Collins et al., 2010) can help address this issue and provide new information for old collections. The application of ZooMS to museum collections can be challenging because many of these objects have a complex biography. After their "first life" (conception, realization, use, reuse, and discard), they are subject to taphonomic processes in their depositional environment and excavation, before becoming part of museum collections, thus living a "second life." MRT acquired its first collection of Longobard funerary objects in 1884 (original documents consulted by A.M. - Anonim. 1878. Testona, Collezione Calandra, *Minuta su elenco degli oggetti barbarici*. Rep 4.7.3. Superintendence ABAP-TO Archive. Turin, Italy; Calandra & Calandra, 1885). This collection included items of worked bone, which were grouped according to their aesthetic qualities. The contextual information for these objects was extremely scarce—as it was common for late 19th century excavations. This means that any taphonomic information provided by depositional context is also missing, and assessment relies solely on the macroscopic and microscopic assessment of the objects. After this, at the beginning of the 21st century, modern stratigraphic excavations of other Longobard sites in Piedmont have yielded many other artifacts, accompanied by detailed information (Brogiolo et al., 2017) that then become part of the collection.

ZooMS is fast, cost-effective, and, when molecular preservation is optimal, can provide genus-level determination for mammalian bone and some species-level attributions (Buckley, 2018). ZooMS usually requires the direct sampling of the objects in order to obtain a small piece of bone (5–30 mg) from which collagen can be extracted and analyzed by matrix-assisted laser desorption ionization–time of flight–mass spectrometry (MALDI-TOF-MS). Museums, however, often have strict conservation policies, requiring the most minimally invasive approaches to analyze their collections. However, these techniques provide not only means of gaining taxonomic information but also insight into the molecular preservation of the item, which is useful for reconstructing the life history of an object, as well as informing curators on the best practices for certain objects.

Triboelectric-based sampling methods, also called "eZooMS" (Fiddyment et al., 2015, 2019, 2020; Teasdale et al., 2017), are based on the phenomenon that collagen fragments may spontaneously adhere to surfaces which become electrically charged with friction, such as erasers, plastic bags, and specific membrane boxes for storing significant museum artifacts. The eraser-based method is especially appropriate for the sampling of non-mineralized materials such as

parchment, although it has been applied to mineralized substrates such as ivory (Coutu et al., 2021). McGrath et al. (2019) developed a triboelectric method based on the simple "rubbing" action of a sampling bag against the object. This "bag method" has been used successfully on relatively recent bone points from 14th to 16th centuries CE Iroquois groups (McGrath et al., 2019) but did not yield results on Middle Palaeolithic Neanderthal *lissoirs* Martisius et al., 2020. More successful results on these objects were obtained sampling collagen from the membrane boxes in which the *lissoirs* were stored.

Other non-invasive or minimally invasive techniques for protein extraction from diverse substrates include the "EVA" membrane (Demarchi et al., 2020; Manfredi et al., 2017), polishing films (Evans et al., 2023) or enzyme functionalized films (Cicatiello et al., 2018; Ntasi et al., 2021), which were not tested in this study. Despite the high potential for the use of polishing films on osseous materials (Coutu et al., 2021; Evans et al., 2023), we chose not to use polishing films in consultation with the museum curator at the MRT, who deemed the removal of a tiny bone chip, which leaves a small, documented and easily traceable scalpel mark (whenever possible, this was carried out on a portion of the object which is not visible) preferable than the potential micro-alteration of a larger portion of the object.

Here we seek to complement these previous studies by employing triboelectric-based approaches alongside minimally destructive, acid-based collagen extraction to identify the most appropriate sampling method for osseous Longobard objects from museum collections. Additionally, we systematically assess the taxonomic identification of the raw materials used for the manufacture of these objects. We also developed a short method of tandem mass spectrometry in order to improve our identification of bovids and cervids. The results of this study provide the first scientific assessment of the raw material used by Longobard artisans (De Marchi, 2014b; Giostra, 2017; Walczer, 2017) and illustrate the importance of museum collections in osteo-archeological research.

We studied every single osseous Longobard item available in the MRT museum collection, which include combs, buttons, needles, semi-worked plaquettes, and osseous panels part of a composite container, previously interpreted as a reliquary made entirely of deer antler (Pantò & Pejrani, 2001; Pejrani, 2007). These objects were on display or in storage and come from five archaeological sites in Piedmont, Northern Italy (Figure 1). Provenance and descriptions of the objects are reported in Data S1.

2 | MATERIALS

Thirty-seven osseous objects from the Longobard collection were suitable for sampling, including thirteen combs from the 18th century "Calandra" excavation in Moncalieri Testona (Turin) (Pantò, 2017); one comb, two needles, one semi-worked antler plaquette, and a reliquary case excavated during the 1980s (Centallo, Cuneo) (Pantò & Pejrani, 2001; Pejrani, 2007); thirteen combs and three buttons from a 2000 to 2005 excavation (Collegno, Turin) Giostra, 2004, 2007a; Pejrani, 2017); as well as one comb from Asti (Crosetto, 2012), one from Industria (Monteu da Po, Turin) (Zanda & Pantò, 1999), and one handle from Testona Villa Lancia (Moncalieri, Turin) (Pantò et al., 2014). All items date between the 6th and the 8th centuries CE and were recovered from funerary contexts; thus, they are expected to have both functional and symbolic values Giostra, 2007b; Pejrani, 2017; de Vingo, 2015). Figure 2 illustrates select examples, whereas Figure 5 presents a visual summary of all sampled objects.

The museum's restoration laboratories archives document that the 13 combs from the Calandra excavation in Testona had been heavily restored and consolidated during the 1980s using Paraloid B72 (an ethyl methacrylate [70%] and methyl acrylate [30%] copolymer commonly used in conservation). Although Paraloid B72 does not contain proteins and thus would have not introduced exogenous

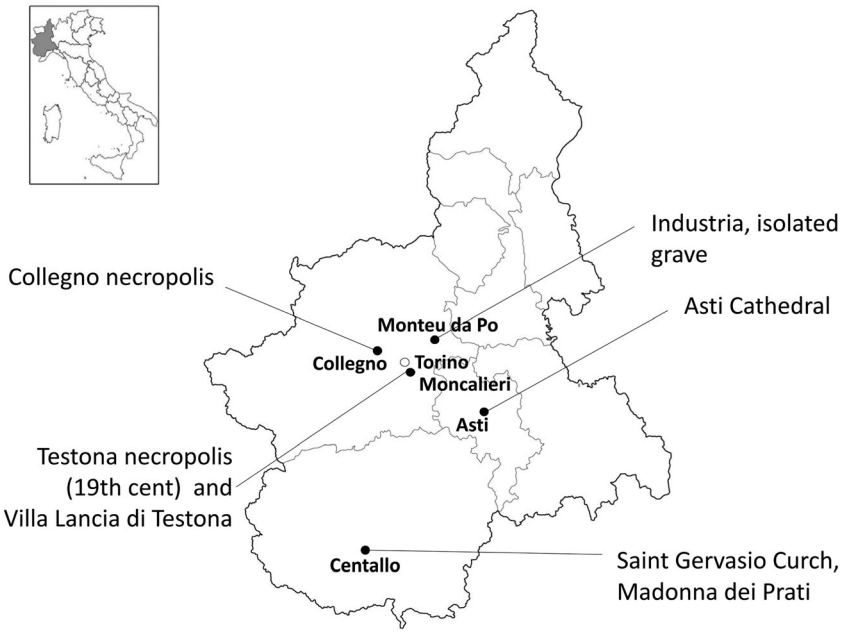

FIGURE 1 Map of the Piedmont region (Italy) with the indication of the Longobard archaeological sites which yielded the worked osseous material culture now part of the Musei Reali collections (Turin, Italy).

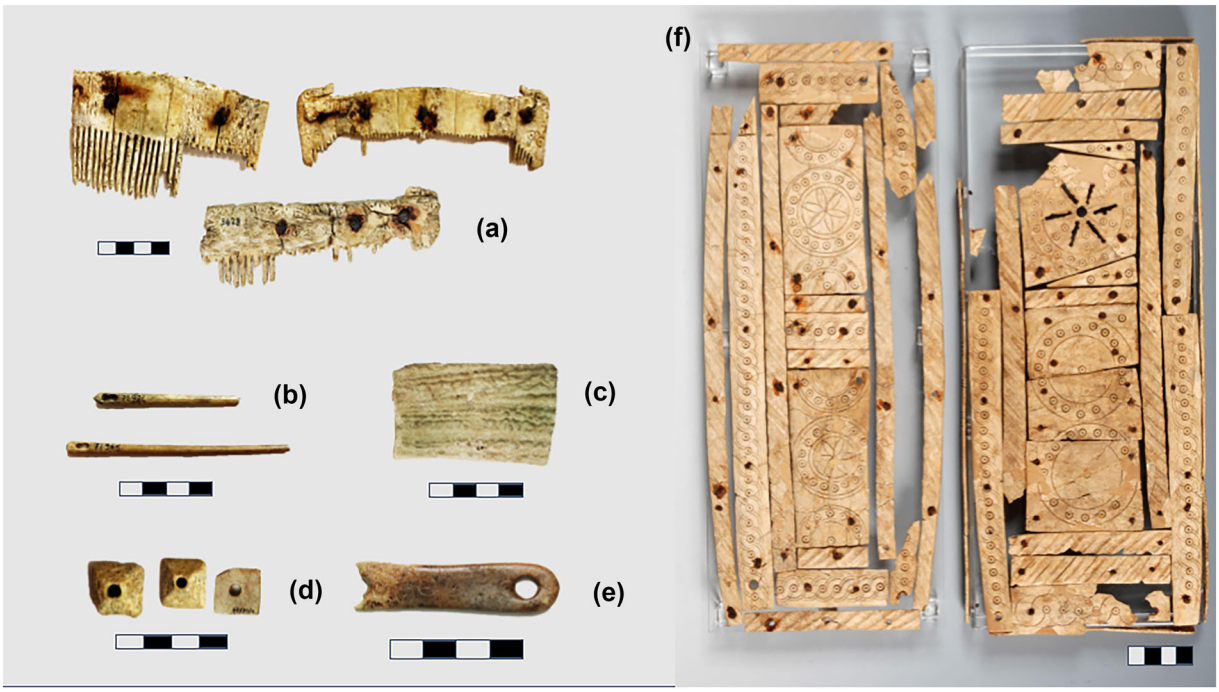

FIGURE 2 (a) Combs Inv. 5626 (left), Inv. 5625 (right) and Inv. 5628 (bottom) from the 19th century excavation of Testona; (b) needles Inv. 71974 and 71965; (c) semi-worked plaquette Inv. 71976 from Centallo; (d) buttons from the 2005 excavation of the Collegno necropolis; (e) handle from a grave in Testona Villa Lancia; and (f) the reassembled case Inv. 52573 from the Centallo 1980s excavation. [Colour figure can be viewed at wileyonlinelibrary.com]

contamination in the objects, collagen-based glues are commonly used in restoration laboratories and cross-contamination during the application is therefore a possibility (Hendy et al., 2018). These combs have been part of the permanent exhibition of the MRT since 1989 and are displayed in glass cabinets without any temperature or humidity control. The finds from Centallo and Industria have also been displayed for more than 30 years under similar conditions. Conversely, the single item from Moncalieri Villa Lancia and the better preserved finds from Collegno have been kept in museum cabinets with humidity control and low-intensity light exposure since 2013. The heavily fragmented combs from the more recent excavations in Collegno, and the single item from Asti, were kept in storage in the warehouses of MRT.

3 | METHODS

3.1 | Sampling methods

All objects were sampled using a non-invasive technique for collagen extraction, that is, the "bag" and/or the "eraser" methods. Wherever possible, a classic minimally invasive sampling approach (i.e., taking a small chip, corresponding to 1–2 mg of the osseous material) was also used to compare the suitability of non-invasive versus invasive techniques for these objects. Data S2 reports all the samples and the extraction techniques applied on each object, whereas a visual summary of the three ZooMS approaches is presented in Figure 3. Sampling was conducted in the Restoration laboratories of MRT. Samples were then transferred to the dedicated paleo-proteomics lab of the University of Turin and handled according to established guidelines (Hendy et al., 2018), including the use of laminar flow hoods and the preparation of procedural blanks. From the 37 archaeological items, a total of 92 samples were collected: on the five objects, we could use all three sampling methodologies (eraser, bag, and minimally invasive). The fragility of some of the items hampered the use of the eraser method, as we had to avoid exerting too much pressure.

3.1.1 | "Bag" method

According to McGrath et al. (2019), the triboelectric effect (friction between the sample and a plastic storage bag) can be exploited to recover sufficient collagen for further analyses. The Longobard objects, which were not on display, were stored in the museum deposits inside clean plastic bags; therefore, these objects were simply transferred to a new, clean bag and the original bag was used for collagen extraction (this is referred to as the "original bag" method). Other items were taken directly from the museum display cabinets; therefore, the "forced bag" method was tested, whereby each object was transferred into a new polyethylene bag (alimentary use, biologically clean, with zip-lock). Over a period of 4 h, each object was periodically rubbed within the bag (1 min of gentle rubbing every 15 min). After this, the objects were left in the bag overnight, and the following morning the objects were placed again in their original display

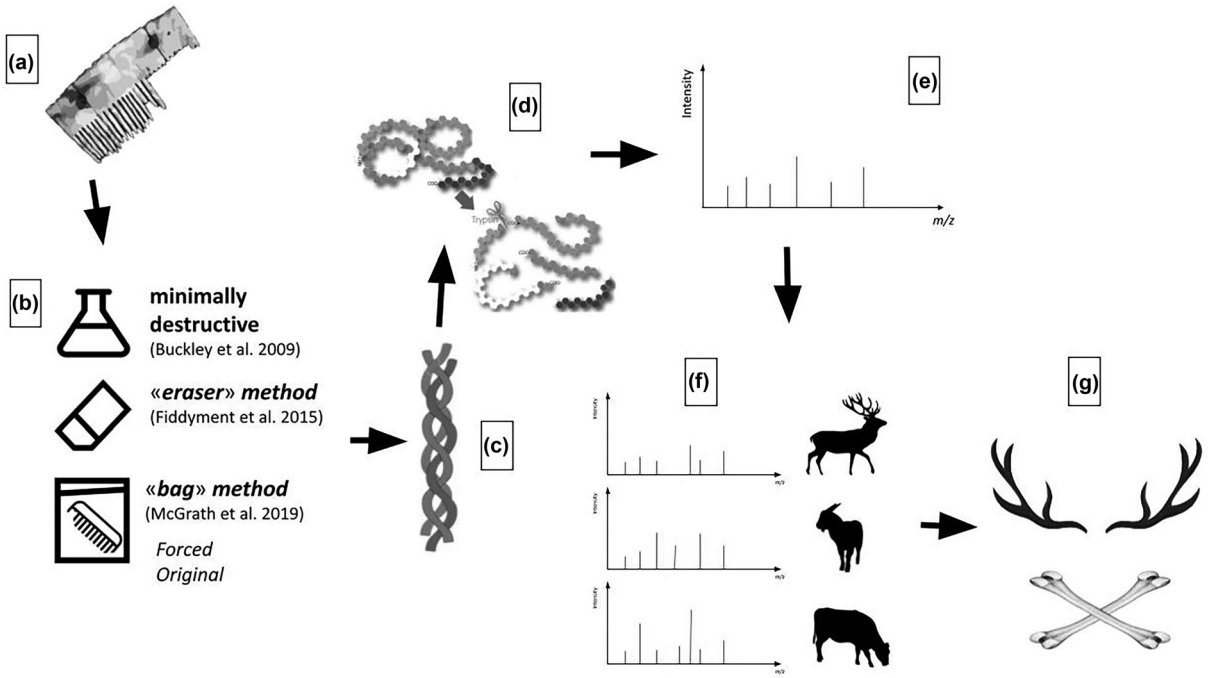

FIGURE 3 Illustration of the ZooMS method: (a) from a sample, (b) using three different sampling methods, (c) collagen is extracted. In the lab, collagen strands are digested into smaller fragments, called (d) peptides. (e) When peptides are analyzed by MALDI-TOF-MS, it is possible to identify the taxon, based on the characteristic (f) peptide mass fingerprint allowing interpretation of the raw materials used to produce the (g) archaeological objects. Credits: (a) Musei Reali pic modified by A.M.; (b) icons by E.H.; (c/d) icons modified after Collins et al., 2010; (f) icons from PhyloPic: cow by Katy Lawler, deer by Ferran Sayol, goat by Jody Taylor; and (g) from Freepic and Vecteeze).

cabinets. In the laboratory, 1 mL of 50 mM ammonium bicarbonate solution was added to each bag, and residual proteins were gelatinized by heating at 65°C for 60 min. The solution was then transferred to clean Lo-Bind microcentrifuge tubes prior to digestion.

3.1.2 | "Eraser" method

Following Fiddyment et al. (2015, 2019), a polyvinyl eraser (Faber Castell) was cut into small portions and gently rubbed on the surface of the objects, collecting the *erdu* into Lo-Bind microcentrifuge tubes. Around 500 µL of 50 mM ammonium bicarbonate solution (pH ~ 7.8) were added to each tube prior to digestion. This left no marks on the bone surface that were visible to the naked eye.

3.1.3 | Minimally invasive sampling

In some cases, the presence of loose/detached fragments allowed us to remove small chips (< 2 mg), or a small quantity of sterile powder (< 1 mg), which was scraped from less visible parts of the objects using a scalpel. Bone chips or powders were collected into clean Lo-Bind microcentrifuge tubes and further processed in the laboratory according to well-established protocols. Bone chips were treated using the method of Buckley et al. (2009), with slight modifications: samples were demineralized using 500 µL 0.6 M HCl to recover the acid soluble matrix (ASM) and the acid insoluble matrix (AIM). The AIM was gelatinized by heating in a Thermalshake (WVR) at 65°C for 60 min, and then both fractions were ultra filtered (Nanopall centrifuge filters 3KDa MWCO) in order to obtain collagen fragments, which were re-suspended in a final volume of 500 µL and 50 mM ammonium bicarbonate before digestion.

Resampling

In some instances, resampling of the object was necessary (Data S2) in order, for example, to clarify the presence of contamination sources or to establish a direct comparison between sampling methodologies.

3.2 | Analytical methods

3.2.1 | MALDI-TOF-MS

For all samples, regardless of the extraction method, digestion was carried out by adding 2 µL of a 0.5 µg/µL trypsin solution (Promega, proteomics grade) and heating the samples at 37°C overnight. Digestion was stopped by adding 10% trifluoroacetic acid (TFA) to a final TFA concentration of 0.1%. Peptide digests were then purified using C18 solid-phase extraction tips (Pierce), following the manufacturer's instructions, evaporated to dryness and stored at −18°C. Immediately before the analysis, dried peptides were re-suspended in 10 µL TFA solution (0.1%) and mixed 1:1 with α-cyano-4-hydroxycinnamic acid

matrix solution (1%, prepared in 50% acetonitrile/0.1% TFA [v/v/v]). Around 0.9 µL aliquots were spotted directly on a MBT Biotarget 96 MALDI plate (Bruker).

Peptides from each sample were spotted three times (when analyzed for the first time) or two times (when replicas), then analyzed on a bench-top Microflex LRF MALDI TOF mass spectrometer (Bruker Daltonics, Germany). Samples were analyzed in reflector mode, using the following parameter settings: ion source 1, 18.96 kV; ion source, 2 16.02 kV; lens voltage 9.05 kV; reflector 20.01 kV; and laser power 22–28%. The spectrum collected for each sample resulted from the sum of 1000 laser shots. Mass range for detection was set at 800–4000 *m/z*, and peptide masses below 650 Da were suppressed. Peptide calibration standard #8206195 (Bruker Daltonics, Germany), a mixture of seven peptides (Angiotensin II $m/z = 1046.541$, Angiotensin I $m/z = 1296.685$, Substance P $m/z = 1347.735$, Bombesin $m/z = 1619.822$, ACTH (1–17 clip) $m/z = 2093.086$, ACTH (18–39 clip) $m/z = 2465.198$, and Somatostatin $m/z = 3147.471$) was used for external mass calibration to maximize mass accuracy. Procedural blanks were included in the analysis, and a list of common contaminants/spurious peaks was derived (reported in Data S2).

Raw text files for each spectrum were exported and further analyzed using mMass, an open access mass spectrometry interpretation tool (Niedermeyer & Strohalm, 2012). All of the resulting spectra were processed by performing the baseline correction (precision: 100%, relative offset: 10–30%). Peak picking was carried out as follows: *S/N* threshold ≥3, picking height of 100%, deisotoping using standard mMass parameters (isotope mass tolerance 0.1 *m/z*; isotope intensity tolerance 50.0%, isotope mass shift 0, remove isotopes, remove unknown). In some cases, an internal calibration was also applied using known reference masses.

3.2.2 | ZooMS markers for identification

Peaks from each spectrum were compared against published publicly available lists of ZooMS marker peaks (ZooMS Markers: Published Data by Sam Prasslee. *Google Docs* [online]) to achieve taxonomic identification. Animal species unrelated to local biogeography of medieval northern Italy (e.g. African bovid taxa) were excluded, although they possess the same collagen markers of local (European) bovids (full list in the Data S1). Distinguishing between closely related taxa using collagen fingerprinting is complicated when molecular preservation is poor, and markers are not detected in the MALDI-TOF spectrum. The case of family Bovidae and Cervidae is a case in point: they share a part of their evolutionary path (Chen et al., 2019) and therefore collagen similarity is high.

Table 1 illustrates the markers currently available for separating out different taxa which we considered probable. These could include domestic animals, such as cattle (*Bos taurus*), sheep (*Ovis aries*), goats (*Capra hircus*), horse (*Equus caballus*), and donkey (*Equus asinus*). Among the wild animals, we considered auroch (*Bos primigenius*), bison (*Bison bonasus*), chamoix (*Rupicapra rupicapra*), ibex (*Capra ibex*), roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), fallow deer (*Dama dama*), elk (*Alces alces*), and reindeer (*Rangifer tarandus*).

3.2.3 | NanoHPLC-tandem MS

A short method for nanoHPLC-tandem mass spectrometry was set up in order to improve the identification rate of the samples, at least at family level (Bovidae vs Cervidae). An Ultimate 3000 HPLC instrument (Thermo Scientific, Bremen, Germany) coupled through a nanoESI source to an orbitrap Fusion TRIBRIB high-resolution mass spectrometer (Thermo Scientific, Bremen, Germany) was used for sample analysis. The chromatographic separation was achieved with a reverse phase nano column (PepMap RSLC C18, 3 µm, 100 Å, 75 µm × 15 cm, Thermo Scientific) preceded by a nano-pre-concentration column (C18 PepMap trap cartridge 100 Å, 5 µm, 0.3 mm × 5 mm; Thermo Scientific, Milan, Italy). The eluents were formic acid 0.1% aqueous solution (solvent A) and acetonitrile: formic acid 0.1% aqueous solution 80:20 (solvent B) in a gradient ramp as follows: from 5% of B maintained for 5 min (to pre-concentrate) and increased to 90% of B in 25 min. Then the column went back to the initial conditions in 1 min and reconditioned for 10 min. Flow was set to 300 nL/min; injection volume was 3 µL. The pre-concentration step was reached with 100% of trifluoroacetic acid, 0.05% in water/acetonitrile, and 98/2 at a flow rate of 5 µL/min. This was carried out in backflush mode, and the pre-concentration column went back to the initial condition 10 min before the end of the separation run.

The nano C18 column was directly connected to a nanoESI source set with the following parameters: spray positive voltage 2000 V and ion transfer tube temperature 275°C. Full scan spectra were acquired in the range of *m/z* 500–17,000 with a resolution of 60 k. A dedicated MS^2 experiment was set up for each unique peptide belonging to Bovidae or Cervidae. In particular, for Bovidae, we selected the peptide with $m/z = 790.8868$ ($z = 2$), and for Cervidae the peptide with $m/z = 775.8815$ ($z = 2$). The spectra of these two protonated molecular ions were acquired in the range of *m/z* 150–1600 with a resolution of 50 k. HCD (High Collision induced Dissociation) activation mode was selected, with a collision energy of 30% and 2 Da as isolation window.

The MS^2 of the peptides were used to evaluate the composition of the Longobard combs by comparing them with those obtained in silico using the Protein Prospector MS-Product software (v 6.3.1, University of California, San Francisco, USA; Tables S2, S3, and Figure S4).

For those samples with relatively high abundance of Bovidae and Cervidae peptides (such as PALTO 528 and PALTO 624), the coverages of *y*- and b-ions were very good (Tables S1 and S2). Thanks to the high resolving power of the Orbitrap Fusion mass spectrometer (60 K), it was possible to recognize the fragment ions. On the contrary, for samples with relatively low abundance of selected peptides,

TABLE 1 Summary of the peptide markers useful to distinguish among the most common taxa exploited as raw materials in Longobard times.

Taxonomic grouping		Peptide markers (*m/z*) (nomenclature of Brown et al., 2021)										Species included
		α1 508	α2 978	α2 978 (+16)	α2 484	α2 502	α2 292	α2 793	α2 454	α1 586	α1 586 (+16)	α2 757	α2 757 (+16)	
Bovidae	Bovinae	1105	1192	1208	1427	1580	1648	2131	2792	2853	2869	3017	3033	*Bison* sp., *Bos primigenius*, *Bos taurus*
	Caprinae with sheep and chamois	1105	1180	1196	1427	1580	1648	2131	2792	2883	2899	3017	3033	*Ovis aries* and *Rupicapra rupicapra*
	Caprinae with goat and ibex	1105	1180	1196	1427	1580	1648	2131	2792	2883	2899	3077	3093	*Capra hircus* and *Capra ibex*
Cervidae	All species except reindeer and roe deer	1105	1180	1196	1427	1550	1648	2131	2792	2883	2899	3017	3033	*Alces alces*, *Cervus elaphus*, and *Dama dama*
	Roe deer	1105	1180	1196	1427	1550	1648	2131	2792	2883	2899	3043	3059	*Capreolus capreolus*
	Caribou/reindeer	1105	1150	1166	1427	1580	1648	2131	2792	2883	2899	3077	3093	*Rangifer tarandus*
Equidae		1105	1182	1198	1427	1550	1649	2145	2820	2883	2899	2983	2999	*Equus caballus* and *Equus asinus*

the coverage of fragment ions was reduced. However, by comparing the retention time and the MS/MS of peptides, the identification was confident.

4 | RESULTS

4.1 | Selection of the sampling method

Table 2 summarizes the number of samples (including subsamples) analyzed for each sampling method and the success rate for each method (i.e., the proportion of samples that were successfully identified to taxon).

The four sampling methods, original bag, forced bag, eraser, and bone chip, varied in terms of proportion of the samples that could be identified to taxon, as well the taxonomic resolution of identifications. Peptide mass fingerprints obtained through the "original bag" sampling allowed taxonomic discrimination in only three out of 10 cases. Discrimination between bovid and cervid families was possible only in one case: sample 497 - Inv. 93801 (cervid). The other two samples (sample 515 - Collegno T 38 and sample 498 - Collegno T92) yielded markers common to *R. rupicapra*, *O. aries*, *C. hircus*, *C. ibex* (belonging to the Caprinae sub-family of the Bovidae family), as well as to *C. capreolus*, *C. elaphus*, *A. alces*, and *D. dama* (belonging to the Cervidae family). No identification was possible in any of the 10 samples acquired using the forced bag method. We are surprised that the *forced bag* method did not yield any successful results given the excellent results obtained in other studies (e.g., McGrath et al., 2019). We hypothesize that this might be because of the effect of consolidants which fixed collagen strands to the objects' surface. Conversely, the eraser method could recover these strands. The 30% success rate of the "original bag" method was also lower than expected. In this case, we are unsure if the positive results were because of the fact that the friction between sample/bag occurred over a long period, or because these samples lost micro-particles (some "dust" was visible in the bags), which contained collagen.

With regard to the *eraser method*, of the 35 samples tested, 16 failed to produce spectra with sufficient resolution to identify specimens to taxon. When only *m/z* 1105.6 and *m/z* 1427.7, were observed in the MS data, spectra were considered as unidentifiable as these markers are common to many species. Eleven samples produced spectra that provided discrimination between cervids ($n = 5$) and bovids ($n = 6$), and in a further eight samples, only markers common to both bovids and cervids were observed.

The *minimally invasive method* provided the highest proportion of identifiable spectra with the best taxonomic resolution. Only two spectra failed to provide sufficient resolution for taxonomic identification. Among the identifiable spectra, 25 provided family-level, or better, taxonomic identification of the samples, including 18 samples interpreted as Cervidae, four as Bovinae (i.e., *Bos* or *Bison*), one as *Equus* sp, and two as *Homo sapiens*. Bovidae and Cervidae could not be distinguished in 10 samples. A visual summary of the results is reported in Figure 4.

TABLE 2 Summary of the number of samples (including subsamples) analyzed for each sampling method and the success rate for each method (i.e., the proportion of samples that were successfully identified to taxon).

Method	*N* of samples	Identified to taxon	Failed	Percent identified	Percent of identifications at or below family level
Original bag	10	3	7	30%	33%
Forced bag	10	0	10	0%	-
Eraser	35	19	16	54.3%	58%
Bone chip	37	35	2	94.6%	71%

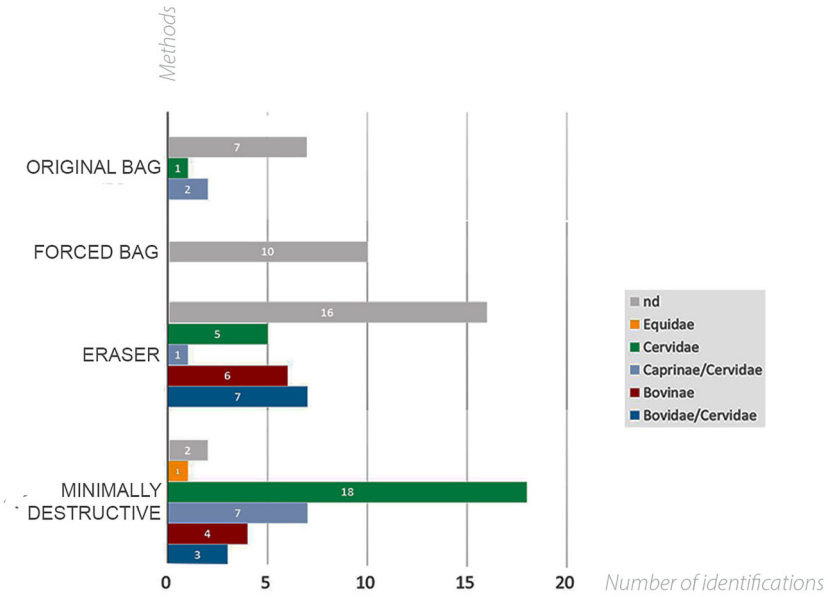

FIGURE 4 Summary of the taxonomic resolution achieved with each subsampling method. Nd means no taxonomic identification was possible. [Colour figure can be viewed at wileyonlinelibrary.com]

4.2 | Taxonomic ID of archaeological samples

As some artifacts were subsampled multiple times, the highest resolution taxonomic identification was taken as the "integrated ID" for the object (Data S2). In the following paragraph, we describe each category of objects and the materials used for their manufacture. Some authors (De Marchi, 2014b; De Vingo & Fossati, 2001; Walczer, 2017) have highlighted the abundant use of deer antler in Longobard craft manufacture, as well as the secondary and opportunistic use of bones from slaughtered animals. Given the size of these objects, we expected that large mammals such as *C. elaphus* and *B. taurus* would be the most common source of raw materials for these items. We assume that most, if not all, of the artifacts identified as bovine belong to the species *B. taurus* rather than *B. primigenius* or *B. bonasus* as the latter two species are likely to have been rare or extirpated locally (Baker, 1994; Rokosz, 1995; Rottoli, 2014). Of the 37 objects studied, all but seven were identified to taxon. A summary of the raw materials used in the manufacture of these objects is presented in Figure 5.

4.2.1 | Needles ($n = 2$)

A total of five subsamples were analyzed from two needles, employing both eraser and forced bag methods. No samples produced spectra suitable for taxonomic identification.

4.2.2 | Buttons ($n = 3$)

Three pyramidal buttons were analyzed, two from Collegno T146 and one from T53. All three were made using bovine bone, most likely *B. taurus*.

4.2.3 | Handle ($n = 1$)

The results from the analyses on the Villa Lancia handle were inconclusive as the spectra did not allow the discrimination of bovids and cervids.

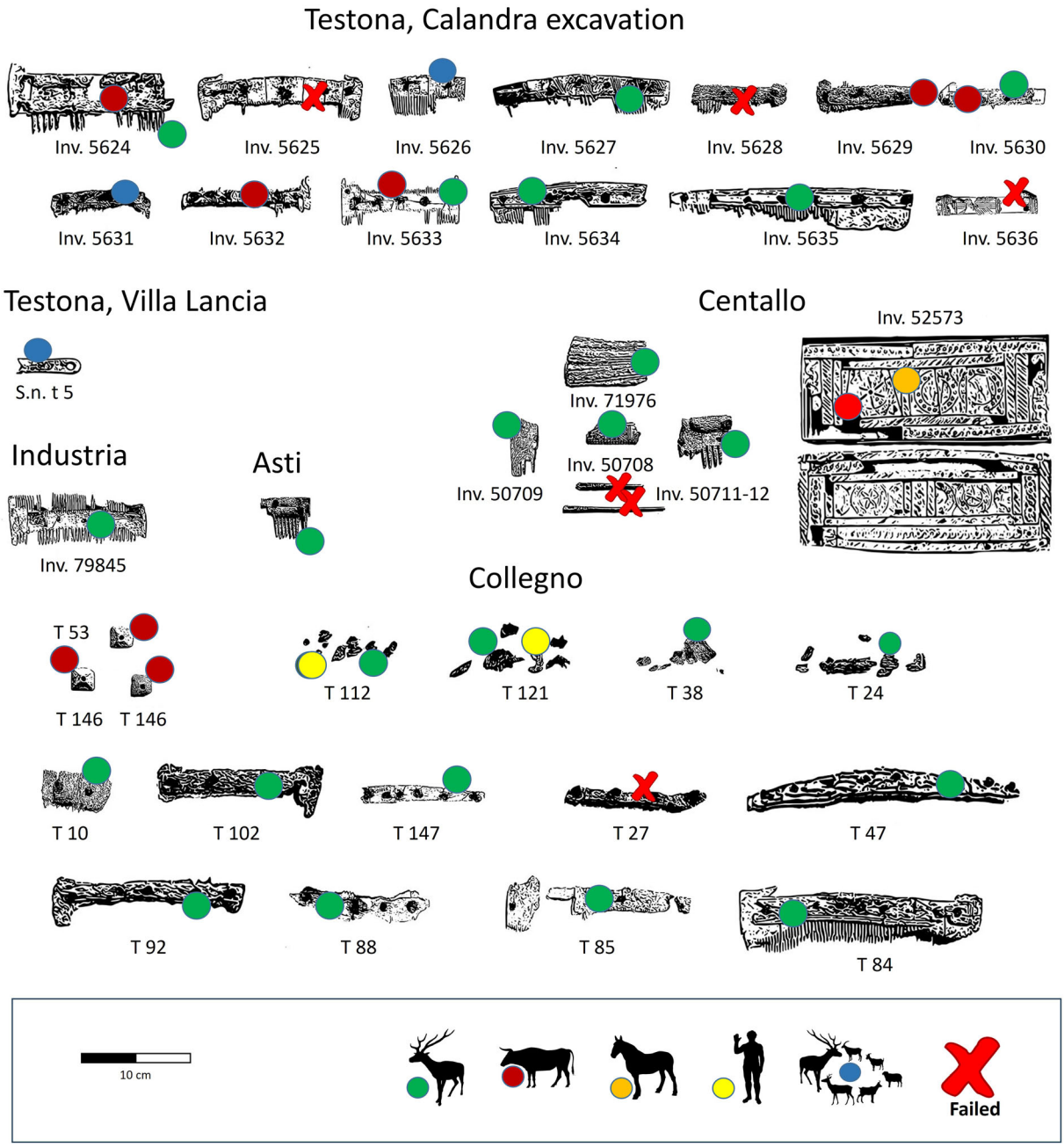

FIGURE 5 Summary of taxonomic identification of the raw materials used to manufacture the Longobard artifacts. All objects are represented to scale except Inv. 52573 which measures 33 × 13.7 × 1.5 cm. Organism silhouettes are from PhyloPic (https://www.phylopic.org/ ; T. Michael Keesey, 2023). [Colour figure can be viewed at wileyonlinelibrary.com]

4.2.4 | Semi-worked antler ($n = 1$)

The semi-worked plaquette (Inv. 71976) has clear morphological features that are typical of deer antler. Three extraction methods were tested; the eraser and the acid-extracted bone chip were both consistent with the identification of the plaquette as Cervidae, whereas the forced bag method provided no identifiable spectra.

4.2.5 | Combs ($n = 30$)

Eighteen objects (the combs from Testona, Centallo, Collegno, and Asti) were made using cervid bone and/or antler. Two combs from the Calandra Collection from Testona attest markers compatible with Bovinae (*B. taurus)* bone. Unfortunately, in three samples identified as either Cervidae or Bovidae by MALDI-MS analysis, peptides for both

Cervidae and Bovidae were identified with LC–MS/MS (Inv. 5624, Inv. 5630, and Inv. 5633). This contradictory result could be explained by the use of bovine glues (cow bone collagen) for restoring the finds. This possibility was highlighted by Ntasi et al. (2022), who assessed the proteomic signatures of common glues used in the restoration of cultural heritage materials.

An additional two samples (bone chips) yielded peaks at $m/z = 1477.7$, 2869.4, 2115.1, and 2957.4, which are specific markers of Primates, such as *Homo*, chimpanzee, and bonobo. These two samples are badly-preserved combs from graves number T112 and T121 of the Collegno necropolis (Data S1): only small fragments of the comb are preserved and the finds were identified thanks to the presence of the metal nails, which were similar to those used to produce combs. The use of human bone in past crafts has sometimes been documented (e.g., McGrath et al., 2019); therefore, we repeated the sampling and the analysis to verify the possibility that Longobard artisans may have employed human bone to craft their objects. Subsample PALTO 609 (grave T121) came from a portion of spongy bone, which was confirmed to be human by one of us (R.B.) and that was probably erroneously placed in the comb's sample bag. Conversely, subsample PALTO 508, taken from a different portion of the other object from grave T121 yielded the marker at $m/z = 1550.8$ typical of cervids. Therefore, the most probable explanation is that the previous sample refers to a part of the human skeleton present in the grave.

4.2.6 | Case from Centallo ($n = 1$)

Item Inv. 52573 is an intricate case constructed from multiple small bone plaquettes (Figures 2f and 4). During restoration, 45 distinct pieces were re-assembled to form this object. Previous scholars identified deer antler as the raw material for the plaquette based on morphological characteristics (Pejrani, 2007). In this study, one sample, PALTO 607, was taken from a plaquette with a different texture to the others, indicative of cortical bone, and was identified by proteomics as made from Bovinae raw material (probably *B. taurus*). Another sample was taken from another small plaquette that was similar in appearance and texture to the other plaquettes, and peptide markers referring to Equid were retrieved(*Equus* sp., PALTO 539).

5 | DISCUSSION

Protein identification by mass spectrometry is a valuable tool to obtain information on ancient remains and a variety of cultural heritage artifacts (Demarchi, 2023; Giuffrida et al., 2018; Warinner et al., 2022). However, one of the main challenges to obtaining high-quality biomolecular fingerprints is the preservation status of the items. Organic materials in bone degrade overtime, at various rates depending on exposure to heat, moisture, bacteria, and acidity, among other things (Hedges, 2002). These processes can continue during storage of materials after excavation. While little information about the depositional context of the Longobard objects is known, the soil from the Collegno excavations is said to be acidic (Pejrani Baricco, 2004), and the archaeological sites are situated in a moist, temperate climate.

A first interesting result of the present study was the lack of direct correlation between visually assessed preservation and the quality of the collagen fingerprint obtained. Frequently, artifacts that were considered badly preserved because of fragmentation and erosion of surface features were easily identified using ZooMS. This is despite the fact that the excavation reports of Collegno specifically mention that soil acidity had compromised the preservation of a variety of organic materials (Pejrani Baricco, 2004). A second important finding regards the impact of the restoration treatment: ZooMS yielded some high-quality data also for some of the legacy items which had been more heavily restored during the late 1980s, including consolidation using Paraloid B72 (from Testona and Centallo).

5.1 | Sampling strategies

With regard to the relationship between taxonomic assessment and sampling method, the spectra from only four out of 55 MALDI-TOF collagen samples obtained by triboelectric-based methods yielded peaks pertaining to high molecular weight (HMW) peptides (i.e., m/z 3017.4 or 3033.4), whereas HMW peptides were observed in the spectra of 34 out of 37 bone chip samples. Because HMW peptides are often more informative (Martisius et al., 2020; McGrath et al., 2019; Sinet-Mathiot et al., 2021) when trying to distinguish closely related taxa, the minimally invasive bone-chip method can provide higher taxonomic resolution. However, non-invasive methods have obvious advantages in museum settings (Evans et al., 2023; Martisius et al., 2020; McGrath et al., 2019; Ntasi et al., 2021). Among the procedures we tested, the eraser method clearly outperformed the "bag" approaches. It yielded suitable spectra in 54% of the cases, compared to only 30% of the "original bag" samples. The "forced bag" was always unsuccessful. Probably, this is because of the different time of contact between the object and the bag: the forced bag method allows only a short contact, which may not be sufficient for some objects, particularly those that have been heavily treated during restoration.

Although the eraser method does not perform as well as the minimally destructive method, sampling one item multiple times using the eraser method can improve the taxonomic resolution and reproducibility of proteomic research. For example, 11 items in this study were sampled twice using the eraser method, and for nine of these items, one of the two eraser samples produced spectra which provided a taxonomic resolution higher than the other. However, Sinet-Mathiot et al., (2021) highlighted the impact of the eraser sampling on some Palaeolithic artifacts, which produced micro-traces similar to prehistoric use-wear; therefore, proposing that the eraser method should not be considered an appropriate sampling approach for Palaeolithic bone surfaces. In our specific case, the manufacturing process used to produce Longobard combs is well known (De Marchi, 2014b, 2017; de Vingo, 2009; Giostra, 2007a; Walczer, 2017), and we ensured that

the sampling procedure was clearly detailed in the restoration report for each object. Given the results of our study, we will implement a future sampling strategy at MRT which will be preferentially based on the eraser method, with the micro-sampling being used whenever the former approach is deemed unsuitable. The eraser method seems to be a good trade-off between invasivity and quality of the information obtained.

5.2 | The importance of biomolecular methods for reconstructing the second life of objects (object biography)

The analyses performed highlighted some interesting details pertaining to the objects' biographies. Two subsamples from the case from Centallo (Inv. 53573) revealed that the case included plaquettes made with bone from cattle and horse, in addition to the plaquettes originally identified from morphological characteristics as deer antler. While one of the plaquettes was sampled because of its unique texture, the other (horse) plaquette was not clearly different to the others that composed the case, suggesting the possibility that other plaquettes may also have been made from other animal resources. That said, the small triangular shape of the two sampled plaquettes may also suggest that they could derive from the repair of the case, after its manufacture.

Traces of bovine collagen were found together with deer collagen on items from the Testona 18th century excavation (Inv. 5624, Inv. 5630, and Inv. 5633). The use of bovine glues for conservation purposes until the 1980s was frequent, their composition had been studied in other proteomic research (Ntasi et al., 2022), and indeed, their use was confirmed by the MRT restorers (personal communication). ZooMS in this case helped to reveal a part of the object's biography, which is the use of the abovementioned glues not recorded into the restoration report in the museum archive. This provides a valuable tool for reconstructing conservation practices at risk of being lost with the retirement of the museum professionals who conducted the restoration.

5.3 | When to choose a high-resolution LC–MS2 technique to distinguish between taxonomic groups

We performed tandem MS analyses on 11 samples (four eraser and seven bone chips). While ZooMS by MALDI-TOF-MS is now a well-established, straightforward, and cost-effective method for taxonomic identification, alternative tandem mass spectrometry based approaches (e.g., SPIN in Rüther et al. (2022) may provide accurate taxonomic data on protein-poor samples exploiting the higher resolving power of Orbitrap mass analyzers. Such analyses are less cost-effective and typically require more complex data analysis workflows

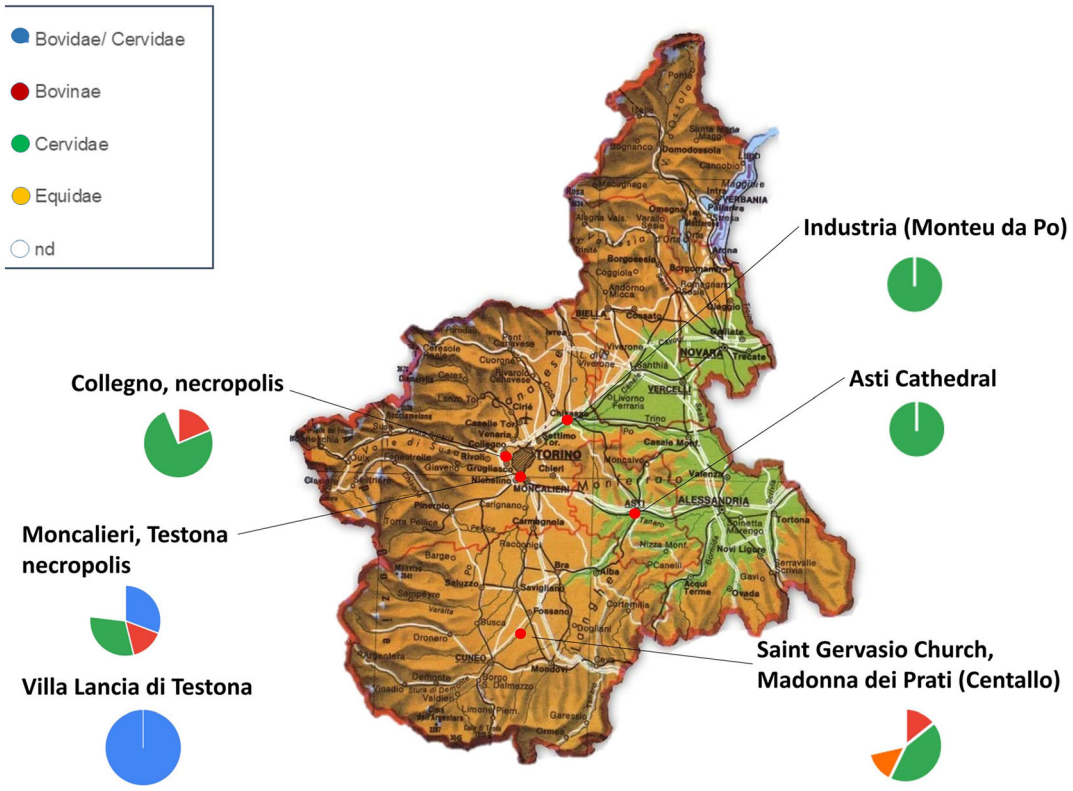

FIGURE 6 Pie chart showing the source of osseous raw materials for each site. The physical map of Piedmont highlights the mountainous setting of the region. [Colour figure can be viewed at wileyonlinelibrary.com]

than MALDI-TOF-MS. However, when characteristic taxon-specific peptides are known and a simple alternative (e.g. cervid vs bovid) is sought, then a short LC–MS/MS analysis with targeted fragmentation of desired precursor ions, such as the one used here, can provide an important complement to ZooMS-based identification.

5.4 | The use of multiple animal resources in Longobard sites

The general results of the analyses confirmed the widespread use of deer resources as frequently encountered in literature (De Marchi, 2014b; Giostra, 2004; de Vingo, 2015), but the analyses we presented may also be read in accordance with a wider use of sheep and goat after the end of the Late Roman world (Brogiolo & Chavarría, 2020; Rottoli, 2014; Salvadori, 2011) (Figure 6). Here we present some peculiars results from Collegno. The results obtained from this necropolis, in fact, highlight that Longobard artisans chose bovine bone when making buttons (small, hard, and durable), while the majority of combs were made using deer antler or bone. Collegno lies at the foot of the Susa valley and it is highly probable that migrating deer moved through that valley during the season in which they shed their antlers, thus providing local artisans with plenty of easily available raw materials. The comb from Industria, during Longobard times a control garrison located east of Turin on the river Po route, did not yield clear-cut results (Table S1), but given the results comprising *O. aries*, *R. rupicapra*, *A. alces*, *C. elaphus*, *D. dama*, we cannot discount the use of low-cost readily-available local materials (De Marchi, 2014a). The comb from Asti, coming from a church and burial context, was clearly made with deer antler. Three different fragments presumed to belong to the same comb from Centallo (Inv 50708, 50709, and 50711–12) were all identified as cervid, thus supporting the interpretation of a single object.

The consistent use of cattle bone to produce buttons and deer bone/antler for combs suggests that specific animal resources were often chosen for particular uses. One object, the case from Centallo, was composed of multiple animal resources, minimally including deer antler, cattle, and horse bone. Horses are considered to have had great symbolic importance for the Longobard people (Bedini & Petiti, 2014), and so it is possible that the inclusion of multiple animal resources was a symbolic one.

6 | CONCLUSIONS

Testing sampling methods for performing ZooMS on worked osseous materials, which can be effectively integrated in routine museum archeology procedures in a cost–benefit perspective, was one of our intended aims. This work allowed us to set some guidelines which may be useful for daily museum practice, ultimately increasing the informative potential of finds which are currently displayed and stored according to traditional typological criteria, without accounting for different aspects of their history.

With regard to the triboelectric-based collagen sampling for ZooMS, which are ideal in museum settings, only the eraser method yielded results of adequate quality, which can be improved slightly by taking multiple rubbings from the same sample. The integration of well-established MALDI-TOF-MS with a short "targeted" method of nanoHPLC-MS/MS has allowed us to achieve taxonomic identification of most of the objects analyzed. Importantly, this study has confirmed the use of different raw materials in the manufacture of Longobard combs and other grave goods, including Bovidae bones as well as deer antler. Given the geographical location of the sites, all taxa identified were likely local and the raw materials readily available. Finally, successful extraction of bone collagen suggests that future work may consider ancient DNA analysis to gather additional information about the animals used in the manufacture of these objects, and the ZooMS analysis presented here would allow curators to employ a targeted sampling strategy for future research.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Zenodo at https://zenodo.org/deposit/8099321.

OPEN RESEARCH

All MALDI-TOF data are available at the following link https://zenodo.org/deposit/8099321

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