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ORIGINAL PAPER

New insights into Neolithic milk consumption through proteomic analysis of dental calculus

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Abstract

There has long been debate over the origins of dairy consumption within European populations. Whilst it was previously assumed that lactase persistence (LP) was under positive selection following the advent of agriculture, recent genetic studies of prehistoric human remains have revealed LP may have only emerged in Europe in the last 4000 years. These findings stand in contrast to organic residue analysis of Neolithic pottery indicating the utilisation of dairy products, and zooarchaeological mortality profiles consistent with dairying herds at Neolithic sites. The recent discovery of the milk protein β-lactoglobulin (BLG) within human dental calculus presents a new method via which to explore dairy product consumption in the archaeological past. Here, we apply shotgun proteomic analysis to dental calculus samples from three British Neolithic sites, revealing the earliest identification of BLG in human dental calculus to date. The presence of BLG peptides in individuals who are unlikely to possess LP provides new insight into dairying in the British Neolithic, suggesting the potential processing of milk by Neolithic populations to reduce the lactose content of dairy products.

Keywords Dairying · β-lactoglobulin · Dental calculus · Neolithic · Britain

Introduction

The British Neolithic is a period which has long been characterised by the arrival and adoption of agriculture and perceived associated sedentary lifestyle, alongside the emergence of new forms of material culture and the construction of a range of monumental forms. Most recently, more support for the hypothesis that this 'Neolithic package' was brought to Britain by incoming European farmers has been recognised (Brace et al. 2019). Importantly, the Neolithic heralds a significant shift in subsistence, characterised by the introduction of domesticates into Britain, which included emmer wheat (*Triticum dicoccum*), einkorn wheat (*Triticum momococcum*) and barley (*Hordeum vulgare*), alongside emerging animal husbandry of domesticated species—predominately cattle, pig, sheep and goats (Thomas 1999; Rowley-Conwy 2004; Brown 2007). Our understanding of Neolithic subsistence has been obtained from a range of archaeological evidence, such as the faunal remains of domesticated animals (e.g., Albarella and Serjeantson 2002; Mulville and Grigson 2007), evidence of cereal cultivation—including carbonised cereal grains (e.g., Robinson 2000; Jones and Legge 2008), stable isotope analysis of skeletal material (e.g., Richards and Hedges 1999; Hedges et al. 2006; Richards 2008; Stevens et al. 2012; Schulting 2013), and organic residue analysis of pottery (e.g., Copley et al. 2008; Craig et al. 2015). Nevertheless, whilst we know that British Neolithic diets appear to be (isotopically) dominated by C3 terrestrial plant resources and terrestrial animals, with little to no marine input,

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there are still aspects of Neolithic subsistence which remain unclear. The contribution of wild plants, cereals, dairy and aquatic foodstuffs to the diet have all been debated (e.g., Robinson 2000; Thomas 2003; Richards et al. 2003; Milner et al. 2004; Rowley-Conwy 2004), and the contribution of crop-derived protein to Neolithic diets has also been suggested to typically be underestimated (Bogaard et al. 2013). The potential variability or homogeneity within British Neolithic diets is also still not fully understood.

Dental calculus, the mineralised bacterial biofilm of dental plaque, is consistently found within Neolithic skeletal assemblages and is now known to be a rich source of ancient biomolecules, notably ancient DNA and proteins (Warinner et al. 2014a, b), which can provide new insights not only into the oral microbiome but also past diet. Metaproteomic analysis of Neolithic dental calculus may therefore provide a new approach to early prehistoric dietary reconstruction, by providing direct and diagnostic evidence of consumed foodstuffs, including the consumption of milk or dairy products. Here we apply metaproteomic analysis to human dental calculus samples from three British Neolithic sites to assess the prevalence of dietary and milk proteins, and its efficacy as a method via which to increase our understanding of Neolithic diets. To date, the calculus from only a single Middle Neolithic individual has yet been analysed using metaproteomic methods (Mays et al. 2018). To our knowledge, this study represents the oldest human dental calculus samples analysed proteomically to date globally.

Dietary proteins within dental calculus

The periodic mineralisation of dental plaque to form calculus serendipitously entraps and preserves ancient biomolecules and microdebris, providing a direct record of consumed foodstuffs. A small number of studies have now explored dietary proteins preserved within human dental calculus. To date, evidence of putative plant proteins originating from oats (*Avena sativa*), peas (*Pisum sativum*) and Brassicaceae in archaeological dental calculus have been recovered, as well as a putative faunal blood haemoglobin (HBB) protein, and the milk proteins lactoperoxidase (LPO) and α-S1-casein (CSN1S1) (Hendy et al. 2018a; Jersie-Christensen et al. 2018; Mays et al. 2018). The detection of these proteins showcases how metaproteomic analysis can provide a new direct way of determining dietary information from the archaeological past. The most frequently detected dietary protein within metaproteomic studies of dental calculus has however thus far been the milk protein β-lactoglobulin (BLG).

Lactase persistence and the origins of milk consumption

Within the archaeological literature, there has long been a debate over the origins of milk drinking and dairy product consumption (Leonardi et al. 2012). Milk is a significant nutritional resource, containing fats, sugars and proteins, as well as vitamins, minerals and essential amino acids (Bovenhuis et al. 2013). Lactose, a type of disaccharide sugar, is the main carbohydrate in milk, comprising 3.8–5.3% of total content. In order for humans to digest lactose however, it must be broken down by the β-galactosidase lactase-phloritzin hydrolase (more commonly known simply as 'lactase'; EC 3.2.1.108), which allows it to be absorbed within the intestine (Luinge et al. 1993; Vesa et al. 2000; Ségurel and Bon 2017). As infants, humans have the ability to break down lactose. However, after weaning, the body naturally stops producing lactase, unless the individual has a genetic mutation which allows for its continued production into adulthood—known as lactase persistence (LP). Indeed, LP is thought to be the clearest example of gene-culture coevolution—the idea that cultural practices can alter the genome (Bersaglieri et al. 2004; Leonardi et al. 2012). Currently, around one third of the global population carry an LP mutation, with the highest frequencies (over 75% of the population) being found in Europe, East Africa, West Africa and the Middle East (Ségurel and Bon 2017), the same areas of the globe which have experienced a long history of dairying.

Previously, it has been suggested that the ability to digest raw milk must have provided a selective advantage and that LP would have been under positive selection following the advent of cattle, sheep and goat management and domestication (Cavalli-Sforza 1973; Beja-Pereira et al. 2003; Nielsen et al. 2007). It has generally been assumed that European Neolithic populations began dairying—and therefore also consuming milk products—soon after the introduction of agriculture into the region. These ideas have been traditionally supported by a number of lines of archaeological evidence, particularly organic residue analysis of Neolithic pottery showing the presence of milk lipids (e.g., Copley et al. 2003, 2005a, b; Craig et al. 2005; Salque et al. 2012, 2013; Cramp et al. 2014a, b; Smyth and Evershed 2015), and zooarchaeological analyses and mortality profiling of domesticated fauna (e.g., Legge 2005; Mulville et al. 2005; Vigne 2008; Greenfield and Arnold 2015). In almost all cases, these studies show that dairying was practised as soon as domesticated ruminants were available, i.e. at the start of the Early Neolithic period.

It was therefore commonly assumed that increased reliance on dairy products from the Early Neolithic would have driven the selection of LP during subsequent millennia. At least five different allelic variants have been identified as causative of LP globally, suggesting

convergent evolution (Tishkoff et al. 2007; Jones et al. 2015; Liebert et al. 2017). Initial investigations of the European LCT gene responsible for LP (C-13910 > T; −13910*T allele (rs4988235)) suggested that selection favouring the LP allele may have begun in the Neolithic in conjunction with the spread of agriculture and increased use of domesticated animal species. These early studies using statistical approaches via simulation modelling (e.g., Gerbault et al. 2009; Itan et al. 2009), modern microsatellite diversity studies (e.g., Coelho et al. 2005) or modern allele frequencies and extended haplotypes (e.g., Bersaglieri et al. 2004; Myles et al. 2005) suggested that the LCT gene most likely emerged in Europe with the Neolithic transition (Bersaglieri et al. 2004; Coelho et al. 2005; Gerbault et al. 2011).

Advances in next-generation sequencing technologies and the emergence of full genome characterisation of archaeological samples however now suggest that LP may not have been prevalent in the Neolithic, but instead present only in very low frequencies across the population, if present at all. The absence of LP in European Neolithic populations has now been noted in a number of studies (e.g., Burger et al. 2007; Gamba et al. 2014; Witas et al. 2015; Olalde et al. 2018; Brace et al. 2019), and Allentoft et al. (2015) suggest that LP had a very low frequency (5–10%) even in Bronze Age European populations, indicating that LP is the result of more recent positive selection. Similarly, a large-scale study by Mathieson et al. (2015) suggests that LP in Europe only became under strong selection within the last 4000 years. Most recently, none of the 67 British Neolithic individuals analysed in Brace et al. (2019) exhibited LP.

The current genetic evidence, therefore, suggests that European Neolithic populations did not possess LP, and as such, could not have easily digested large quantities of unprocessed dairy products or raw milk. Together, the combined genetic, organic residue analysis and zooarchaeological evidence suggests that whilst people were likely practising dairying in the Neolithic, that either (1) these products served a non-dietary function (i.e., were not being consumed); (2) that dairy products must have been consumed in very small quantities; or (3) were alternatively processed in such a way as to remove the lactose (e.g., through the production of cheeses or other processed dairy products).

The recent discovery of the preservation of the milk protein β-lactoglobulin (BLG) in human dental calculus (Warinner et al. 2014a) provides a method for detecting milk consumption in the archaeological past. Composed of 162 amino acid residues, BLG is the dominant protein within the whey fraction of milk, belonging to the lipocalin family of proteins (Kontopidis et al. 2004; Wal 2004; Monaci et al. 2006). The amino acid sequence of BLG differs between species, meaning it can be used as a species-specific indicator (Kontopidis et al. 2004), but is crucially not found within human milk (Crittenden and Bennett 2005; Restani et al. 2009), thereby excluding a host origin when detected in human dental calculus. BLG is only found in milk, making it a 'tissue'-specific biomarker, and it is more resistant to microbial attack and enzymatic degradation than other milk proteins (Warinner et al. 2014a). Finally, it has been proposed that BLG could be used as a proxy for lactose, given that they partition together in the whey fraction of milk during processing (Warinner et al. 2014a). If correct, the presence of BLG in Neolithic calculus may provide an additional source of information on Neolithic dairy consumption and the origins of raw milk consumption. Although the presence of BLG in dental calculus has been explored in samples dating back to the Bronze Age (Warinner et al. 2014a) and a single individual from the Middle Neolithic (Mays et al. 2018), applications of metaproteomic analyses to early prehistoric calculus have not yet been widely explored.

Here we present the results of shotgun proteomic analysis by liquid chromatography-tandem mass spectrometry (LC/MS-MS) on human dental calculus samples from three British Neolithic sites (Hambledon Hill, Hazleton North and Banbury Lane; Fig. 1), to explore the presence and prevalence of dietary proteins more generally, and milk proteins specifically, within Neolithic populations. Sites chosen for the analysis date to the Early and Middle Neolithic, a period during which there is extensive evidence of domesticated fauna in the UK and of the utilisation of dairy. The three sites represent a range of site types, for which we have varying amounts of palaeodietary information, and which show differing degrees of evidence for dairying at the site level. Based on recent genetic analyses, individuals from all three sites are unlikely to have had LP (Brace et al. 2019). The goal of the analysis was to explore the presence of dietary proteins within calculus of an Early and Middle Neolithic date in the UK. Furthermore, if milk proteins were detected within the samples, we hoped to further explore the dichotomy in the British archaeological record between the apparent usage of dairy through material culture evidence and zooarchaeological remains, and the seeming absence or very low genetic prevalence of LP.

Materials and methods

Ten dental calculus samples from three British Neolithic sites were analysed using a previously published shotgun metaproteomic approach utilising liquid chromatography-tandem mass spectrometry (LC/MS-MS) (Warinner et al. 2014a, b) (Table 1; Supplementary Information). The ten dental calculus samples analysed comprised of three individuals from the site of Hambledon Hill, five individuals from Hazleton North, and two individuals from Banbury Lane (Fig. 1). Hambledon Hill is an early Neolithic monument

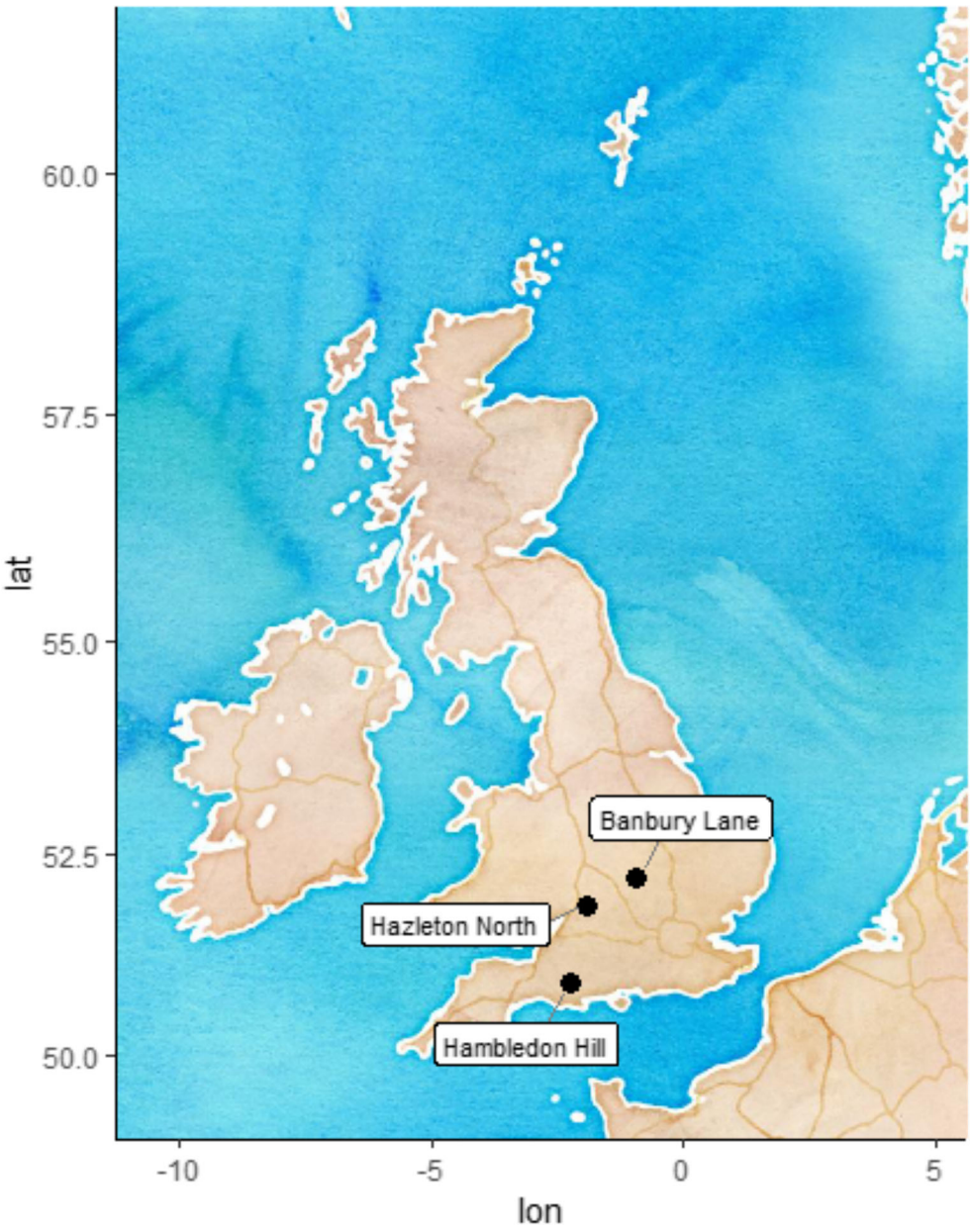

Fig. 1 Site locations of dental calculus samples analysed (map created by the authors using the R package ggmap (Kahle and Wickham 2013))

complex in Dorset, comprising of two causewayed enclosures, two long barrows and a range of other outer earthworks. The sampled individuals were recovered from both the main enclosure, dated to 3680–3310 cal. BC, and the second, smaller causewayed enclosure (known as the Stepleton enclosure), which is of a similar date, 3650–3370 cal. BC (Mercer and Healy 2008) (see Supplementary Information, Table S1). Hazleton North is a Cotswold-Severn chambered tomb near Cheltenham, Gloucestershire, dating to c.3800–3620 cal. BC (Saville et al. 1987; Meadows et al. 2007). The five individuals sampled for dental calculus here come from a number of different areas within the trapezoidal lateral long cairn (see Supplementary Information, Table S2). Finally, Banbury Lane is a Middle Neolithic triple-ditched monument, located in Northampton (Holmes 2012). The individuals sampled within this study all derive from a large pit of disarticulated human remains dug into the entrance to the innermost ditch of the monument, which has been dated to c.3360–3090 cal. BC (Holmes 2012) (see Supplementary Information, Table S3). All individuals analysed from the three sites were adults, apart from one individual from Hambledon Hill identified as infant/juvenile (HH74 HB3/ HH3) by McKinley (2008) (Table 1 and Tables S1–S3). Furthermore, the majority of the skeletal material sampled was disarticulated or fragmentary, although one articulated skeleton from both Hambledon Hill and Hazleton North respectively was analysed (Tables S1 and S2). Due to this, few of the individuals included within this study could be osteologically sexed. Although not all of the 10 individuals

Table 1 Samples utilised for proteomic analysis. Lab codes detailed here were created by the author for ease of analysis. Osteological information was obtained from McKinley (2008), Rogers (1990) and Caffell and Holst (2012)

Site	Finds number	Lab code (this study)	Age	Sex	Calculus location	Weight used/mg
Hambledon Hill	HH74 HB3	HH3	Infant/juvenile	Unknown	Maxillary left M2	15.4
Hambledon Hill	HH77 610	HH610	Older adult	Unknown	Mandibular left I2	15.3
Hambledon Hill	ST81 3188	HH3188	Mature adult	Female	Mandibular left M3	17.3
Hazleton North	HN81 4786	HN4786	25–35 years	Unknown	Maxillary right I2	17.9
Hazleton North	HN81 5037-1 (skeleton 1)	HN5037-1	33–45 years	Male	Mandibular right M3	21.6
Hazleton North	HN81 7387	HN7387		Male	Mandibular left I2	33.3
Hazleton North	HBG81 7656	HN7656	25–35 years	Unknown	Mandibular left PM1 and PM2 (combined)	18.0
Hazleton North	HN82 11456	HN11456	35–45 years	Unknown	Mandibular right M2	23.1
Banbury Lane	BL132.17	BL132.17	Adolescent/young adult	Unknown	Maxillary left M1	2.5
Banbury Lane	BL309.12	BL309.12	26–35 years	Unknown	Maxillary left M1	14.7

have been directly AMS dated, they all come from secure and well-dated archaeological contexts. Additional information on each of the individuals studied can be found in the Supplementary Information (Tables S1–S3).

Dental calculus was removed from archaeological specimens using a sterile dental scaler and stored in sterile 2.0-mL tubes. Peptides were extracted from decalcified dental calculus using a filter-aided sample preparation (FASP) protocol modified for degraded samples (Cappellini et al. 2014) according to previously published protocols (Warinner et al. 2014a, b). The extracted peptides were then analysed using shotgun protein tandem mass spectrometry (MS/MS) at the Central Proteomics Facility, Target Discovery Institute, Oxford on a Q-Exactive tandem mass spectrometer (see Supplementary Information).

Data analysis

Raw tandem mass spectra were converted to searchable Mascot generic format using Proteowizard version 3.0.7518 using the 200 most intense peaks in each mass spectrum. MS/MS ion database searching was performed on Mascot (Matrix Science™, version 2.5.1 (Perkins et al. 1999)), against all available sequences in UniProt and the Human Oral Microbiome Database (HOMD) (as in Warinner et al. 2014a). Searches were run twice, first as tryptic with one missed cleavage and a peptide tolerance of 10 ppm (Table S6), and then as semi-tryptic allowing up to two missed cleavages and a peptide tolerance of 10 ppm (Table 2). For both searches, MS/MS ion tolerance was set to 0.07 Da. Post-translational modifications were set as carbamidomethylation (fixed modification), and acetyl (protein N-term), deamidated (NQ), glutamine to pyroglutamate, methionine oxidation and hydroxylation of proline (variable modifications). Searches were performed against a decoy database to estimate protein false discovery rates. Mascot search results were filtered to a false discovery rate of less than 5% and an ion score of > 25. Searches for dietary proteins were conducted based on the strategy reported in Hendy et al. (2018b). First, the protein results were filtered to include only those proteins that were represented by at least two peptides, before assigning Mascot protein family identifications to broad taxonomic classifications of mammalian, plant or microbial sources. A conservative approach was taken, and any protein identified in our blank controls or injection blanks was assigned to the 'contaminant' category; these were principally derived from trypsin, human collagens and human keratins. For all putative dietary derived proteins, BLASTp was used to verify all peptide matches, and taxonomic assignment is reported based on the consensus peptide assignments for each individual (see Supplementary Information).

To validate whether the identified dietary proteins were derived from ancient endogenous proteins as opposed to modern contaminants, levels of deamidation were assessed following the method proposed in Mackie et al. (2018). The raw MS/MS files were run on MaxQuant (Cox and Mann 2008) version 1.6.2.6a against bovid β-lactoglobulin, as well as against a custom database including bovid and horse milk proteins, human salivary proteins, and common laboratory contaminant proteins (Supplementary Information, SI File 1). Bulk deamidation rates were assessed in the identified peptides using intensity-based calculations—for example, if a protein is 2% deamidated, this means that, for its summed intensities, only 2% of the total sum relates to peptides with deamidated residues. All parameters were default, apart from using a semi-tryptic search strategy with dependent peptides enabled, and the score cut-off for modified and unmodified peptides was set to 60. Carbamidomethyl (C) was added as a fixed modification, whilst variable modifications included oxidation (M), acetyl (protein N-term), deamidation (NQ), Gln->pyro-Glu, Glu->pyro-Glu and hydroxyproline.

Table 2 BLG peptides identified within dental calculus samples. All sequences have been verified for specificity by conducting a protein BLAST (blastp) search against the NCBI nr database, and sequences that uniquely match BLG are marked with an asterisk (*). Only samples with at least one spectrum uniquely matching BLG are considered BLG+. This excludes spectra with a Mascot ion score < 25. Peptides identified more than once are followed by parentheses indicating the total number of observations within tryptic (T) and semi-tryptic (ST) searches. Italicised samples represent those represented by only a single peptide. Where peptides were observed in tryptic and semi-tryptic searches, only the semi-tryptic MASCOT score is presented (tryptic peptide MASCOT scores are presented in Table S6). Species identification indicated with a caret symbol (^) is done so as among Bovidae, Bovinae (cattle, yak and buffalo) are distinguished from Caprinae (sheep and goats) by N → D at residue 71. However, because N deamidation to D is a common post-mortem modification, it is uncertain if the D at this residue is authentic or a damage artefact, and therefore species identification can only be to the level of Bovidae. Similarly, species identification indicated with a number sign (#) is done so as among Bovidae, Bovinae (cattle, yak and buffalo) are distinguished from Caprinae (sheep and goats) by N → D at residue 149. However, because N deamidation to D is a common post-mortem modification, it is uncertain if the D at this residue is authentic or a damage artefact, and therefore species identification can only be to the level of Bovidae

Sample ID	Total spectra aligned to BLG	Identified peptide sequences (no. of peptides and search strategy)	Modifications	Mascot ion score	Peptide taxonomic assignment	Protein taxonomic assignment
HH3	*1*	*R.VYVEELKPTPEGDLEIL.L (1 ST)*^*		*42*	*Bovidae*	*Bovidae*
HH610	36	A.MAASDISLLDAQSAPLR.V (2 ST)*	Deamidated (NQ), Oxidation (M)	71, 51	Pecora	Bovidae
		M.AASDISLLDAQSAPLR.V (2 ST)*	Deamidated (NQ)	73, 71	Pecora	
		A.SDISLLDAQSAPLR.V (2 ST)*	Deamidated (NQ)	56, 60	Pecora	
		S.DISLLDAQSAPLR.V* (1 ST)	Deamidated (NQ)	62	Pecora	
		R.VYVEELKPTPEGNLEIL.L (4 ST)*^	Deamidated (NQ)	52, 39, 45, 43	Bovidae (Bovinae?)	
		Q.KWENGECAQK.K* (1 ST)	2 Deamidated (NQ)	47	Bovidae	
		F.KIDALNENK.V* (1 ST)	Deamidated (NQ)	71	Pecora	
		K.VLVLDTDYK.K (2 T, 2 ST)*		69, 68	Pecora	
		K.VLVLDTDYKK.Y (2 T, 2 ST)*		45, 49	Pecora	
		R.TPEVDNEALEK.F (5 T, 3 ST)*#	Deamidated (NQ)	49, 58, 77	Bovidae, but not Capra	
		R.TPEVDNEALEKF.D (8 ST)*#	Deamidated (NQ)	57, 54, 58, 53, 39, 57, 55, 48	Bovidae, but not Capra	
		R.TPEVDNEALEKFDK.A (5 T, 3 ST)*#	Deamidated (NQ)	41, 55, 75	Bovidae, but not Capra	
		R.TPEVDKEALEK.F (1 T)		50	*Capra* sp.	
HN7387	12	A.MAASDISLLDAQSAPLR.V (1 ST)*	Deamidated (NQ), Oxidation (M)	92	Pecora	Bovidae
		A.SDISLLDAQSAPLR.V (1 ST)*		65	Pecora	
		K.VLVLDTDYKK.Y (1 T, 1 ST)*		62	Pecora	
		R.TPEVDDEALEK.F (3 T, 1 ST)*#		61	Bovidae, but not Capra	
		R.TPEVDDEALEKF.D (2 ST)*#		54, 52	Bovidae, but not Capra	
		R.TPEVDDEALEKFDK.A (4 T, 1 ST)*#		70	Bovidae, but not Capra	
HN7656	*1*	*R.TPEVDDEALEK.F (1 T, 1 ST)*#*		*69*	*Bovidae, but not Capra*	*Bovidae*
HN11456	5	R.TPEVDDEALEK.F (2 T, 2 ST)*#		62, 68	Bovidae, but not Capra	Bovidae
		R.TPEVDDEALEKF.D (3 ST)*#		52, 60, 59	Bovidae, but not Capra	
HN4786	6	R.TPEVDNEALEK.F (5 T, 5 ST)*#	Deamidated (NQ)	61, 60, 52, 51, 45	Bovidae, but not Capra	Bovidae
		R.TPEVDNEALEKFDK.A (1 T, 1 ST)*#	Deamidated (NQ)	70	Bovidae, but not Capra	
BL309.12	2	R.TPEVDNEALEK.F (2 T)*#	Deamidated (NQ)	45, 56	Bovidae, but not Capra	Bovidae

Contamination controls

It is important to monitor and test for contamination because bovine proteins are used in some proteomics laboratories as instrument standards (e.g. bovine fetuin), among other purposes. Precautionary measures were taken to reduce the likelihood of contamination from modern protein sources and ensure that the reported proteins were endogenous to the dental calculus (Hendy et al. 2018b), including: (1) the use of a dedicated ancient protein laboratory where modern proteins are not extracted or analysed; (2) the inclusion of blank controls analysed in parallel with the experimental samples to monitor for contamination during protein extraction; (3) the inclusion of injection blanks between each dental calculus sample to monitor for protein carryover during LC-MS/MS analysis; and (4) the analysis of bulk deamidation rates in observed dietary peptides using intensity-based calculations. No milk or other dietary proteins were detected in extraction blanks or injection blanks in this study.

Results and discussion

Proteins were successfully recovered from all 10 of the dental calculus samples analysed, with total protein identifications ranging from 15 to 128 (following quality filtering) (Table S5). The identified proteins were assigned primarily to the human proteome and to microbial taxa commonly found within the oral microbiome, as well as to common contaminants identified in previous dental calculus proteomic analyses (principally trypsin, human keratins and human collagens) (Hendy et al. 2018a; Mackie et al. 2017; Warinner et al. 2014b) (Fig. S1). The only dietary protein identified within the sample set was BLG. The initial Mascot search strategy only considered peptides which conformed to tryptic cleavage patterns, identifying 34 BLG peptides in 6 individuals (Table S6). These identifications were augmented with spectral data searched using semi-tryptic modifications (Table 2), which identified 29 additional BLG peptides (i.e., not detected in the tryptic dataset) and an additional BLG positive individual (HH3). Using this combined dataset, over half of the dental calculus samples ($n = 7$) displayed evidence for BLG peptides (Table 2). BLG peptides were identified in all three assemblages (Hambledon Hill ($n = 2$), Hazleton North ($n = 4$), Banbury Lane ($n = 1$)), although with one individual from both Hambledon Hill and Hazelton North represented by only a single BLG peptide. No BLG peptides were identified within blank controls or injection blanks. Using the combined search strategy, 63 spectra (comprising 14 distinct peptides) were assigned to BLG. For each of the samples which tested positive for BLG, a consensus BLG sequence could be assigned to ruminants of the Pecora infraorder of Artiodactyla, with all seven samples containing Bovidae-specific peptides (see Supplementary Information).

Levels of deamidation were examined using MaxQuant to ensure that the recovered BLG peptides and human oral proteins conformed with damage expected from ancient proteins, as opposed to modern contaminants (Mackie et al. 2018). Whilst asparagine (N) tends to deamidate into aspartic acid (D) relatively rapidly (van Duin and Collins 1998; Robinson and Robinson 2001, 2004), deamidation of glutamine (Q) into glutamic acid (E) is markedly slower and therefore can be used to assess protein degradation in archaeological contexts (van Doorn et al. 2012; Wilson et al. 2012). As expected, contaminant proteins (e.g., human keratins, trypsin, etc.) displayed, on average, low-levels of deamidation, with 90.9% undamaged asparagines and 98.5% undamaged glutamines (Fig. S2). The subset of analysed human proteins displayed variable, but on average, more advanced deamidation, with only 17.7% undamaged asparagines and 33.4% undamaged glutamines across the samples (Fig. S2). The BLG peptides displayed the most advanced levels of deamidation. In total, five individuals displayed BLG peptides with at least one deaminating residue: two individuals (HH610 and HN7387) displayed BLG peptides with both Ns and Qs and three individuals (HN11456, BL309.12, HN4786) displayed peptides with either Ns or Qs. For further two individuals (HH3, HN7656), the detected BLG peptides contained neither Ns nor Qs. In the five individuals where at least one deamidating BLG peptide was observed, the deamidation levels were extremely advanced. On average, only 2% of the asparagines and 5% of the glutamines were undamaged across the samples (Fig. 2). These levels of degradation are more advanced than those reported in previous analyses of BLG from ancient dental calculus proteomes (Mays et al. 2018; Mackie et al. 2017), a result consistent with the greater antiquity of these Neolithic individuals compared to previous studies (e.g., Middle Bronze Age, Roman and Medieval individuals).

Of the two individuals from Hambledon Hill in which BLG peptides were detected here, both indicated BLG deriving from Bovidae (HH3; HH610), a family of ruminants which includes cattle, buffalo, bison, antelopes, sheep, goats and gazelles (Gentry 1992). Therefore, the BLG within the calculus of two individuals at Hambledon Hill may have derived from cattle, sheep or goat milk. However, one tryptic peptide in individual HH610 also suggested the specific presence of goat (*Capra* sp.) milk. These results are supported by the faunal assemblage from the site, with domesticated cattle (*Bos taurus*) being the most dominant species present, followed by smaller numbers of ovicaprids (*Ovis aries* and *Capra hircus*) (Legge 2008). The age and sex structure of the cattle remains at Hambledon Hill have previously been suggested to be indicative of a dairy herd (Whittle 1992, p. 221; Copley et al. 2003), although others have suggested that the restricted

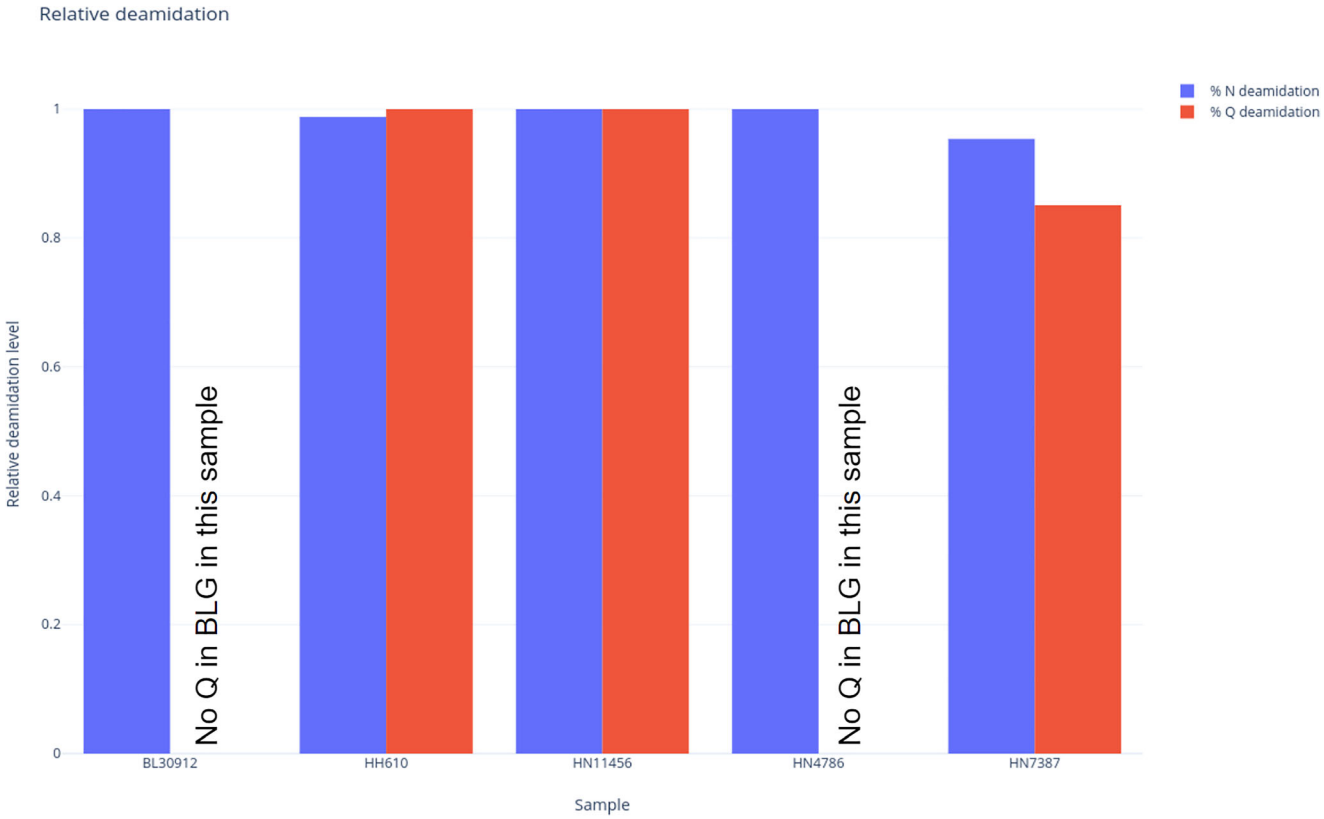

Fig. 2 Deamidation of all β-lactoglobulin peptides in the samples where a deamidating BLG peptide was found, as measured by relative intensity of peptides with deamidated asparagines (N) and glutamines (Q) versus the total intensity of both the deamidated and unmodified version of the peptides

age distribution of the cattle means that the animals were unlikely to have derived from a resident herd (Legge 2008). The high number of cattle skulls has also been interpreted as being representative of an association between the deposition of human and faunal remains (Thomas 1999, p. 28), and parallels between the treatment of human and cattle remains has been suggested to represent 'respectful consumption' of beef (Jones 2007, p. 164). The large-scale deposition of cattle remains, however, alongside large amounts of burnt wheat, barley and hazelnuts, has prompted suggestions of feasting at the site (Jones and Legge 2008; Mercer 2008). Additionally, organic residue analysis of pottery from Hambledon Hill has indicated the presence of both porcine and ruminant fats, and ruminant adipose and dairy fats (Copley et al. 2003, 2005a, b, 2008). The presence of dairy fats in > 25% of potsherds analysed has been suggested to indicate that 'dairying was a very important element of animal husbandry at Hambledon Hill' (Copley et al. 2008, p. 535).

Of the four individuals from Hazleton North in which BLG peptides were detected, all were also specific to Bovidae, but with peptides distinct from Capra, suggesting that individuals from Hazleton North were likely exploiting milk from cattle and/or sheep, but not goat. Stable isotopic analysis of $\delta^{13}C$ and $\delta^{15}N$ on human remains from the site has revealed a diet high in animal protein, supplemented by C3 plants (Hedges et al. 2008). Although Hazleton North does not possess a faunal assemblage akin to that at Hambledon Hill, the remains of domesticated cattle were nonetheless recovered within the chambered areas of the tomb at the site (Levitan 1990), and therefore, is consistent with the utilisation of cattle milk by the individuals at Hazleton North.

One individual from Banbury Lane (BL309.12) was also found to have BLG specific to Bovidae, but not Capra, using the tryptic search strategy. Unlike the other two sites, Banbury Lane has very little faunal material or pottery associated with either the monument itself or the human remains (Holmes 2012).

The combined results obtained here from British sites indicate that Neolithic populations were utilising milk and/or dairy products from either cattle, goat or sheep (or potentially combinations of these). This evidence is consistent with the recent discovery of bovine milk consumption in the Middle Neolithic individual recovered from Stonehenge (Mays et al. 2018). The results obtained here also show varying numbers of BLG spectra detected across all individuals, as has been observed in previous studies (Warinner et al. 2014a; Mays et al. 2018). For example, two individuals analysed here (HH3 and HN7656) exhibited only one BLG-specific

spectrum each, whereas in contrast, individual HH610 from Hambledon Hill had a total of 36 spectra matching BLG peptides (13 unique peptides). The reasons as to why some individuals have a significantly higher number of BLG spectra are still unclear but is something which certainly warrants further future study. Additional study of the abundance of BLG spectra within different dental calculus samples may reveal if this is linked to the amount of dairy consumed by an individual, or instead if it is the result of preservational biases or environments, or the timings and nature of calculus formation. In order to accurately assess this, however, a much broader scale study, with the likely inclusion of modern dental calculus samples from individuals with known diets, would be needed. Further study should also reveal if individuals with no evidence of BLG peptides within their calculus (as observed in this study, and within other proteomic studies (Warinner et al. 2014a; Hendy et al. 2018a)) were truly not consuming dairy products, or if the absence of milk proteins is instead the result of differential calculus formation processes or taphonomic effects.

It is important to note, however, that due to recent discoveries of LP prevalence in the European Neolithic (Burger et al. 2007; Gamba et al. 2014; Witas et al. 2015; Olalde et al. 2018; Brace et al. 2019), it is unlikely that the British Neolithic individuals studied here would have carried the genetic mutation associated with lactase persistence. Human remains from neither Hambledon Hill nor Hazleton North have been subjected to ancient DNA analysis to date, but none of the individuals ($n = 3$) genetically analysed thus far from Banbury Lane have a derived lactase persistence allele (Brace et al. 2019). The presence of BLG within the dental calculus however indicates the consumption of dairy products, as supported by other archaeological evidence for dairying from the period, as discussed above. It is important to note however that BLG may reflect either the regular consumption of small quantities of raw milk—based on the observation that individuals without LP can tolerate up to 240 mL of milk per day with negligible symptoms (Suarez et al. 1995; Swallow 2003)—or alternatively, the consumption of processed milk products with reduced lactose content. Distinguishing between these two consumption scenarios is not possible based on the current data and analytical technique. Nevertheless, as organic residue analysis has revealed that the processing of dairy in pottery vessels was widespread from the Early Neolithic onwards in Britain (Copley et al. 2005b), the likely absence of LP, but presence of BLG, is therefore more consistent with the hypothesis that Neolithic populations were processing milk to remove the lactose, but in such a way that at least some BLG was retained.

Lactose can be removed from or decreased in milk products through a range of processing methods. For example, cheese contains little or no lactose, as it is removed during processing with the whey fraction of the milk. Indeed, 98% of lactose is removed in the whey during most cheese production (Izco et al. 2002). The production of cheese within prehistory has however been suggested to have been beneficial for past populations not only due to the reduced lactose content, thereby making it more readily digestible and suitable for non-LP individuals, but also because it allows for the preservation of milk products in a transportable form (Salque et al. 2013). Lactose content is also known to be much decreased in fermented milk products, such as yoghurt, kefir and buttermilk (Alm 1982; O'Brien 1999). As such, fermented milk products have previously been suggested to be suitable for consumption by lactose intolerant individuals (Alm 1982). Although yoghurt does contain small amounts of lactose, it is believed that this lactose is more easily digested than that found within whole milk, due to hydrolysis and autodigestion of lactose by the yoghurt bacteria—thus improving its absorption and creating a 'lactase activity' in the gastrointestinal tract (Kolars et al. 1984; Savaiano 2014).

BLG is known to be present in processed milk products, but generally in significantly lower quantities than whole milk, and decreases the more the whole milk is processed. BLG is known to be absent or at very low levels in hard cheeses, for example, due to the removal of the whey fraction of the milk during processing. BLG is however present in yoghurts and other fermented milk products, and it has been noted that it is not markedly subject to hydrolysis or proteolysis during the fermentation process (Bertrand-Harb et al. 2003; Tzvetkova et al. 2007). The heating or fermentation of milk will decrease the levels of BLG within it, but to varying degrees dependent upon the type and intensity of processing utilised (Czerwenka et al. 2007; Bu et al. 2013).

The idea of Neolithic milk processing to reduce lactose content has been proposed previously (e.g., in the form of cheese making (Salque et al. 2013)) and is plausible given the recent genetic evidence for the absence of LP in Neolithic populations. Of the three sites analysed here, only the pottery from Hambledon Hill has previously had organic residue analysis undertaken upon it, indicating the exploitation of dairy fats (Copley et al. 2003). Overall, the evidence for dairy consumption (as evidenced through BLG peptides within calculus detected here and lipid residue analyses on pottery), combined with genetic support for the absence of LP, suggests that either British Neolithic populations were processing raw milk in an attempt to remove or reduce the lactose content, or alternatively consuming relatively small quantities of raw milk. As such, these observations open a range of exciting new research avenues exploring how Neolithic populations may have been consuming and processing raw milk, the required technologies needed to do this, the kinds of products which may have been created, the potential variability which may have existed within these processes in

the past, and notions of cuisine. It is conceivable that past populations chose to utilise the milk of different animals purposively, and processed this in different ways, due to cultural reasons or even taste. We can consider the modern regional variability which exists in cheese production in the UK—for example, varying in terms of the types of milk used, the processing methods, if the finished product is a soft or hard cheese, how long the cheese is left to mature for, and often having Protected Designation of Origin or Protected Geographical Indication status (British Cheese Board 2018)—as an indication that similar regional differences may have also existed in the prehistoric past in dairy processing and production. The fact that milk lipids are frequently found in British Neolithic pottery, often with thermally modified compounds, such as long-chain ketones, also suggests that raw milk was processed by heating.

Conclusions

Proteomic analysis of dental calculus obtained from 10 British Neolithic skeletons has revealed the presence of the milk protein β-lactoglobulin (BLG) in seven of the analysed skeletons, providing the earliest direct evidence for the consumption of dairy products in Britain and the earliest identification of BLG in human dental calculus to date. Analysis of deamidation patterns suggests that the BLG is indeed a degraded endogenous protein and not the result of laboratory contamination. Although only a small sample size, the presence of Bovidae-specific peptides within the calculus samples provides new insight into prehistoric patterns of milk consumption and can also be tied into larger discussions regarding dietary variables driving natural selection in humans and gene-culture co-evolution. Whilst previous studies suggest that early Neolithic individuals lacked LP (Brace et al. 2019), future genomic analyses targeting individuals with direct evidence for BLG may provide greater insight into selection for raw milk consumption. Additionally, the indication that the individuals studied here are unlikely to have had LP, but were consuming and utilising dairy products, indicates a range of exciting new research avenues exploring how Neolithic populations may have been consuming and processing raw milk and the potential variability which may have existed within these processes in the past.

The lack of other dietary proteins detected within the Neolithic samples analysed here echoes the results of other metaproteomic studies of dental calculus, suggesting dietary proteins are of low abundance in dental calculus and that BLG appears to have enhanced resistance to degradation within calculus, leading to its long-term survival (Mackie et al. 2017; Mays et al. 2018; Hendy et al. 2018a; Jersie-Christensen et al. 2018). This research however clearly highlights the utility of metaproteomic approaches in exploring prehistoric dairying. To our knowledge, this study represents the earliest direct evidence of milk consumption globally and also the earliest identification of the milk whey protein β-lactoglobulin (BLG) to date.

The presence of Bovidae-specific peptides within the calculus samples analysed here opens up the possibility that British Neolithic populations may have been exploiting multiple species for dairy products including cows, sheep and goat, although zooarchaeological evidence from the sites is most consistent with cattle milk. Future studies targeting a greater diversity of British Neolithic sites may indicate distinctions between the utilisation of different ruminant species for milk exploitation, and whether this varied on a population level (i.e., between sites), or was more closely aligned to social constructs. Despite the small sample size, the detection of BLG in seven of the ten tested individuals may provide a tentative glimpse into the prevalence of dairy consumption. The detection of BLG in the majority of individuals across three sites may suggest that milk consumption was not confined to a small number of individuals within Neolithic society but was more likely a widespread dietary practice—a hypothesis that certainly warrants further investigation with a larger number of individuals, archaeological sites and burial contexts. With further future work, the idea that we may be able to distinguish if certain members of a society consumed differential amounts of dairy products or dairy from different animals—for example, along the lines of sex, gender, age or social standing—is truly fascinating and would provide a unique new insight into British Neolithic culture and social structure. Furthermore, if milk is such an accessible foodstuff in the Neolithic, what was the underlying driver of the observed selection pressure for lactase persistence? Increased genetic testing of archaeological remains in the future, including the analysis of individuals with evidence of BLG, may provide more insight into milk consumption and processing, and the gene-culture co-evolution of LP.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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