Direct evidence of milk consumption from ancient human dental calculus

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Milk is a major food of global economic importance, and its consumption is regarded as a classic example of gene-culture evolution. Humans have exploited animal milk as a food resource for at least 8500 years, but the origins, spread, and scale of dairying remain poorly understood. Indirect lines of evidence, such as lipid isotopic ratios of pottery residues, faunal mortality profiles, and lactase persistence allele frequencies, provide a partial picture of this process; however, in order to understand how, where, and when humans consumed milk products, it is necessary to link evidence of consumption directly to individuals and their dairy livestock. Here we report the first direct evidence of milk consumption, the whey protein β-lactoglobulin (BLG), preserved in human dental calculus from the Bronze Age (ca. 3000 BCE) to the present day. Using protein tandem mass spectrometry, we demonstrate that BLG is a species-specific biomarker of dairy consumption, and we identify individuals consuming cattle, sheep, and goat milk products in the archaeological record. We then apply this method to human dental calculus from Greenland’s medieval Norse colonies, and report a decline of this biomarker leading up to the abandonment of the Norse Greenland colonies in the 15th century CE.

Milk is a major nutritional resource. In addition to being a source of clean liquid (milk is 80–90% water), milk solids contain approximately 25–55% sugar (lactose), 25–45% fat, and 5–35% protein (caseins and whey proteins), as well as calcium, potassium, and B-vitamins. Adoption of animal milk consumption by humans typically requires behavioral adaptations, such as culturing and curdling techniques, to remove or reduce the lactose content of milk in order to make dairy products digestible after infancy. Additionally, populations with long pastoralist traditions in Europe and India, East Africa, and the Arabian peninsula have also independently evolved lactase persistence (LP), a genetic adaptation in the regulation of the lactase gene (LCT) that allows continued adult digestion of milk2–4 (Fig. 1). LP is hailed as one of the clearest examples of gene-culture co-evolution in humans4, yet many fundamental aspects of its evolution remain unknown5–9 and the socioeconomic context and scale of prehistoric and historic dairying are only poorly understood.

The ability to directly identify milk consumption patterns in past populations would thus advance understanding of human dietary ecology, evolution, and cultural agency. However, previous attempts to directly measure milk consumption using bone calcium isotopes have proven unsuccessful10. Other milk biomarkers offer only indirect lines of evidence. For example, isotopic inference of milk lipids from pottery residues11 is the most widely used approach to identify dairying, but this method cannot discriminate species of origin, and reuse of communal vessels and exploitation of cervid adipose tissue12 both pose further challenges to interpretation. Milk proteins have also been recovered from food residues13–15, but such finds are exceptional and rare, and
animal bone evidence for the exploitation of secondary products is limited by the availability of large, well preserved assemblages to provide interpretable mortality profiles. To address these problems we turned to dental calculus, a mineralized form of dental plaque that serves as a long-term reservoir of dietary biomolecules and microfossils. Nearly ubiquitous in archaeological populations and sourced directly from the oral cavity, dental calculus presents a unique opportunity to access primary evidence of ancient diets at an individual level. In a recent study, we identified the milk whey protein β-lactoglobulin (BLG) in the dental calculus of a modern Swiss dental patient using tandem mass spectrometry. This protein was also recently identified within preserved kefir cheese curds associated with the mummified remains of Bronze Age Xiaohe pastoralists (ca. 1980-1450 BC) in Xinjiang, China. BLG is a lipocalin within the calycin superfamily of proteins, and it is the dominant whey (milk serum) protein in ruminant milk, making up 11% of the total milk proteins and 50% of the whey proteins. BLG offers many advantages as a milk biomarker including: 1) humans do not produce BLG and therefore the presence of the protein in dental calculus excludes a host origin; 2) BLG is present only in milk and thus it is a specific biomarker for this fluid; 3) BLG is more resistant to enzymatic degradation and microbial proteolysis than other milk proteins; 4) BLG lacks close bacterial orthologs, making it readily identifiable against a background of bacterial proteins; 5) over half of the amino acid residues in BLG are variable among traditional dairy livestock, allowing genus and species discrimination between cattle, buffalo, sheep, goat, horse, donkey, and reindeer, among others (Supplementary Fig. S1); 6) BLG is the dominant protein in the whey fraction of milk and partitions with lactose during dairy processing, thereby making BLG a superior proxy for lactose than caseins or milk fats, which separate from the lactose-rich whey fraction during cheese and butter production; 7) and finally, because BLG is identified directly from protein sequence data, uncertainties arising from indirect detection methods, such as isotopic analysis, are minimized.

This study seeks to employ shotgun protein analysis by liquid chromatography-tandem mass spectrometry to identify dairy consumption in the archaeological record. In this study, we analyzed 92 archaeological dental calculus samples selected from regions (Europe and northern Southwest Asia) and without (Central West Africa) long-standing dairying traditions for the presence of BLG (Fig. 1; Table 1). After establishing that BLG can be detected in the dental calculus of archaeological populations from dairy-consuming regions, we then applied this approach to 6 additional medieval dental calculus specimens from the Norse Greenland sites Brattahlíð (Qassiarsuk) and Sandnes (Kilaarsarfik). These sites date...
to a period during which a major dietary shift from ruminant dairy to marine resources has been previously hypothesized on the basis of bone stable isotope and zooarchaeological evidence. We confirm that a decline in BLG observed at these sites is consistent with a dietary shift, and specifically diminished access to dairy products, leading up to the abandonment of the Norse Greenland colonies in the 15th century CE.

**Results**

**Lactase Persistence in Present Day Populations.** To assist in the selection of archaeological samples for analysis, we generated an updated interpolated contour map of present-day LP frequency in Europe, northern Southwest Asia, and Africa (Fig. 1) using recently published LP genotype data. We then selected archaeological dental calculus specimens from sites located within regions with high (Northern Europe), moderate (Central Europe), low (northern Southwest Asia), and very low (Central West Africa) present-day LP frequencies.

**BLG in Europe and northern Southwest Asia.** Seventy-four dental calculus samples were selected from 20 sites in Northern Europe (Britain, Denmark, and Norway; n = 40), Central Europe (Germany, Hungary, and Italy; n = 38), and northern Southwest Asia (Armenia and Russia, n = 6) dating from the Bronze Age (ca. 3000 BCE) through the 19th century CE (Table 1; Supplementary Table S1). Approximately one quarter (25.7%) of the Eurasian dental calculus samples tested positive for BLG peptides (Fig. 1). In total, 229 spectra (representing 37 unique peptide sequences) from the Eurasian dataset were assigned to BLG (Table 1; Supplementary Table S2), resulting in a reconstruction of 72% of the protein (Fig. 2). For each of the 19 Eurasian dental calculus samples that tested positive for BLG, the consensus BLG sequence could be assigned to ruminants of the Pecora infraorder of Artiodactyla, and 18 positive for BLG, the consensus BLG sequence could be assigned to ruminants of the Pecora infraorder of Artiodactyla, and 18

**Absence of BLG in Central West Africa.** Eighteen dental calculus samples were selected from a 19th century cemetery on the island of St. Helena, located approximately 2,000 km west of Angola in the southern Atlantic Ocean. The cemetery contains the remains of Central West Africans originating from a region with traditionally very low or no milk consumption (Fig. 1; Supplementary Table S1). As expected, BLG peptides were not identified in any of the West African samples (Fig. 1; Table 1).

**BLG in Norse Greenland.** Recent isotopic and faunal evidence suggests that Greenland Norse settlements shifted from an economy initially based on dairy to one increasingly reliant on marine mammals after the onset of the Little Ice Age ca. 1250 CE. To test this hypothesis, we analyzed dental calculus from individuals buried at Tjodhildes Church, an early cemetery (ca. 985–1250 CE) at the Eastern settlement landnám site of Brattahlíð (Qassiarsuk) established by Erik the Red, the founder of the Norse Greenland colonies, and at Sandnes (Kilaarsarfik), a high-status farm and church in the Western Settlement that continued to be in use until the abandonment of the settlement in the 15th century CE. The individuals analyzed from Tjodhildes Church (n = 2) exhibited strong evidence of dairy consumption (Fig. 3), with a total of 38 spectra matching BLG peptides (12 unique peptides including one Bos-specific sequence). Because these two individuals had not been previously analyzed isotopically, we then performed carbon and

### Table 1 | Proteomic results of archaeological dental calculus samples analyzed in this study

| Region                  | Dates                  | N     | Individuals (spectra) |
|-------------------------|------------------------|-------|-----------------------|
| Northern Europe         |                        |       |                       |
| Britain                 | ca. 800 BCE to 1895 CE | 33    | 11 (128)              |
| Denmark                 | ca. 3000–1500 BCE      | 2     | 0 (0)                 |
| Norway                  | 1100–1700 CE          | 5     | * (43)                |
| Central Europe          |                        |       |                       |
| Germany                 | ca. 3000 BCE to 1200 CE| 9     | 0 (0)                 |
| Hungary                 | ca. 3000–1500 BCE      | 2     | 1 (38)                |
| Italy                   | ca. 2700 BCE to 200 CE | 17    | 4 (11)                |
| Northern Southwest Asia |                        |       |                       |
| Armenia                 | ca. 2000–700 BCE       | 4     | 1 (2)                 |
| Russia                  | ca. 3000–1500 BCE      | 2     | 1 (8)                 |
| Central West Africa     |                        |       |                       |
| St. Helena              | ca. 1840–1872 CE       | 18    | 0 (0)                 |
| Greenland (Norse)       |                        |       |                       |
| Eastern Settlement      | ca. 890–1230 CE        | 2     | 2 (38)                |
| Western Settlement      | ca. 1290–1430 CE       | 4     | 1 (1)                 |

Notes:
*Extracted proteins from five individuals were pooled for analysis.

Figure 2 | Protein coverage of β-lactoglobulin identified within Eurasian archaeological dental calculus. (a) Human dental calculus from the British Anglo-Saxon site of Norton-on-Tees (sample NEM18, ca. 6th century CE) found to contain seven β-lactoglobulin peptides. (b) Three-dimensional structure of bovine β-lactoglobulin protein, rendered from PDB 3NPO using VMD v.1.9.1<sup>a</sup>. The mapped locations of all BLG peptide sequences identified by tandem mass spectrometry within archaeological dental calculus are shown in red, resulting in a coverage of 72% of the reconstructed consensus BLG protein.
Discussion

Our results show that the milk protein BLG preserves in archaeological dental calculus and can be identified in specimens dating back to at least the Bronze Age (ca. 3000 BCE) in Europe and northern Southwest Asia. Moreover, we demonstrate that BLG is a species-specific milk biomarker that allows cattle, sheep, and goat dairy product consumption to be distinguished. As expected, no BLG was detected in specimens from Central West Africa, where dairy consumption was historically very low or absent and current LP frequencies are very low.

In addition to establishing BLG as a biomarker of milk consumption in the archaeological record, the broad survey of dental calculus BLG conducted in this study reveals previously uncharacterized temporal and geographical complexities in dairy consumption. In Central Europe, for example, it is intriguing given the high prevalence of LP in both modern day (Fig. 1) and medieval German populations that no BLG peptides were detected in Bronze Age or medieval German samples (0/9 individuals) (Table 1). This stands in contrast to the northern Italian and Hungarian samples where BLG peptides were detected at a relatively high frequency (5/11 individuals) (Table 1; Supplementary Table S2). These geographic patterns may reflect different dairy consumption levels or differential usage of high (e.g., soured milk, whey, ricotta, kefir) and low (e.g., cheese, butter) BLG dairy products. Using this approach, it now becomes possible to explore specific cultural, social, and environmental factors influencing past dairy economies at both a population and an individual level.

To explore a specific dairy economy in greater detail, we applied this approach to Norse Greenland to examine the hypothesis that this population underwent a dramatic dietary shift in response to environmental change during the Little Ice Age 23. The medieval Greenland Norse economy was primarily based on animal husbandry and especially dairying. The short growing season and cold climate of Greenland precluded the successful establishment of agriculture but was sufficient for ruminant pastoralism. Like other Scandinavian populations, the medieval Norse utilized nearly all by-products of dairy processing, including BLG-rich whey 28,29. The use of sour whey (su˚r) to pickle meats is described in the Norse Icelandic sagas 30, and Scandinavian whey “cheese” (e.g., myost, gjetost, and brunost), an evaporated whey concentrate with an exceptionally high lactose content (30–55%), was also likely consumed, but unfortunately the sagas lack detail on specific “cheese” (ost) types.

Our dental calculus BLG results confirm that dairy products were consumed by individuals buried in the early Norse cemetery at Tjodhildes Church (ca. 985–1250 CE), and bone collagen stable isotopic values from the same individuals are consistent with a terrestrial diet. By contrast, BLG was very low or absent in the dental calculus of individuals buried at the Sandnam site, which was occupied until ca. 1430 CE, and bone stable isotope values from these individuals indicate a dietary shift toward marine resources.

Diminished access to dairy products and a collapse of dairy herds would have had a strongly negative effect on the Norse economy, removing not only a major source of storable nutrition but also impeding the ability to preserve other perishable foods, such as meats, thereby exacerbating food instability especially during the winter months. Our findings support the hypothesis that climate change and the consequent decline of dairy herds contributed to the decline and ultimate abandonment of the Norse Greenland colonies in the 15th century CE.

In this study, we identify the protein β-lactoglobulin in archaeological dental calculus and demonstrate that it is a species-specific milk biomarker and an indicator of dairy consumption in the archaeological record. Nearly ubiquitous and obtained directly from the oral cavity of individuals, dental calculus provides a novel approach to detecting patterns of milk consumption and the dietary variables driving recent natural selection in humans.

Methods

Interpolated contour map of present day lactase persistence. Lactase persistence frequency data was estimated from allele frequency data assuming dominant inheritance and was taken, where possible, from full sequencing of the lactase
enhancer to include all 5 published known functional LP variants with 14 additional data taken from genotyping of the individual SNPs, where informative, as recorded in the GLAD database (http://www.ucl.ac.uk/mace-lab/resources/glad/) but revised and updated to include more recent publications through March 2014.32 Latitude and longitude of the data points were taken as near as possible to the collection sites where these were known. Where country alone was known these were estimated using major cities. The tree was constructed in R (v 3.1.0, 2014-04-10, “SpeciesTree”) using the partstat package and included weighting for sample size. Interpolation smoothing was conducted at the lowest non-overlapping bandwidth (value of sigma) allowable from the heterogeneous data available. Interpolation may be inaccurate where there are few data points, and it should be noted that neighboring populations with different ancestry and life-style, in Africa particularly, sometimes have very different allele frequencies.

Samples and MS/MS analysis. Dental samples (n = 98) were obtained from diverse historic human populations in Eurasia, Africa, and Greenland dating from the Bronze Age to the present (Supplementary Table S1). Dental calculus was removed using a dental scaler and stored in sterile 2.0 mL tubes until further analysis. Tryptic peptides were extracted from decalcified dental calculus using a filter-aided sample preparation (FASP) protocol modified for degraded samples according to previously published protocols.20 The extracted peptides were then analyzed using shotgun protein tandem mass spectrometry (MS/MS) to detect the presence of β-lactoglobulin. MS/MS analysis of samples was performed at three independent laboratories in Switzerland, the UK, and Denmark.

Functional Genomics Center Zurich at the University of Zurich and Swiss Federal Institute of Technology. Samples from Greenland and Germany (Z1, Z2, Z7, Z9, and Z28) were used in mass spectrometry in Functional Genomics Center Zurich (FGCZ) using an LTQ-Orbitrap VELOS mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) coupled to a Dionex nanoLC-200 UPLC system (Eksigent Technologies, Dublin, CA, USA). Solvent composition at the two columns was 0.2% formic acid, 1% acetonitrile for column A and 0.2% formic acid, 100% acetonitrile for column B. Peptides were loaded on a self-made tip column (75 μm × 80 mm) packed with reverse phase C18 material (AQ, 3 μm 200 Å, Bischoff GmbH, Leonberg, Germany) and eluted with a flow rate of 250 nL per min by a gradient from 0.8% to 4.8% B in 2 min, 35% B at 57 min, 48% B at 60 min, 97% at 65 min. Full-scan MS spectra (300–1700 m/z) were acquired in the Orbitrap with a resolution of 75 000 at m/z 400 after accumulation to a target value of 1.000 000. Higher energy collision induced dissociation (HCD) MS/MS spectra were recorded in data dependent manner in the Orbitrap with a resolution of 7500 at m/z 400 after accumulation to a target value of 100 000. Precursors were isolated from the ten most abundant precursors and fragmented with a fixed injection time of 0.07 Da. Based on previous observations of ancient proteome degradation,21 we set post-translational modifications were as carbamidomethylation (fixed modification) and acetyl (protein N-term), deamidated (NQ), glutamine to pyroglutamate, methionine oxidation and hydroxylation of proline (variable modifications). Mascot search results were filtered using an ion score cut-off of 15 and significance threshold of p < 0.05. BLAST was used to verify matches to β-lactoglobulin, and taxonomic assignment is reported based on the consensus peptide assignments for each individual. The three-dimensional structure of bovine β-lactoglobulin protein, rendered from PDB 3NPO using Visual Molecular Dynamics software22 VMD v1.9.1, http://www.ks.uiuc.edu/Research/vmd/current/.

Bone collagen stable isotope analysis. Bone collagen from Tjodhilde’s Church individuals KAL1052 and KAL1064 was prepared for stable isotope δ13C and δ15N analysis as previously described.23 Duplicate collagen specimens (1 mg) were measured using a Sercon 20–22 Isotope Ratio Mass Spectrometer coupled to a Sercon GSL Elemental Analyser in the Department of Archaeology at the University of York. The results for KAL1052 are as follows: δ13C, −18.7352; δ15N, 8.12409, 8.128799; C/N, 3.31. The results for KAL1064 are as follows: δ13C, −19.0134; δ15N, 8.1551. Carbon isotopic values are reported relative to Pee Dee Belemnite (PDB); nitrogen isotopic values are reported relative to AIR. Mean bone collagen δ13C and δ15N for each sample are presented in Figure 3. Stable isotopic data for the Sandnes samples and the remaining Tjodhilde’s Church samples were obtained from the literature.24,25

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