Proteomic Analyses On an Ancient Egyptian Cheese and Biomolecular Evidence of Brucellosis

Enrico Greco[†‖]*, Ola El-Aguizy[‡], Mona Fouad Ali[§], Salvatore Foti[†], Vincenzo Cunsolo[†], Rosaria Saletti[†], Enrico Ciliberto[†]

[†] Department of Chemical Sciences, University of Catania, viale A. Doria 6-I, 95125 Catania, Italy egreco@unict.it, sfoti@unict.it, vcunsolo@unict.it, rsaletti@unict.it, cilibert@unict.it.

[‡] Head of the Cairo University Excavation Mission, Faculty of Archaeology, Cairo University 12613 Giza, Egypt olaelaguizy@cu.edu.eg.

[§] Head of Conservation Department, Faculty of Archaeology, Cairo University 12613 Giza, Egypt monalyeg@yahoo.com.

ABSTRACT: The material analyzed in this study is probably the most ancient archaeological solid residue of cheese ever found to date. The sample was collected during the Saqqara Cairo University excavations in the tomb of Ptahmes dated to XIX dynasty[1, 2]. Our biomolecular proteomic characterization of this archaeological sample shows that the constituting material was a dairy product obtained by mixing sheep/goat and cow milk. The interactions for thousands of years with the strong alkaline environment of the incorporating soil rich in sodium carbonate and the desertic conditions did not prevent the identification of specific peptide markers which showed high stability under these stressing conditions. Moreover, the presence of *Brucella melitensis* has been attested by specific peptide providing a reasonable direct biomolecular evidence of the presence of this infection in the Ramesside period for which only indirect paleopathological evidence has been so far provided[3, 4]. Finally, it's worth noting that, although proteomic approaches are successfully and regularly used to characterize modern biological samples[5], their application in ancient materials is still at an early stage of progress, only few results being reported about ancient food samples[6]. In the absence of previous relevant evidences of cheese production and/or use, this study, undoubtedly has a clear added value in different fields of knowledge ranging from archaeometry, anthropology, archaeology, medicine history to the forensic sciences.

The tomb of Ptahmes, mayor of Memphis and high-ranking official under the Pharaohs Sethi I and Ramses II (1290-1213 BC) of the XIX dynasty was rediscovered in 2010 after a part of it was revealed in 1885 and lost under the sands at the end of the 19th century[7-17]. Now the site is just partially excavated and published[1, 2, 18, 19]. It is located in the south of the Causeway of the Pharaoh Unas which yields a number of tombs dated to the New Kingdom. The sample in this study was discovered during the excavation procedures of Cairo University in the season 2013/2014. While cleaning the sand around the southern outer wall of the tomb, in a corner formed from the difference in width between the forecourt and the second court, in an area corresponding to one of the lateral storehouses, a big number of broken jars was found. One of these contained a solidified whitish mass, while a canvas fabric which might have covered it or used for its preservation was found in one of the fragments of the jar. (fig.1). The characteristic of the canvas made it unsuitable for containing liquid or, in general, non solid-materials. The sample (PTAH_1) was accurately collected in order to avoid any kind of contamination.

Fig. 1. Jar and canvas discovered inside the tomb of Ptahmes, Mayor of Memphis during the XIX dynasty (photo by the authors)

A first investigation was performed in order to study the microscopic structure of the sample. In the fig. 2 the SEM (FEDSEM LEO Supra 55VP with Zeiss GEMINI column) image shows the heterogeneity of the material that contains both crystalline and amorphous species. The crystals were analyzed by Bruker D5005 X-Ray Diffractometer and the primary com-

pound was sodium carbonate in the *trona* phase[20, 21]. The origin of this specie is clearly driven by the extensive presence of this salt in the surrounding area where the rare but present rainfalls induce periodic cycles of solubilisation, diffusion and recrystallisation of the sodium carbonate. Such a highly alkaline environment reacted with almost all the fats present in the solid residue causing saponification. For this reason, a proteomic analysis was found to be more suitable for the recognition of the nature of the sample.

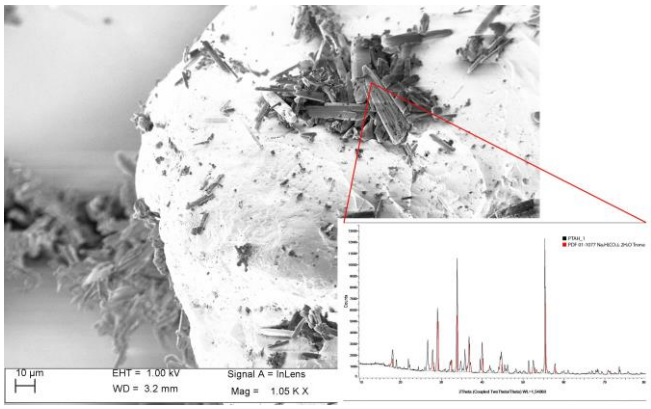

Fig. 2. SEM image and XRD of the sample PTAH_1 and PDF reference of trona phase[20, 21]

In order to analyse the amorphous components the sample were dissolved in aqueous 0.1% Trifluoroacetic Acid (TFA) at a concentration of 1mg/mL (pH 2.6). Protein concentration, determined using fluorometric assay, resulted 170 µg/mL. The sample was desalted and purified from non-protein contaminants using the PlusOne 2-D Clean-Up kit (GE Healthcare Life Sciences) according to the manufacturer's recommendations and dissolved in 20 mM ammonium bicarbonate (pH 8.3) at a concentration of 1 mg/mL and then reduced, alkylated and digested with porcine trypsin as previously reported[22]. The resulting solution was diluted 1:2 in 5% aqueous Formic Acid (FA) and analyzed by UHPLC/High Resolution nanoESI-MS/MS.

Mass spectrometry data were acquired on an Orbitrap Fusion Tribrid (Q-OT-qIT) mass spectrometer (ThermoFisher Scientific, Bremen, Germany) equipped with a ThermoFisher Scientific Dionex UltiMate 3000 RSLC nano system (Sunnyvale, CA), as previously described[23]. LC/MS/MS data were analyzed and searched against the comprehensive (all species) UniProt protein sequences database (April 2017 release, containing 554241 entries) [24] using integrated PEAKS de novo sequencing software (v. 7.0, Bioinformatics Solutions Inc., Waterloo, ON Canada) and Mascot algorithm (Matrix Science, London, UK, version 2.5.1), as previously reported[22]. Peptide spectral matches (PSM) were validated using a Target Decoy PSM Validator node based on q-values at a 0.1% False Discovery Rate (FDR). Proteins that contained same peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Proteomic analysis allowed the characterization of about five hundred peptides coming from more than 90 proteins with different organism origin. Most of them were from human and represented a background that comprised keratins, skin and saliva-associated proteins probably due to contamination. Taking into account that sample PTAH_1 was supposed to be an ancient dairy product, it is important to note that nine peptides were from *Bovidae* milk proteins (cow, sheep, goat or buffalo). Six of them were from caseins (α_{s1}-, β- and κ-), whereas the remaining three peptides belong to two proteins (i.e. lysozyme and serum albumin) typically present in the whey fraction of milk and milk-derived foods[25, 26, 27] (Table 1). In detail, four peptides, FVVAPFPEVFR, YIQKEDVPSER, YLGYLEQLLR and YNVPQLEIVPK allowed the identification of an α_{s1}-casein; all these peptides are common to the ovine (UniProt Accession No. P18626) and caprine (Acc. No. P04653) α_{s1}-casein. On the other hand, the sequence YLGYLEQLLR is also related to the cow (Acc. No. P02662) and water buffalo (Acc. No. 062823) species. β-casein was identified by the characterization of the peptide YPVEPFTESQSLTLTDVEK, a sequence trait common to sheep (Acc. No. P11839) and goat (Acc. No. P33048) species. Finally, the peptide YIPIQYVLSR, shared between sheep (Acc. No. P02669), goat (Acc. No. P02670), cow (Acc. No. P02668) and water buffalo (Acc. No. P11840) species, allowed the identification of a κ-casein. As above reported, three peptides were markers of two cow proteins normally present in milk and dairy products. In detail, cow milk lysozyme (Acc. No. Q6B411) was identified by the characterization of the peptide STDYGIFQINSR; cow serum albumin (Acc. No. P02769) was identified by the characterization of peptides KVPQVSTPTLVEVSR and LFTFHADICTLPDTEK.

Albumin, which occurs in many body tissues and secretions, is not synthesized in the mammary gland but it is presumed to enter the milk by leaky junctions between the mammary epithelial cells or by uptake with other components such as immunoglobulins. Lysozyme, another protein that is typically found in milk whey fraction, is an enzyme belonging to the glycoside hydrolases and it is known to be a natural antimicrobial agent.

Table 1. Selected proteins present in the sample PTAH_1 and identified by database search of MS data (details in the text).

Proteins	Protein Coverage (%)	Protein score (%)	Peptide score [a] (%)	Supporting peptides (*species*)	Monoisotopic m/z (*z*)		Δm (ppm)
					Measured	Calculated	
α_{s1}- casein	20	98.8					
			99.9	FVVAPFPEVFR (*sheep; goat*)	654.3610 (*2+*)	654.3610	0
			99.9	YLGYLEQLLR (sheep; goat; cow; buffalo)	634.3561 (*2+*)	634.3559	0.3
			88.8		455.2332 (*3+*)	455.2333	-0.2
			99.1	YIQKEDVPSER (*sheep; goat*) YNVPQLEIVPK (*sheep; goat*)	650.3688 (*3+*)	650.3690	-0.3
β-casein	9	59.5					
			97.5	YPVEPFTESQSLTLTDVEK *(sheep; goat)*	1092.0400 (*2+*)	1092.0414	-1.3
κ-casein	5	61.6					
			99.8	YIPIQYVLSR (sheep; goat; cow; buffalo)	626.3585 (*2+*)	626.3584	0.2
Lysozyme	8	61.7					
			99.9	STDYGIFQINSR *(cow)*	700.8441 (*2+*)	700.8439	0.3
Serum Albumin	5	81.7					
			98.0	KVPQVSTPTLVEVSR *(cow)*	547.3174 (*3+*)	547.3174	0
			89.4	LFTFHADICTLPDTEK [b] *(cow)*	636.6456 (*3+*)	636.6451	0.8
Protein RecA	2	55.6					
			94.5	IGSIKER (Brucella melitensis biotype 1)	401.7428 (*2+*)	401.7427	0.2

a) Percentage confidence score is used to reflect the probability that this peptide-spectrum match is correct. The percentage score is calculated in accordance with the empirical calculation used in PeptideProphetTM[28].
b) Cysteine residue is carbamidometilated.

Altogether, these data confidently suggested that the investigated archaeological organic sample represents a cheese-like product obtained using bovine milk mixed with milk from ovine (goat or sheep).
Among the hundreds peptides identified in the ancient sample, no proteins or peptides from *Lactobacillus kefiranofaciens* and other lactic acid bacteria (usual microbial signature characteristic for kefir or kefir-like fermentation[6]) were found. Moreover, the sequence IGSIKER (*see Table 1*) allowed the identification of a protein (Acc. No. P65975) from *Brucella melitensis* biotype 1. It is interesting to note that *Brucella melitensis* is the main cause of brucellosis in human, and represents a natural pathogen for sheep and goats[29].
It should be noted that this amino acid trait is common to proteins from other bacteria, such as a hypothetical protein from *Coxiella burnetii* (e.g. NCBI Acc. No. WP_098953193) another gram-negative bacterium that mostly affect ruminants[30]. On the light of this evidence it is not possible to exclude *a priori* that this peptide could be related to *Coxiella burnetii*. However, it should be noted that if the peptide IGSIKER arises from the protein RecA of *Brucella melitensis*, it represents a theoretical tryptic fragment generated by two specific cleavages at level of the Arg^{238}-Ile^{239} and Arg^{245}-Asp^{246} bonds. On the contrary, the peptide IGSIKER may be generated from a hypothetical protein of *Coxiella burnetii* if we hypothesized an unspecific tryptic cleavage at the Thr^{292}-Ile^{293} bond. So that, taking into account that less of 5% (corresponding to about twenty-five peptides) of all the identified peptides was generated by unspecific tryptic cleavages and in order to satisfy the principles of parsimony (i.e. Occam's razor) the sequence IGSIKER may be reasonably related to the *Brucella melitensis*.
In conclusion, even if very ancient kefir or milk or dairy residues, coming from North African[31], Chinese[6, 27] and European[32] excavations have been found and analyzed, the present sample represent the oldest solid cheese so far discovered (3200 BP).
The results here obtained show how proteomic investigation of ancient materials may provide valuable contributions for their characterization. In particular, the present work evidences the capability of these approaches in order to identify not only the milk components preserved in the ancient dairy material, but also the unambiguous detection of different milk species employed in cheese ancient manufacturing.
Moreover, up to date, only indirect signs of Brucellosis have been discovered on Egyptian archaeological pelvic and hip bones such as sacroiliitis, spondylitis and osteoarticular lesions dated 750 BC[3, 33, 34]. So that, the identification of a peptide sequence which may be related to the *Brucella melitensis* in our investigation, could represent the first biomolecular direct evidence of this disease during the pharaonic period, even if requires additional investigations in order to be exhaustively and conclusively confirmed.

AUTHOR INFORMATION

Corresponding Author

* email: egreco@unict.it
ORCID

Enrico Greco: 0000-0003-1564-4661
Mona Fouad Alì: 0000-0001-6631-5003
Salvatore Foti: 0000-0002-4007-6582
Vincenzo Cunsolo: 0000-0002-2349-5494
Rosaria Saletti: 0000-0002-1990-6764
Enrico Ciliberto: 0000-0002-4583-0759

Present Addresses

‖ Peking University, College of Environmental Science and Engineering, 5 Yiheyuan Rd, Haidian Qu, Beijing Shi, China, 100080.

Author Contributions

E.C. conceived and planned the project. E.C. and E.G. wrote the paper. E.G., S.F., V.C. and R.S. performed analytical work and data analysis, and O.e.A. and M.A. directed the excavations procedures and sampling of archaeological materials. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest or conflict interest.

ACKNOWLEDGMENT

The authors gratefully acknowledge the Bio-Nanotech Research and Innovation Tower (BRIT; PON project financed by the Italian Ministry for Education, University and Research, MIUR), the Ministry of State for Antiquities of Egypt, Mr. Aurélien Tafani and Ms. Allison Wu for the article revision. This research was partly supported by 'Piano della Ricerca di Ateneo 2016-2018' of the University of Catania, Italy.

REFERENCES

(1) El-Aguizy, O. Bulletin de l'Institut Française d'Archéologie Orientale (BIFAO) 2010, 110, 13-34
(2) Staring, N., Bulletin de Institut Français d'Archéologie Orientale (BIFAO) 2015, 114, 2, 455-518
(3) Pappas, G.; Papadimitriou P., International Journal of Antimicrobial Agents 2007, 30, s. 1, 29-31
(4) Bourke, J. B. Medical History 1971, 15(4), 363–375.
(5) D'Ambrosio, C.; Arena, S.; Salzano, A. M.; Renzone, G.; Ledda, L.; and Scaloni, A. Proteomics, 8: 3657-3666.
(6) Yang, Y.; Shevchenko, A.; Knaust, A.; Abuduresule, I.; Li, W.; Hu, X.; Wang, C.; Shevchenko, A., Journal of Archaeological Science 2014, 45, 178-186.
(7) Davies, N., The Mastaba of Ptahhetep and Akhethetep at Saqqareh. 2 Parts. Egypt Exploration Fund: London, 1900, 2 Pl. XVII.
(8) Saleh, M., Three Old-Kingdom Tombs at Thebes, Philipp von Zabern: Mainz am Rhein 1977, n.68.
(9) Davies, N., The Rock Tombs of Deir el-Gebrawi, 2 Vols. Egypt Exploration Fund: London, 1902, Pl. XIX.
(10) Darby, W. J.; Ghalioungui, P.; Grivetti, L., Food: the Gift of Osiris, 2 Vols. Academic Press: London, 1977, 760-772.
(11) Zaky, A.; Zaky I,; Annales du Service des Antiquités de 1'Egypte 1942, 41, 295-313.
(12) Wilson, H., Egyptian Food and Drink, Shire Publications Ltd: Aylesbury, 2008, 47;
(13) Darby, W. J.; Ghalioungui, P.; Grivetti, L., Food: the Gift of Osiris, 2 Vols. Academic Press: London, 1977, 772-75.
(14) Curtis, R. I., Ancient food technology, Brill: Leiden, Boston, Köln, 2001, 173.
(15) Lubbock J., The Journal of the Anthropological Institute of Great Britain and Ireland 1875, 4 , 215-222.
(16) Anthes, R., Zeitschrift für Ägyptische Sprache und Altertumskunde 1931, 67, 1, 2–9.
(17) Berlandini, J., Bulletin de l'Institut Française d'Archéologie Orientale (BIFAO) 1982, 82, 85-103.
(18) El-Aguizy O., Bulletin of the Egyptian Museum 2007, 4, 41-50.
(19) El-Aguizy O., Bulletin de l'Institut Française d'Archéologie Orientale (BIFAO) 2007, 107, 1-4.
(20) Pabst, A. Amer. Mineral. 1959, 44, 274–281.
(21) Anthony, J. W.; Bideaux, R. A.; Bladh, K. W.; Nichols M., C., Handbook of Mineralogy, Mineralogical Society of America: Chantilly 2003. vol. V.
(22) Gallina, S.; Cunsolo, V.; Saletti, R.; Muccilli, V.; Di Francesco, A.; Foti, S.; Lorenzten, A. M.; Roepstorff, P., Amino Acids 2016, 48 (7), 1569-1580.
(23) Cunsolo, V.; Fasoli, E.; Di Francesco, A.; Saletti, R.; Muccilli, V.; Gallina, S.; Righetti, P. G.; Foti, S., J Proteom 2017, 152; 58-74.
(24) UniProt: the universal protein knowledgebase, Nucleic Acids Research 2017, 45, D1, D158–D169.
(25) Cunsolo, V.; Muccilli, V.; Saletti, R.; Foti S., Eur. J. Mass Spectrom. 2011, 17, 305-320.
(26) Cunsolo, V.; Saletti, R.; Muccilli, V.; Gallina, S.; Di Francesco, A.; Foti, S., Food Res. Int. 2017, 99, 41-57.
(27) Xie, M.; Shevchenko, A.; Wang, B.; Shevchenko, A.; Wang, C.; Yang, Y., Quaternary International 2016, 426, 158-165.
(28) Keller, A.; Nesvizhskii, A. I.; Kolker, E.; Aebersold, R., Anal. Chem. 2002, 74(20), 5383-5392.
(29) Young, E., Clinical Infectious Diseases 1995, 21(2), 283-289.
(30) Helmy, Y. A.; El-Adawy, H.; Abdelwhab, E. M., Pathogens 2017, 6, 33
(31) Dunne, J.;, Evershed, R. P.; Salque, M.; Cramp, L.; Bruni, S.; Ryan, K.; Biagetti, S.;Di Lernia, S., Nature 2012, 486, 390–394.
(32) Salque, M.; Bogucki, P. I.; Pyzel, J.; Sobkowiak-Tabaka, I.; Grygiel, R.; Szmyt, M.; Evershed, R. P., Nature 2013, 493, 522–525.
(33) Moreno, E., Frontiers in Microbiology 2014, 5 (213).
(34) Hodgkins, J. M., Am.J.Phys.Anthropol. 2002, S36, 11.