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Corresponding Author: Professor Lucia Guidi,

Corresponding Author's Institution: University of Pisa

First Author: Costanza Ceccanti

Order of Authors: Costanza Ceccanti; Gabriele Rocchetti; Luigi Lucini; Gianluca Giuberti; Marco Landi; Stefano Biagiotti; Lucia Guidi

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Cover Letter

UNIVERSITA' DI PISA

DIPARTIMENTO DI SCIENZE AGRARIE, ALIMENTARI E AGRO-AMBIENTALI

Department of Agriculture, Food and Environment

Direzione
Administrative Staff
Via del Borghetto, 80
I- 56124 Pisa (Italy)
Tel. +39 050 2216090
Fax +39 050 2210606
e-mail: disaaa@agr.unipi.it
website: www.agr.unipi.it

Direttore
Director
Prof. Alberto Pardossi
e-mail: direttoredisaaa@unipi.it

Responsabile Amministrativo
Administrative Responsible
Dott.ssa Paola Giovannetti
e-mail: paola.giovannetti@unipi.it

Pisa, March 4, 2020

The Editor of

Food Chemistry

Dear **Editor**,

I'm sending you the manuscript "**Comparative phytochemical profile of the Elephant garlic (*Allium ampeloprasum* var. *holmense*) and the common garlic (*Allium sativum*) from the Val di Chiana area (Tuscany, Italy) before and following in vitro gastrointestinal digestion** by Ceccanti et al. for submission on the international journal Food Chemistry.

Thank you in advances for your cooperation.

Sincerely yours,

Lucia Guidi

Dott.ssa Lucia Guidi
Dipartimento di Scienze Agrarie, Alimentari e Agro-Ambientali
Università di Pisa
Via del Borghetto 80
56124 Pisa
Phone +39 050 2216613
FAX +39 050 2216630
Email: lucia.guidi@unipi.it

Highlights

- Elephant garlic (EG) had two-fold higher total phenolic content than common garlic (CG).
- Lipid-derived molecules, polyphenols and amino acids were the discriminative compounds in the raw bulbs.
- Sulphur-containing compounds were found in lower amounts in EG, underling the higher digestibility of this bulb.
- 4-hydroxybenzoic acid 4-*O*-glucoside, 5-nonadecylresorcinol and tryptophan were proposed as main biomarkers of EG consumption.

Comparative phytochemical profile of the Elephant garlic (*Allium ampeloprasum* var. *holmense*) and the common garlic (*Allium sativum*) from the Val di Chiana area (Tuscany, Italy) before and following *in vitro* gastrointestinal digestion

Costanza Ceccanti[a], Gabriele Rocchetti[b], Luigi Lucini[b], Gianluca Giuberti[b], Marco Landi[a,c], Stefano Biagiotti[d], Lucia Guidi[a,c]*

[a] Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy

[b] Department for Sustainable Food Process, Università Cattolica del Sacro Cuore, Via Emilia Parmense 84, 29122 Piacenza, Italy

[c] Interdepartmental Research Center Nutrafood "Nutraceuticals and Food for Health", University of Pisa, via del Borghetto 80, 56124 Pisa, Italy

[d] Telematic University Pegaso, Piazza Trieste e Trento, 48 -80132 Napoli, Italy

*Corresponding author: c.ceccanti3@studenti.unipi.it

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Abstract

This study is aimed to comparatively investigate the phytochemical profile of the common garlic (*Allium sativum* L.; CG) vs the *Allium ampeloprasum* var. *holmense*, named Elephant garlic (EG), collected in the Val di Chiana area (Tuscany, Italy), focusing on the nutritional and phytochemical properties. The results reported a lower amount of fibres in EG, underling the higher digestibility of this bulb, confirmed also to the lower sulphur containing compounds found in EG rather than in CG. Untargeted metabolomic profiling followed by supervised and unsupervised statistics allowed to depict the differences in phytochemical composition among the two bulbs, both as raw bulbs, processed following the in vitro gastrointestinal digestion process. Typical sulphur-containing compounds, such as alliin and N-gamma-glutamyl-S-allylcysteine, could notably be detected in lower amounts in EG. During the in vitro gastrointestinal digestion process, EG maintained a distinct phytochemical signature. Our findings support the distinct sensorial attributes of the bulbs.

Key words: UHPLC-QTOF mass spectrometry; food metabolomics; *in vitro* digestion; polyphenols; sulphur compounds.

41 **1.Introduction**

42 The genus *Allium* includes about 700 bulbous species characterised by high diversity,
43 considering physiology and morphology aspects. Regarding that, the elephant garlic (*Allium*
44 *ampeloprasum* var. *holmense* (Mill.) Asch. et Graebn.) is classified as a type of leek (*Allium*
45 *ampeloprasum* var. *porrum* (L.) J. Gay) but, at the same time, it is considered as the common garlic
46 (*Allium sativum* L.) in terms of shape and flavour, although being three times as large as the
47 common garlic (Kim et al., 2018a). Moreover, *A. sativum* is the economically most important
48 species belonging to the *Allium* genus and has been used for long time as food and in
49 pharmacology. This bulbous species owes its importance as antifungal, antibacterial, antiviral,
50 antitoxic and anticancer agent (Rattanachaikunsopon & Phumkhachorn, 2009; Kim et al., 2018a).
51 The bioactive compounds of garlic are reported to have a biological effect on human metabolism
52 (i.e. antithrombotic and fibrinolytic effects in blood, reducing effect of LDL cholesterol level)
53 (Steiner, Khan, Holbert & Lin, 1996). These bioactive compounds of common garlic can be divided
54 into sulphur-containing compounds and sulphur-free polyphenolic compounds. The sulphur-
55 containing compounds (i.e. alliin and its derivatives) are the main responsible for the antimicrobic
56 activity, as well as of the peculiar sensorial attributes of this bulb. In contrast, the sulphur-free
57 polyphenolic compounds play an important role to prevent the oxidative damage caused by ROS
58 (Reactive Oxygen Species) (Ma et al., 2011).

59 Nowadays, elephant garlic has been proposed as a substitute of common garlic in cooking (fresh
60 or processed) because its flavour is very close to that of common garlic but with a milder impact on
61 human breath and a better digestibility than common garlic (Block, 2011). For this reason, the
62 elephant garlic is named “kissingarlic”, “garlic for people who don’t like garlic” and “garlic-like”
63 (Lu, Ross, Powers, Aston & Rasco, 2011). In Val di Chiana, an area located in Tuscany (centre of
64 Italy), with peculiar weather and soil characteristics, elephant garlic was joined to the list of
65 Traditional Agri-food Products of the Tuscany Region (Executive Decree Tuscany Region, n. 1569
66 of April 4th, 2016

https://www.aglionevaldichiana.net/public/Documenti/Decreto_Regione_Toscana.pdf) and later to the list of Traditional Agri-food Products of Italy (G.U. n.143 of June 21th, 2016 https://www.aglionevaldichiana.net/public/Documenti/Decreto_MiPAAF.pdf) with the name "Aglione della Valdichiana".

In nutritional and nutraceutical terms, the elephant garlic features suggest a reduction in the content of sulphur-compounds among its bioactive substances, although few studies have been carried out for its characterization to date. Kim et al. (2018b) evaluated the organo-sulphur compounds in *Allium* species, showing a high content of γ-glutamyl peptides in the elephant garlic and the highest alliin content in the common garlic. Therefore, they reported a higher content of sulphur-containing volatile compounds in common garlic rather than in the elephant garlic, with the presence of 13 sulphur compounds found in garlic against 6 sulphur compounds found in the elephant garlic, even though in very low concentrations. Moreover, Najda, Błaszczyk, Winiarczyk, Dyduch and Tchórzewska (2016), analysing elephant garlic and common garlic from Poland, found a higher polyphenol content and antioxidant activity in elephant garlic bulb rather than in common garlic bulb. These authors did not find peculiar differences in terms of polyphenol profile between the two bulbs. In contrast, Lu, Ross, Powers, Aston and Rasco (2011), analysing American garlics and elephant garlics, found lower antioxidant activity in the elephant garlic rather than in the common garlic and a polyphenol content very similar in both the bulbs. The comparison of these last two studies suggests that the origin of both the bulbs may play a pivotal role in determining the polyphenol profile.

Holistic approaches like metabolomics could be very useful to describe and to differentiate the profile of bioactive compounds in garlic and elephant garlic, as well as to ensure geographical traceability (Maietti et al., 2012). This last aspect becomes relevant with the view of a hypothetic award of PDO (Protected Designation of Origin), as proposed for "Aglione della Valdichiana". In fact, the quali- quantitative differences in secondary metabolites, such as sulphur compounds and polyphenols, can better discriminate the bulbs hence identifying potential counterfeits thus ensuring

traceability. Noteworthy, a deep profiling of the phytochemicals in these two bulbs opens the possibility to further investigate nutraceutical properties rather than desired or unpleasant sensorial attributes.

In this regard, combining *in vitro* gastrointestinal digestion process with untargeted metabolomics may be useful to analyse the bioaccessibility of polyphenols and other health-related compounds. To date, limited information on the changes of bioactive compounds in garlic and/or elephant garlic during simulated gastrointestinal processes is present in literature (Bhatt & Patel, 2013; Torres-Palazzolo, Ramirez, Locatelli, Manucha, Castro & Camargo, 2018; Rosen et al., 2001). In the last few years, many research reported the bioaccessibility of health-promoting compounds, considering it as the percentage of compounds from the food sample released during the simulation of digestion (Pérez-Vicente, Gil-Izquierdo, García-Viguera, 2002; Rodríguez-Roque, Rojas-Graü, Elez-Martínez & Martín-Belloso, 2014; Rocchetti, Chiodelli, Giuberti & Lucini, 2018). The combination of *in vitro* gastrointestinal digestion with untargeted metabolomics may provide a better understanding of the main changes occurring to bioactive compounds during simulated gastrointestinal processes (Rocchetti, Chiodelli, Giuberti & Lucini, 2018).

Up to now, no detailed comparative studies of nutritional parameters and nutraceutical compounds and their bioaccessibility during the human digestion of the elephant garlic and the common garlic have been carried out. In this study, for the first time, the nutritional aspects, some mineral elements (including the sulphur) as well as phenols and secondary metabolites from untargeted metabolomics have been comparatively investigated in elephant and common garlic offered by Val di Chiana farmers. Then, the fate of garlic metabolites was investigated for the first time, using an *in vitro* gastrointestinal digestion and then an untargeted metabolomics-based approach, in order to further explore the main differences between the two bulbs from a nutritional standpoint.

2. Materials and methods

2.1. Plant material

Common garlic (CG) and elephant garlic (EG) samples were grown in Val di Chiana area and were offered by local association "Qualità e Sviluppo Rurale srl" to the Department of Agriculture, Food and Environment (DAFE) of the University of Pisa during June 2019. Both the bulbs derived from the same farm and have been cultivated in the same climatic and edaphic conditions to exclude the contribution of pedo-climatic conditions. Cloves of EG and CG were randomly selected, manually peeled and chopped in small pieces. A part of the bulb material was lyophilized for the determination of some mineral elements, whereas the fibre, the sugar contents and the untargeted metabolomics based on high-resolution mass spectrometry; the other part was freeze-dried and stored at –80 °C until analysis. All the analyses were carried out in triplicate.

2.2. Proximate analysis

A part of freeze-dried material was weighed and oven-dried at 65 °C till constant weight and the percentage of dry matter (% DM) was calculated. Then, the dried samples were used for the determination of phosphorous, potassium, calcium and magnesium concentration in both the analysed bulbs. Dry tissues were mineralized for 60 min at 220 °C using a solution of HNO_3:$HClO_4$ (2.5:1 *v/v*). Phosphorus concentration was determined colorimetrically using an Ultrospec 2100 Pro spectrophotometer (GE Healthcare Ltd., Little Chalfont, UK), following the Olsen method, whereas K, Ca and Mg with an atomic absorption spectrophotometer (Varian AA 24FS, Australia). Results were expressed as % P, K, Ca and Mg. Nitrogen, carbon, hydrogen and sulphur determinations were obtained from an Elementar Vario MICRO cube instrument.

Sugar (sucrose and glucose) quantification was carried out according to Yusof, Rasmusson and Galindo (2016) and Sotelo, Pérez, Najar-Rodriguez, Walter and Dorn (2014) with minor modifications. Sucrose and glucose were determined using K-SUFRG commercial kit (Megazyme, Wicklow, Ireland), following the manufacturer's protocol. Results were expressed as $g \cdot 100\ g^{-1}$ dry weight (DW).

143 Protein determination was performed using the spectrophotometer and the Protein Assay Kit II®
144 (Bio- Rad). Using a bovine serum albumin standard curve, the results were expressed as mg protein
145 per g fresh weight (FW).
146 The crude fibre and the fibre fractions [neutral detergent fibre (NDF) and acid detergent fibre
147 (ADF), acid detergent lignin (ADL), hemicellulose and cellulose] were analysed according to the
148 method described by Van Soest, Robertson and Lewis (1991) using the instrument ANKOM
149 (ANKOM 65 rpm agitation). Results were expressed as percentage (%).
150 *2.3. Total phenolic content*
151 For total phenolic extraction, the fresh material (1 g) was finely ground in a mortar, suspended in
152 4 mL 80% aqueous methanol (*v/v*), and placed in an ultrasonic water bath (Digital ultrasonic
153 Cleaner, DU-45, Argo-Lab, Modena, Italy) at 4 °C for 30 min. For the determination of total
154 phenolic content, the solution was centrifuged at 10,000 *g* for 7 min and an amount of the
155 supernatant was added to Folin-Ciocalteu reagent, following Folin-Ciocalteu method described by
156 Dewanto, Wu, Adom and Liu (2002) with slight modifications. Consequently, 1.25 mL Na_2CO_3 7%
157 (*w/v*) were added to the solution and samples were incubated for 90 min in dark conditions. The
158 increase of absorbance was measured spectrophotometrically at 760 nm wavelength against a blank.
159 The total phenolic content was expressed as milligrams equivalents of gallic acid per g of fresh
160 weight (mg GAE g^{-1} FW).
161 *2.4. Extraction and untargeted metabolomic profiling by UHPLC-QTOF mass spectrometry*
162 One gram of each lyophilized samples was homogenized in 10 mL of 0.1% HCOOH in 80%
163 (*v/v*) methanol solution using a homogenizer-assisted extraction system (Ultra-Turrax, IkaT25,
164 Staufen, Germany) as previously reported (Rocchetti, Bhumireddy, Giuberti, Mandal, Lucini &
165 Wishart, 2019). Afterwards, samples were centrifuged at 6,000 *g* for 15 min at 4 °C and then
166 supernatants were filtered with 0.22 nm cellulose syringe filters in vials which were stored at –18
167 °C until analysis.

Subsequently, the untargeted metabolomic profile of both bulbs was investigated using UHPLC-ESI-QTOF mass spectrometry, as previously described (Rocchetti, Bhumireddy, Giuberti, Mandal, Lucini & Wishart, 2019). Briefly, chromatography was carried out in the reverse phase mode using an Agilent Zorbax eclipse plus C18 column and a water-acetonitrile gradient solution (from 6% up to 90% acetonitrile in 33 min) for separation. For mass spectrometry detection, QTOF was operated in positive scan mode to acquire ions in the range 50–1200 *m/z*. A volume of 6 µL of each extracted sample was injected using nitrogen as both sheath gas (10 L min^{-1} at 350 °C) and drying gas (8 L min^{-1} at 330 °C). The annotation of garlic and elephant garlic metabolites was achieved using the software Profinder B.07 from Agilent Technologies, according to the "find-by-formula" algorithm. In particular, the annotations were recursively achieved against the comprehensive database FoodDB (www.fooddb.ca), one of the most comprehensive databases available in literature for untargeted studies in food metabolomics and using the entire isotopic profile with a maximum of 5 ppm for mass accuracy. Therefore, in our experimental conditions, a Level 2 of compound identification was achieved as set out by the COSMOS Metabolomics Standards Initiative (Rocchetti, Giuberti, Busconi, Marocco, Trevisan, & Lucini, 2020; Salek, Neumann, Schober, Hummel, Billiau & Steinbeck, 2015; Schrimpe-Rutledge, Codreanu, Sherrod & McLean, 2016). The obtained dataset was further used for statistics and chemometrics.

2.5. Simulated in vitro gastrointestinal digestion process

The *in vitro* gastrointestinal digestion, simulating the oral, gastric and pancreatic digestion phases, was applied to lyophilized samples according to the static method detailed by Minekus et al. (2014). Samples (250 mg) were homogenized with 175 µL simulated salivary fluid (SSF) (Minekus et al., 2014) at pH 7.0, 25 µL salivary α-amylase (from human saliva Type IX-A, Sigma) solution made up in SSF electrolyte stock solution, 1.25 µL $CaCl_2$ 0.3 M and 48.75 µL water. The oral step was run at 37 °C for 2 min. Then, the oral bolus samples were mixed (ratio 1:1) with 375 µL simulated gastric fluid (SGF) at pH 3.0, 80 µL porcine pepsin stock solution (25,000 U mL^{-1}) made up in SGF electrolyte stock solution (pepsin from porcin gastric mucosa, Sigma), 0.25 µL $CaCl_2$ 0.3

194 M, 10 µL HCl 1 M to adjust pH to 3.0, and rest of volume with water. The gastric phase was
195 carried out for 2 h at 37 °C. Then, gastric chyme was mixed (1:1) with 550 µL simulated intestinal
196 fluid (SIF) electrolyte stock solution consisting at pH 7.0, 250 µL of a pancreatin solution (800 U
197 ml^{-1}) based on pancreatin α-amylase activity made up in SIF electrolyte stock solution (pancreatin
198 from porcine pancreas, Sigma), 125 µL fresh bile (160 mM in fresh bile), 2 µL $CaCl_2$ 0.3 M, 7.5 µL
199 NaOH 1 M to adjust pH to 7.0, and water filling the rest of the volume. The intestinal phase was
200 carried out for 2 h at 37 °C. At selected time points (i.e., gastric and pancreatic phases)
201 corresponding digestion sample tubes for each material were cooled on ice to stop the reaction. The
202 experiment was performed in triplicate. Finally, to depict the fate of bioactive compounds during
203 the *in vitro* gastrointestinal digestion, digested samples were prepared and analysed using an
204 untargeted UHPLC-ESI/QTOF mass spectrometry as above reported for the undigested materials.
205 *2.6. Statistical analysis and chemometrics*
206 Results of proximate analysis, minerals and total phenolic content were compared with Student *t*-
207 test ($P \leq 0.05$). Data are expressed as mean ± standard deviation. This statistical analysis was
208 performed using GraphPad (GraphPad, La Jolla, CA, USA).
209 Metabolomic data were interpreted using Agilent Mass Profiler Professional B.12.06 (from
210 Agilent Technologies). Compounds were filtered by abundance and by frequency (only those
211 compounds with an area > 5000 counts and appearing in 100% of samples in at least one condition
212 were considered), normalized at the 75^{th} percentile and baselined to the median of each compound
213 in all samples. The unsupervised hierarchical cluster analysis (HCA – Euclidean distance) was then
214 used to naively group samples, according to intrinsic similarities in metabolomic profile (Rocchetti,
215 Bhumireddy, Giuberti, Mandal, Lucini & Wishart, 2019). Afterwards, the dataset was exported into
216 SIMCA 13 (Umetrics, Malmo, Sweden), Pareto scaled and elaborated for orthogonal partial least
217 squared discriminant analysis (OPLS-DA) supervised modelling, considering the combination of
218 "sample type x digestion phase" as class membership criterion. Finally, the variable importance in
219 projection (VIP analysis) method was used to evaluate the discrimination potential of the different

220 metabolites (i.e., those compounds possessing a VIP score > 1) and a Fold-Change analysis (FC >
221 5) was combined with ANOVA ($P < 0.01$, Bonferroni multiple testing correction) in Volcano plot
222 to point out differential metabolites between raw common garlic and elephant garlic samples.

223 **3.Results and discussion**

224 *3.1. Nutritional results*

225 Results of the proximate analysis of CG and EG cloves cultivated in Val di Chiana area were
226 reported in Table 1. No differences were found in sugars (glucose and fructose) content of both the
227 analysed bulbs, whereas a higher protein content was recorded in EG than in CG. Even the dry
228 matter and the moisture contents were different comparing both the bulbs, with EG reporting a
229 higher moisture content than CG (Table 1). NDF and cellulose resulted significantly lower in the
230 EG as compared to CG, underling the superior digestibility of this bulb (Baer, Rumpler, Miles &
231 Fahei, 1997). These differences probably reflect the differences in variety and species among the
232 two analysed bulbs as well as the different conditions of growth. Mineral content in the bulb
233 samples differed significantly for P and K exclusively and these minerals were lower in the EG as
234 compared to the CG (Table 1). An interesting result is the higher content of the sulphur element
235 found in EG (+ 41%) as compared with CG. Mineral results are comparable with the findings in
236 garlic of Sajid, Butt, Shehzad and Tanweer (2014) and Odebunmi, Oluwaniyi and Bashiru (2010).

237 *3.2. Phytochemical discrimination of raw samples*

238 The first result that need to be highlighted in terms of functional components is represented by
239 the total phenolic content, which was found significantly higher in EG than in CG (Fig. 1). This
240 result is in agreement with the findings by Lu, Ross, Powers, Aston and Rasco, (2011) whilst
241 contrasting outcomes that have been provided by Najda, Błaszczyk, Winiarczyk, Dyduch and
242 Tchórzewska (2016), which reported very low total phenolic content in both bulb types. Thereafter,
243 untargeted metabolomics based on UHPLC-QTOF mass spectrometry was used to investigate in a
244 comprehensive way the differences and similarities in the phytochemical of the two bulb samples,
245 both before and after the *in vitro* gastrointestinal digestion process. Overall, this approach allowed

246 to putatively annotate 2745 mass features that were classified according to the database FoodDB,
247 together with individual abundances and composite mass spectra (Table S1). As first evaluation, a
248 Volcano plot was produced to compare raw EG *vs* CG by coupling ANOVA ($P \leq 0.01$) and Fold-
249 Change (cut-off ≥ 5) analysis (Table S2). As it can be observed, 161 metabolite species were found
250 to discriminate EG and CG, thus suggesting distinctive chemical fingerprints of the raw matrices
251 before *in vitro* gastrointestinal digestion. Interestingly, only the 22% of the discriminant markers
252 were found to be down-accumulated in EG when compared with CG; among these compounds, we
253 found typical compounds characterizing garlic such as alliin (belonging to α-amino acids), together
254 with two isomeric dipeptides, namely N-gamma-glutamyl-*S*-allylcysteine and N-gamma-glutamyl-
255 *S*-cis-(1-propenyl)cysteine. The down-accumulation of sulphur, containing compounds such as N-
256 gamma-glutamyl-S-allylcysteine and N-gamma-glutamyl-S-cis-(1-propenyl)cysteine, characterized
257 EG (Table S2) and could explain the higher total phenolic content observed in EG compared to CG
258 (Fig. 1) since Phan, Netzel, Chhim, Netzel and Sultanbawa (2019) reported that the total phenolic
259 content can decrease with the increase in organosulfur compounds and terpenoid substances in
260 mature garlic bulbs. In addition, a significant down-accumulation of 6 polyphenols and 8 prenol
261 lipids was outlined in EG when compared with CG (Table S2).
262 Overall, it has been suggested that the biological and health promoting properties of garlic
263 primarily derived from its polyphenols and organosulfur compounds (Phan, Netzel, Chhim, Netzel
264 & Sultanbawa, 2019). These trends were confirmed by Kim et al. (2018b), reporting a high level of
265 bioactive γ-glutamyl peptides in both EG and CG. However, considering that organosulfur
266 compounds are extremely unstable and susceptible to further transformation into volatile
267 compounds (such as allicin and diallyl-sulfides), recent attention has been placed on polyphenols
268 due to their potential role in health-related benefits to humans. On the other hand, 125 additional
269 compounds (i.e., the remaining 78% of discriminant metabolites outlined by Volcano plot analysis)
270 were proposed in this work as chemical markers of EG. The most represented classes among the
271 discriminant markers were those of steroids and derivatives (27 compounds), glycero- and

glycerophospho- lipids (24 compounds), prenol lipids (13 compounds), polyphenols (12 compounds), amino acids and derivatives (8 compounds), organooxygen compounds (8 annotations) and fatty acyls (7 compounds). Therefore, our findings revealed a large presence of compounds belonging to lipids and steroids (mainly saponins) in EG. These results are not surprising; in fact, plants belonging to the genus *Allium* have been previously reported as a good source of bioactive saponin compounds, responsible for many of their reported pharmacological activities (e.g., antiproliferative, antifungal and antispasmodic activities). In this regard, previous studies (Lanzotti, 2005; Petropoulos, Fernandes, Ntatsi, Petrotos, Barros &Ferreira, 2018) showed that saponins characterized by furostane, spirostane, cholestane, and oleane-type structures are widely represented in *Allium*, thus confirming our findings.

Additionally, an abundance of N-gamma-L-glutamyl-L-methionine and eruboside B was noticed in the EG sample (Table S1). N-gamma-L-glutamyl-L-methionine belongs to the organosulphur compounds with important biological effects (lipid-lowering, antidiabetic, anticancer, anti-asthmatic, antiplatelet and anti-atherosclerotic activities) already described by Kim et al. (2018b), while eruboside B is a typical garlic compound that improve the antimicrobic properties of *Allium* vegetables (Nakamoto, Kunimura, Suzuki & Kodera, 2020). However, it is important to take into account that genotype has a great impact on the metabolomic profile of garlic and elephant garlic bulbs (Najda, Błaszczyk, Winiarczyk, Dyduch & Tchórzewska, 2016); therefore, both genotype and pedoclimatic conditions represent two critical parameters that need to be taken always into account to reach the quality improvement of the final products.

3.3. In vitro gastrointestinal digestion and discrimination of both bulb samples

Moreover, once the differences between EG and CG were represented in the raw matrices, multivariate statistics (based on both unsupervised and supervised methods) were used to depict the changes occurring during the *in vitro* gastrointestinal digestion process of both bulbs. On this matter, unsupervised hierarchical cluster analysis (i.e., HCA) carried out on the UHPLC-QTOF mass spectrometry data allowed to identify a clear separation trends (Fig. 2), outlining a strong

298 impact of each digestion phase (i.e., both gastric and pancreatic phases) on the phytochemical
299 composition of both bulb samples. Initial differences in phytochemical profiles between EG and CG
300 may be notably conserved, even during the digestion process. The results from unsupervised
301 statistics suggest that a further application of the OPLS-DA score plot would help to point out the
302 most discriminant compounds, driving the trends observed. The OPLS-DA score plot illustrating
303 modification of the metabolite profiles, moves from raw samples to digested samples and it is
304 provided in Fig. 3. This supervised model allowed to confirm the unsupervised findings (Fig. 2).
305 Firstly, a confirmation of the differences existing on the raw matrices was noticed on the left part of
306 the graph, confirming the results of Volcano plots previously discussed (Table S2). Besides, the
307 second latent vector t[2] showed a clear impact of both gastric and pancreatic phases of digestion,
308 driven also by the different matrix incubated (i.e., EG *vs* CG) (Fig. 3).
309 Furthermore, a variable selection method (VIP; variable importance in projection) was used to
310 reduce the numbers of variables and better explain the differences observed over the *in vitro*
311 digestion process (Table 2). Markers, that assigned a VIP score > 1, are organized in chemical class
312 and reported together with the Log Fold-Change (FC) values for each main comparison (i.e., EG *vs*
313 CG on raw, gastric and pancreatic samples) (Table 2). Overall, 85 compounds matched this
314 criterion (including some isomeric compounds), mainly belonging to the classes of polyphenols,
315 amino acids, benzenoids, sulphur containing compounds, fatty acyls, glycerophospholipids,
316 heteroaromatic compounds, indoles, prenol lipids, pyrrolizines, quinolines, steroids and derivatives,
317 tetrahydrofurans and other compounds. Overall, when considering the comparison EG *vs* CG during
318 the gastric phase of digestion, the most affected compounds were found to be hovenidulcigenin A (a
319 prenol lipid; LogFC = –18.55), LysoPC(18:1(11Z)) (a glycerophospholipid; LogFC = –18.36) and
320 4-[(2-hydroxy-1-naphthalenyl)azo]benzenesulfonic acid (a benzenoid; LogFC = –9.28). Until
321 today, the bioactive and pharmacological role of the hovenidulcigenin A and 4-[(2-hydroxy-1-
322 naphthalenyl)azo]benzenesulfonic acid has not been established in literature, while the LysoPC
323 species play an important role as lipid mediators in cellular responses and pathophysiology; in fact,

they are involved in the activation of inflammatory responses and their potential role as vaccine has been discussed (Wi, Seo, Cho, Nam & Park, 2014).

Moreover, the gastric phase of digestion mainly affected CG polyphenols composition. In fact, the discriminant compounds, namely 4-hydroxybenzoic acid 4-*O*-glucoside, phloretin and butein/naringenin were characterized in EG during the gastric phase by LogFC values > 2. The 4-hydroxybenzoic acid is a phenolic acid which can be converted into more useful compounds such as resveratrol, muconic acid, gastrodin, ubiquinone with a wide variety of biological and pharmaceutical activities as antibacterial, antioxidant, anticancer, hypolipidemic, prevention of heart diseases activities (Wang, Bilal, Hu, Wang, & Zhang, 2018); phloretin is one of the best known dihydrochalcone with antifungal, antiviral, anti-inflammatory, estrogenic, anticancer and estrogenic activity and able to improve the fluidity of biological membranes, increasing the penetration of drugs (Behzad, Sureda, Barreca, Nabavi, Rastrelli, & Nabavi, 2017) and butein/naringenin are flavonoids with important healthy roles such as antioxidant, antitumor, cardioprotective, antiviral and antibacterial activity (Salehei et al., 2019; Bordoloi et al., 2019).

Finally, analysing the results of the pancreatic phase, EG was characterized by an overall down-accumulation of several lipid-derived compounds, mainly belonging to fatty acyls (6 annotated compounds) and steroids (4 annotated compounds). Afterwards, the polyphenol 4-hydroxybenzoic acid 4-*O*-glucoside showed significantly higher LogFC values (i.e., 9.95) in EG when compared to CG also during pancreatic digestion phase. Other metabolites characterizing EG during pancreatic phase of digestion were pantoyllactone glucoside (fatty acyls), (2S,4S)-monatin (alpha amino acid), followed by several isomeric compounds classified as "other compounds" (Table 2). The OPLS-DA model allowed to detect also discriminant compounds characterizing the raw bulbs; for example, AS 1-5 (belonging to the class of organic compounds known as glycosyl-n-acylsphingosines and typical in garlic) was found to characterized raw EG (LogFC = 19.91) sample, but it was heavily affected by the *in vitro* digestion process (Table 2).

Regarding the potential biomarkers proposed in this work, 4-hydroxybenzoic acid 4-*O*-glucoside, 5-nonadecylresorcinol and tryptophan were found to be among the most representative compounds in the undigested EG (Table 2). According to literature, phenolic acids such as 4-hydroxybenzoic acid 4-*O*-glucoside can be released from the food matrix in the stomach, further enhancing their release and absorption. The absorption of phenolic acids from beverages occurs at a higher extent than from solid food matrices. In fact, most of phenolic acids exist as conjugated or bound to dietary fibre, thus reaching the colon and becoming available for further metabolism by gut microbiota (Rocchetti et al., 2019; Mosele, Maciá & Motilva, 2015). In addition, hydrolysis by intestinal or microbial esterases can promote the release of phenolic acids in the intestine, supporting their absorption across the gastrointestinal barrier and enter the peripheral blood circulation. Therefore, besides a clear species effect, our findings suggested that consuming EG could be a valid strategy to promote the bioaccessibility of bioactive phenolic acids, such as 4-hydroxybenzoic acid 4-*O*-glucoside. Another phenolic compound namely 5-nonadecylresorcinol is an alkylresorcinol with a cytotoxic activity, and it is able to significantly inhibit the growth of various cell lines, such as lung cancer cells, human epithelial cells, breast cancer cells, epithelioid cervix carcinoma cells and human central nervous system tumour cell line (Liu, Winter, Stevenson, Morris & Leach, 2012). The tryptophan is an essential amino acid for the human health because of human body is not able to synthetize it. Recently, Li et al., (2019) reported a high antioxidant activity of this amino acid and the addiction of tryptophan to the walnut protein-derived peptides had a potential in the inhibition of xanthine oxidase, a critical enzyme in human health, because of its ability to catalyse the oxidation of hypoxanthine to xanthine and xanthine to uric acid. Therefore, the inhibition of the xanthine oxidase may be able to alleviate the development of hyperuricemia (Li et al., 2019). The different accumulation of health-related compounds in both analysed bulbs, during the *in vitro* gastrointestinal digestion process, supports the potential exploitation of EG as source of bioactive compounds with important biological and pharmacological roles in addition to CG.

Conclusions

This study is aimed at comparing phytochemical compounds of CG and EG from the Val di Chiana area (Tuscany, Italy), with a focus on bioactive compounds. In fact, despite these two bulbs could seem similar in shape and aspect, they belong to different *Allium* species. The differences were reflected also in the proximate results and produced two distinctive metabolomic profiles. Firstly, the EG samples showed a lower fibre content, which is supportive for a higher digestibility of this bulb, confirmed successively to the lower sulphur-containing compounds found in EG rather than in CG. The total phenolic content definitely resulted two-fold higher in the EG than in the CG. Finally, the untargeted metabolomic approach using UHPLC-QTOF mass spectrometry allowed to identify a higher number of organosulphur compounds in CG than in EG. These sulphur-containing metabolites are responsible for several biological effects of *Allium* vegetables, in addition to some unpleasant attributes that garlic leaves in breath of humans. This opens the possibility to use EG to replace CG with food tasting purposes. The untargeted metabolomic approach also identified 125 key metabolites which were most representative in raw EG, mainly including lipid-derived molecules, polyphenols and amino acid derived compounds. In addition, clear differences were outlined between EG and CG during *in vitro* gastrointestinal digestion process, underling a higher impact of the gastric phases on the phytochemical modifications, with 4-hydroxybenzoic acid 4-*O*-glucoside, 5-nonadecylresorcinol and tryptophan proposed as biomarker of the consumption of EG. Taken together, the present findings indicate distinct phytochemical profiles among EG and CG, with distinct bioactive and functional properties and sensorial attributes. Our dataset also contributes to identify some putative biomarkers that could be exploited for the traceability of *Allium ampeloprasum* var. *holmense*.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Costanza Ceccanti: Conceptualization, Data curation, Formal analysis, Writing - original draft. **Gabriele Rocchetti**: Conceptualization, Methodology, Investigation, Formal analysis, Writing - review & editing. **Marco Landi**: Conceptualization - review & editing, Supervision. **Gianluca Giuberti**: Methodology, Investigation, Formal analysis, Writing- review & editing. **Luigi Lucini**: Validation - review & editing, Supervision. **Stefano Biagiotti**: Resources, Supervision. **Lucia Guidi**: Validation - review & editing, Supervision.

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Appendix A. Supplementary data

The following are the Supplementary data to this article:

- Table S1
- Table S2

References

1. Baer, D. J., Rumpler, W. V., Miles, C. W., & Fahey J. G. C. (1997). Dietary fiber decreases the metabolizable energy content and nutrient digestibility of mixed diets fed to humans. *The Journal of Nutrition*, *127*(4), 579–586.
2. Behzad, S., Sureda, A., Barreca, D., Nabavi, S. F., Rastrelli, L., & Nabavi, S. M. (2017). Health effects of phloretin: from chemistry to medicine. *Phytochemistry reviews*, *16*(3), 527–533.
3. Bhatt, A. & Patel, V. (2013). Antioxidant activity of garlic using conventional extraction and in vitro gastrointestinal digestion. *Free Radicals and Antioxidants*, *3*(1), 30–34.

4. Block, E. (2011). Challenges and artifact concerns in analysis of volatile sulfur compounds. In Block E. (Eds.), *Volatile sulfur compounds in food* (pp. 35–63). American Chemical Society.
5. Bordoloi, D., Monisha, J., Roy, N. K., Padmavathi, G., Banik, K., Harsha, C., Wang, H., Kumar, A. P., Arfuso, F., & Kunnumakkara, A. B. (2019). An investigation on the therapeutic potential of butein, a tretrahydroxychalcone against human oral squamous cell carcinoma. *Asian Pacific Journal of Cancer Prevention*, *20*(11), 3437–3446.
6. Dewanto, V., Wu, X., Adom, K. K., & Liu, R. H. (2002). Thermal processing enhances the nutritional value of tomatoes by increasing total antioxidant activity. *Journal of Agricultural and Food Chemistry*, *50*(10), 3010–3014.
7. Kim, J. W., Jeong, D., Lee, Y., Hahn, D., Nam, J. O., Lee, W. Y., Hong D. H., Kim, S. R., Ha, & Y. S. (2018a). Development and metabolite profiling of elephant garlic vinegar. *Journal of Microbiology and Biotechnology*, *28*(1), 50–58.
8. Kim, S., Kim, D. B., Jin, W., Park, J., Yoon, W., Lee, Y., Kim, S., Lee, S., Kim, S., Lee, O. H., Shin, D., & Yoo, M. (2018b). Comparative studies of bioactive organosulphur compounds and antioxidant activities in garlic (*Allium sativum* L.), elephant garlic (*Allium ampeloprasum* L.) and onion (*Allium cepa* L.). *Natural product research*, *32*(10), 1193–1197.
9. Lanzotti, V. (2005) Bioactive Saponins from *Allium* and *Aster* Plants. *Phytochemistry Reviews*, *4*, 95–110.
10. Li, Q., Shi, C., Wang, M., Zhou, M., Liang, M., Zhang, T., Yuan, E., Wang, Z., Yao, M., & Ren, J. (2019). Tryptophan residue enhances in vitro walnut protein-derived peptides exerting xanthine oxidase inhibition and antioxidant activities. *Journal of Functional Foods*, *53*, 276–285.
11. Liu, L., Winter, K. M., Stevenson, L., Morris, C., & Leach, D. N. (2012). Wheat bran lipophilic compounds with in vitro anticancer effects. *Food Chemistry*, *130*(1), 156–164.

12. Lu, X., Ross, C. F., Powers, J. R., Aston, D. E., & Rasco, B. A. (2011). Determination of total phenolic content and antioxidant activity of Garlic (*Allium sativum*) and Elephant Garlic (*Allium ampeloprasum*) by attenuated total reflectance–fourier transformed infrared spectroscopy. *Journal of Agricultural and Food Chemistry, 59*(10), 5215–5221.
13. Ma, Y., Song, D., Wang, Z., Jiang, J., Jiang, T., Cui, F., & Fan, X. (2011). Effect of ultrahigh pressure treatment on volatile compounds in garlic. *Journal of Food Process Engineering*, *34*(6), 1915–1930.
14. Maietti, A., Tedeschi, P., Stagno, C., Bordiga, M., Travaglia, F., Locatelli, M., Arlorio, M., & Brandolini, V. (2012). Analytical traceability of melon (*Cucumis melo* var *reticulatus*): Proximate composition, bioactive compounds, and antioxidant capacity in relation to cultivar, plant physiology state, and seasonal variability. *Journal of Food Science, 77*(6), C646–C652.
15. Minekus, M., Alminger, M., Alvito, P. M., Ballance, S., Bohn, T. O. R. S. T. E. N., Bourlieu, C., Carrière F., Boutrou, R., Corredig, M., Dupont, D., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D. J., Ménard, O., Recio, I., Santos, C. N., Singh, R. P., Vegarud, G. E., Wickham, M. S., Weitschies, W., & Brodkorb, W. (2014). A standardised static in vitro digestion method suitable for food–an international consensus. *Food & Function*, *5*(6), 1113–1124.
16. Mosele J. I., Maciá, A., & Motilva, M. J. (2015) Metabolic and microbial modulation of the large intestine ecosystem by non-absorbed diet phenolic compounds: A review. *Molecules*, *20*, 17429–17468
17. Najda, A., Błaszczyk, L., Winiarczyk, K., Dyduch, J., & Tchórzewska, D. (2016). Comparative studies of nutritional and health-enhancing properties in the “garlic-like” plant *Allium ampeloprasum* var. *ampeloprasum* (GHG-L) and *A. sativum*. *Scientia Horticulturae*, *201*, 247–255.

18. Nakamoto, M., Kunimura, K., Suzuki, J. I.; & Kodera, Y. (2020). Antimicrobial properties of hydrophobic compounds in garlic: allicin, vinyldithiin, ajoene and diallyl polysulfides. *Experimental and Therapeutic Medicine, 19*(2), 1550–1553.
19. Odebunmi, E. O., Oluwaniyi, O. O., & Bashiru, M. O. (2010). Comparative proximate analysis of some food condiments. *Journal of Applied Sciences Research*, *6*(3), 272–274.
20. Pérez-Vicente, A., Gil-Izquierdo, A., & García-Viguera, C. (2002). *In vitro* gastrointestinal digestion study of pomegranate juice phenolic compounds, anthocyanins, and vitamin C. *Journal of Agricultural and Food Chemistry*, *50*(8), 2308–2312.
21. Petropoulos, S.A., Fernandes, Â., Ntatsi, G., Petrotos, K., Barros, L., & Ferreira, I. C. F. R. (2018) Nutritional value, chemical characterization and bulb morphology of Greek garlic landraces. *Molecules*, *23*, 319.
22. Phan, A. D. T., Netzel, G., Chhim, P., Netzel, M. E., & Sultanbawa, Y. (2019). Phytochemical characteristics and antimicrobial activity of Australian grown garlic (*Allium sativum* L.) Cultivars. *Foods*, *8*(9), 358.
23. Rattanachaikunsopon, P., & Phumkhachorn, P. (2009). Antimicrobial activity of elephant garlic oil against Vibrio cholerae in vitro and in a food model. *Bioscience, Biotechnology, and Biochemistry*, *73*(7), 1623–1627.
24. Rocchetti, G., Giuberti, G., Busconi, M., Marocco, A., Trevisan, M., & Lucini, L. (2020). Pigmented sorghum polyphenols as potential inhibitors of starch digestibility: An in vitro study combining starch digestion and untargeted metabolomics. *Food Chemistry*, *312*, 126077.
25. Rocchetti, G., Lucini, L., Giuberti, G., Bhumireddy, S. R., Mandal, R., Trevisan, M., & Wishart, D. S. (2019). Transformation of polyphenols found in pigmented gluten-free flours during in vitro large intestinal fermentation. *Food Chemistry*, *298*, 125068.
26. Rocchetti, G., Bhumireddy, S. R., Giuberti, G., Mandal, R., Lucini, L., & Wishart, D. S. (2019). Edible nuts deliver polyphenols and their transformation products to the large

intestine: An in vitro fermentation model combining targeted/untargeted metabolomics. *Food Research International*, *116*, 786–794.

27. Rocchetti, G., Chiodelli, G., Giuberti, G., & Lucini, L. (2018). Bioaccessibility of phenolic compounds following *in vitro* large intestine fermentation of nuts for human consumption. *Food Chemistry*, *245*, 633–640.
28. Rodríguez-Roque, M. J., Rojas-Graü, M. A., Elez-Martínez, P., & Martín-Belloso, O. (2014). *In vitro* bioaccessibility of health-related compounds from a blended fruit juice–soymilk beverage: Influence of the food matrix. *Journal of Functional Foods*, *7*, 161–169.
29. Rosen, R. T., Hiserodt, R. D., Fukuda, E. K., Ruiz, R. J., Zhou, Z., Lech, J., Rosen, S. L, & Hartman, T. G. (2001). Determination of allicin, S-allylcysteine and volatile metabolites of garlic in breath, plasma or simulated gastric fluids. *The Journal of Nutrition*, *131*(3), 968S–971S.
30. Sajid, M., Butt, M. S., Shehzad, A., & Tanweer, S. (2014). Chemical and mineral analysis of garlic: a golden herb. *Pakistan Journal of Food Sciences*, *24*(1), 108–110.
31. Salehi, B., Fokou, P. V. T., Sharifi-Rad, M., Zucca, P., Pezzani, R., Martins, N., & Sharifi-Rad, J. (2019). The therapeutic potential of naringenin: a review of clinical trials. *Pharmaceuticals*, *12*(1), 11.
32. Salek, R. M., Neumann, S., Schober, D., Hummel, J., Billiau, K., & Steinbeck, C. (2015) Coordination of Standards in MetabOlomicS (COSMOS): Facilitating integrated metabolomics data access. *Metabolomics*, *11*, 1587–1597.
33. Schrimpe-Rutledge, A. C., Codreanu, S. G., Sherrod, S. D., & McLean, J. A. (2016) Untargeted metabolomics strategies – Challenges and emerging directions. *Journal of the American Society for Mass Spectrometry*, *27*, 1897–1905.
34. Sotelo, P., Pérez, E., Najar-Rodriguez, A., Walter, A., & Dorn, S. (2014). Brassica plant responses to mild herbivore stress elicited by two specialist insects from different feeding guilds. *Journal of Chemical Ecology*, *40*(2), 136–149.

35. Steiner, M., Khan, A. H., Holbert, D., & Lin, R. I. (1996). A double-blind crossover study in moderately hypercholesterolemic men that compared the effect of aged garlic extract and placebo administration on blood lipids. *The American Journal of Clinical Nutrition*, *64*(6), 866–870.

36. Torres-Palazzolo, C., Ramirez, D., Locatelli, D., Manucha, W., Castro, C., & Camargo, A. (2018). Bioaccessibility and permeability of bioactive compounds in raw and cooked garlic. *Journal of Food Composition and Analysis*, *70*, 49–53.

37. Van Soest, P. V., Robertson, J. B.; & Lewis, B. A. (1991). Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *Journal of Dairy Science*, *74*(10), 3583–3597.

38. Wang, S., Bilal, M., Hu, H., Wang, W., & Zhang, X. (2018). 4-Hydroxybenzoic acid—a versatile platform intermediate for value-added compounds. *Applied Microbiology and Biotechnology*, *102*(8), 3561–3571.

39. Wi, S. J., Seo, yS., Cho, K., Nam, M. H., & Park, K. Y. (2014). Lysophosphatidylcholine enhances susceptibility in signaling pathway against pathogen infection through biphasic production of reactive oxygen species and ethylene in tobacco plants. *Phytochemistry*, *104*, 48–59.

40. Yusof, N. L., Rasmusson, A. G., & Galindo, F. G. (2016). Reduction of the nitrate content in baby spinach leaves by vacuum impregnation with sucrose. *Food and Bioprocess Technology*, *9*(8), 1358–1366.

551 **Figure Captions**

552 **Figure 1**. Total phenolic content of common garlic and elephant garlic. Data were compared
553 with Student *t*-test ($P \leq 0.05$). Significance ***: $P \leq 0.001$.

554

555 **Figure 2.** Unsupervised hierarchical cluster analysis (HCA) based on Fold-Change heat map
556 (similarity: Euclidean; linkage rule: ward) for raw and *in vitro* digested elephant garlic (EG)
557 and common garlic (CG) samples.

558

559 **Figure 3.** Supervised OPLS-DA prediction model for raw and *in vitro* digested elephant
560 garlic (EG) and common garlic (CG) samples.

561

562

563 **Table 1**. Proximate analysis and minerals of edible garlic and elephant garlic cloves. Data were compared with Student *t*-test ($P \leq 0.05$).

564 Significance ns: not significant; *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$ for the interaction of factors.

Proximate composition		Unit	Garlic (*Allium sativum*)	Elephant garlic (*Allium ampeloprasum* var. *holmense*)	Significance
Protein		g 100 g^{-1} FW	0.98±0.05	1.22 ± 0,26	*
Carbohydrate	Glucose	g 100 g^{-1} DW	3.55±0.52	3.36 ± 0.14	ns
	Sucrose		0.11±0.04	0.11 ± 0.03	ns
Dietary fibre	Neutral detergent fibre (NDF)	%	9.98±0.16	8.46 ±0.30	**
	Acid detergent fibre (ADF)		9.09±0.40	8.58 ± 1.09	ns
	Acid detergent lignin (ADL)		3.91±0.34	3.25 ± 0.31	ns
	Hemicellulose		0.48±0.33	0.55 ± 0.12	ns
	Cellulose		2.06 ± 0.23	1.29 ± 0.04	**
Minerals	Nitrogen (N)	%	2.64 ± 0.26	1.88 ± 0.06	**
	Carbon (C)		41.29 ± 0.26	42.38 ± 0.28	**
	Hydrogen (H)		6.56 ± 0.02	6.68 ± 0.04	*
	Sulphur (S)		0.38 ± 0.01	0.65 ±0.18	*
	Phosphorus (P)		1.17 ± 0.04	0.59 ± 0.01	***
	Potassium (K)		1.04 ± 0.11	0.76 ± 0.02	*
	Calcium (Ca)		0.21 ± 0.01	0.23 ± 0.02	ns
	Magnesium (Mg)		0.05 ± <0.01	0.06 ± <0.01	ns
Moisture content		%	62.13 ± 0.57	67.23 ± 0.67	***
Dry matter		%	37.87 ± 0.57	32.77 ± 0.67	***

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Table 2. Bioactive compounds identified by VIP (Variable Importance in Projection) selection method following OPLS-DA model, in the raw matrix of elephant garlic and common garlic and during the *in vitro* gastrointestinal digestion of the two analysed bulbs. Compounds are provided together with VIP scores (measure of variable's importance in the OPLS-DA model) and Log Fold-Change in raw material and during different phases (gastric and pancreatic phases) of the *in vitro* digestion. [1]ns: not significative.

Class	Metabolites	VIP score (OPLS-DA)	LogFC [EG *vs* CG] raw	LogFC [EG *vs* CG] gastric	LogFC [EG *vs* CG] pancreatic
Polyphenols	4-Hydroxybenzoic acid 4-O-glucoside	1.02±0.56	11.42	2.16	9.95
	Rosmarinic acid	1.06±0.25	–18.70	–0.83	ns
	Sinapoylspermine	1.22±0.23	[1]ns	–0.37	–17.73
	Bisdemethoxycurcumin	1.22±0.27	0.35	0.64	2.31
	5-Nonadecylresorcinol	1.00±0.53	21.89	ns	ns
	Phloretin	1.06±0.14	0.49	9.80	ns
	Butein/Naringenin	1.24±0.32	ns	11.23	0.01
Aminoacids and derivatives	L-Proline	1.02±0.58	–2.56	–1.01	–6.90
	N-acetyl lysine methyl ester	1.23±0.33	9.24	9.28	–0.33
	Tryptophan	1.23±0.35	19.61	–0.46	–0.29
	Cinnamoylglycine	1.22±0.36	0.57	1.08	–0.30
	(2S,4S)-Monatin	1.22±0.33	0.10	0.64	19.61
Benzenoids	N-Acetylarylamine/ N-benzylformamide/2-Phenylacetamide	1.22±0.32	17.95	1.23	–0.31
	2-(2-Methylpropoxy)naphthalene	1.23±0.33	ns	9.28	–0.33
	4-[(2-Hydroxy-1-naphthalenyl)azo]benzenesulfonic acid	1.23±0.11	0.56	–9.28	ns
	1,1'-[1,12-	1.24±0.35	ns	16.89	0.03

	Dodecanediylbis(oxy)]bisbenzene				
Sulphur containing compounds	N-gamma-glutamyl-S-allylcysteine/ N-gamma-glutamyl-S-trans-(1-propenyl)cysteine	1.01±0.38	–7.01	0.64	2.31
Fatty acyls	Methyl (R)-3-methyl-2-oxopentanoate	1.22±0.19	ns	0.17	20.27
	Pantoyllactone glucoside	1.23±0.27	0.23	0.57	18.85
	Sativic acid/ Pinellic acid/9,12,13-TriHOME/(9S,10E,12S,13S)-9,12,13-Trihydroxy-10-octadecenoic acid/5,8,12-Trihydroxy-9-octadecenoic acid/9,10,13-TriHOME	1.23±0.13	ns	–0.01	–19.08
	Cervonoyl ethanolamide	1.24±0.38	–7.76	7.80	0.16
Glycerophospholipids	LysoPC(18:1(11Z))	1.24±0.36	–0.65	–18.36	–0.15
	LysoPC(20:3(8Z,11Z,14Z))	1.24±0.39	ns	–1.32	0.15
	LysoPC(20:4(8Z,11Z,14Z,17Z))	1.24±0.37	ns	–1.32	–0.01
	1-16:0-2-18:1-phosphatidylcholine	1.25±0.16	0.41	17.52	ns
	1-18:3-2-18:1-phosphatidylcholine	1.25±0.18	0.34	18.55	0.16
	PE-NMe2(16:0/16:0)	1.25±0.32	0.15	–1.01	ns
Heteroaromatic compounds	5-(2-Furanyl)-3,4-dihydro-2H-pyrrole	1.22±0.32	17.96	1.23	–0.31
	3,4-Dihydro-4-[(5-methyl-2-furanyl)methylene]-2H-pyrrole	1.25±0.19	20.92	0.18	0.09
Indoles and derivatives	3-(1H-Indol-3-yl)-2-propenoic acid	1.22±0.36	0.55	1.10	–0.30
	5-Methoxyindoleacetate/ Indolelactic acid/ Methyl 1-methoxy-1H-indole-3-carboxylate/ Methyl oxindole-3-acetate	1.22±0.36	0.57	1.09	–0.30
	Indole-3-ethanol/ Tryptophol	1.25±0.19	20.91	0.18	0.09
Prenol lipids	Hovenidulcigenin A	1.24±0.38	ns	–18.55	0.06
	Hydroxysintaxanthin 5,6-epoxide	1.25±0.34	ns	–1.32	–0.006
Pyrrolizines	2,3-Dihydro-1H-pyrrolizine-5-carboxaldehyde	1.22±0.32	17.96	1.23	–0.31

	1-(2,3-Dihydro-1H-pyrrolizin-5-yl)-2-propen-1-one	1.25±0.19	20.92	0.18	0.09
Quinolines and derivatives	Edulitine	1.22±0.36	0.57	1.08	–0.30
	Graveolinine/ Graveoline	1.24±0.40	ns	1.32	0.19
	6-Methylquinoline	1.25±0.19	20.91	0.18	0.09
Steroids and derivatives	Taurochenodesoxycholic acid	1.22±0.45	ns	18.64	0.26
	Withaphysacarpin/14alpha-Hydroxyixocarpanolide/2,3-Dihydrowithanolide E/ Perulactone B	1.22±0.23	ns	–0.28	–17.4
	Lithocholic acid glycine conjugate	1.24±0.37	21.31	ns	0.19
	Cucurbitacide E	1.24±0.38	ns	–1.32	–0.07
	2-Hydroxyestrone sulfate	1.24±0.32	–18.01	–0.36	0.04
	3b-Hydroxy-5-cholenoic acid	1.24±0.35	ns	–1.32	–0.04
Tetrahydrofurans	Tetrahydrofurfuryl acetate/ Botryodiplodin	1.23±0.19	ns	0.17	20.27
Other compounds	2-Aminoacetophenone	1.22±0.32	17.96	1.23	–0.31
	L-Menthone 1,2-glycerol ketal	1.24±0.39	–16.87	ns	0.19
	Avenalumin II	1.24±0.40	ns	1.32	0.19
	Canavaninosuccinate	1.23±0.28	0.21	0.65	19.61
	4-hydroxysphinganine	1.24±0.39	ns	1.32	0.13
	4-Hydroxycyclohexylcarboxylic acid	1.22±0.19	ns	0.17	20.27
	Dihydro-2,4-dimethyl-6-(2-methylpropyl)-4H-1,3,5-dithiazine	1.23±0.33	9.27	9.30	–0.33
	Ethyl levulinate	1.22±0.19	ns	0.17	20.27
	AS 1-5	1.01±0.53	19.91	ns	ns
	N-(2,5-Dihydroxyphenyl)pyridinium(1+)	1.22±0.35	0.52	1.11	–0.31
	(S)-Pterosin K	1.22±0.29	0.20	0.63	–10.32
	6-Chloro-N-(1-methylethyl)-1,3,5-triazine-2,4-diamine	1.22±0.36	0.56	1.08	–0.30

	Dictyoquinazol C	1.24±0.32	ns	–1.32	0.03
	3-[(5-Methyl-2-furanyl)methyl]-1H-pyrrole	1.25±0.19	20.91	0.18	0.09
	(R)-Boschniakine	1.25±0.19	20.91	0.18	0.09
	Acetyl-methylpyridine derivatives	1.22±0.32	17.96	1.23	–0.32

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Table(s)

Table 1. Proximate analysis and minerals of edible garlic and elephant garlic cloves. Data were compared with Student *t*-test ($P \leq 0.05$).

Significance ns: not significant; *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$ for the interaction of factors.

Proximate composition		Unit	Garlic (*Allium sativum*)	Elephant garlic (*Allium ampeloprasum* var. *holmense*)	Significance
Protein		g 100 g^{-1} FW	0.98±0.05	1.22 ± 0,26	*
Carbohydrate	Glucose	g 100 g^{-1} DW	3.55±0.52	3.36 ± 0.14	ns
	Sucrose		0.11±0.04	0.11 ± 0.03	ns
Dietary fibre	Neutral detergent fibre (NDF)	%	9.98±0.16	8.46 ±0.30	**
	Acid detergent fibre (ADF)		9.09±0.40	8.58 ± 1.09	ns
	Acid detergent lignin (ADL)		3.91±0.34	3.25 ± 0.31	ns
	Hemicellulose		0.48±0.33	0.55 ± 0.12	ns
	Cellulose		2.06 ± 0.23	1.29 ± 0.04	**
Minerals	Nitrogen (N)	%	2.64 ± 0.26	1.88 ± 0.06	**
	Carbon (C)		41.29 ± 0.26	42.38 ± 0.28	**
	Hydrogen (H)		6.56 ± 0.02	6.68 ± 0.04	*
	Sulphur (S)		0.38 ± 0.01	0.65 ±0.18	*
	Phosphorus (P)		1.17 ± 0.04	0.59 ± 0.01	***
	Potassium (K)		1.04 ± 0.11	0.76 ± 0.02	*
	Calcium (Ca)		0.21 ± 0.01	0.23 ± 0.02	ns
	Magnesium (Mg)		0.05 ± <0.01	0.06 ± <0.01	ns
Moisture content		%	62.13 ± 0.57	67.23 ± 0.67	***
Dry matter		%	37.87 ± 0.57	32.77 ± 0.67	***

Table 2. Bioactive compounds identified by VIP (Variable Importance in Projection) selection method following OPLS-DA model, in the raw matrix of elephant garlic and common garlic and during the *in vitro* gastrointestinal digestion of the two analysed bulbs. Compounds are provided together with VIP scores (measure of variable's importance in the OPLS-DA model) and Log Fold-Change in raw material and during different phases (gastric and pancreatic phases) of the *in vitro* digestion. [1]ns: not significative.

Class	Metabolites	VIP score (OPLS-DA)	LogFC [EG *vs* CG] raw	LogFC [EG *vs* CG] gastric	LogFC [EG *vs* CG] pancreatic
Polyphenols	4-Hydroxybenzoic acid 4-O-glucoside	1.02±0.56	11.42	2.16	9.95
	Rosmarinic acid	1.06±0.25	–18.70	–0.83	ns
	Sinapoylspermine	1.22±0.23	[1]ns	–0.37	–17.73
	Bisdemethoxycurcumin	1.22±0.27	0.35	0.64	2.31
	5-Nonadecylresorcinol	1.00±0.53	21.89	ns	ns
	Phloretin	1.06±0.14	0.49	9.80	ns
	Butein/Naringenin	1.24±0.32	ns	11.23	0.01
Aminoacids and derivatives	L-Proline	1.02±0.58	–2.56	–1.01	–6.90
	N-acetyl lysine methyl ester	1.23±0.33	9.24	9.28	–0.33
	Tryptophan	1.23±0.35	19.61	–0.46	–0.29
	Cinnamoylglycine	1.22±0.36	0.57	1.08	–0.30
	(2S,4S)-Monatin	1.22±0.33	0.10	0.64	19.61
Benzenoids	N-Acetylarylamine/ N-benzylformamide/2-Phenylacetamide	1.22±0.32	17.95	1.23	–0.31
	2-(2-Methylpropoxy)naphthalene	1.23±0.33	ns	9.28	–0.33
	4-[(2-Hydroxy-1-naphthalenyl)azo]benzenesulfonic acid	1.23±0.11	0.56	–9.28	ns
	1,1'-[1,12-	1.24±0.35	ns	16.89	0.03

	Dodecanediylbis(oxy)]bisbenzene				
Sulphur containing compounds	N-gamma-glutamyl-S-allylcysteine/ N-gamma-glutamyl-S-trans-(1-propenyl)cysteine	1.01±0.38	–7.01	0.64	2.31
Fatty acyls	Methyl (R)-3-methyl-2-oxopentanoate	1.22±0.19	ns	0.17	20.27
	Pantoyllactone glucoside	1.23±0.27	0.23	0.57	18.85
	Sativic acid/ Pinellic acid/9,12,13-TriHOME/(9S,10E,12S,13S)-9,12,13-Trihydroxy-10-octadecenoic acid/5,8,12-Trihydroxy-9-octadecenoic acid/9,10,13-TriHOME	1.23±0.13	ns	–0.01	–19.08
	Cervonoyl ethanolamide	1.24±0.38	–7.76	7.80	0.16
Glycerophospholipids	LysoPC(18:1(11Z))	1.24±0.36	–0.65	–18.36	–0.15
	LysoPC(20:3(8Z,11Z,14Z))	1.24±0.39	ns	–1.32	0.15
	LysoPC(20:4(8Z,11Z,14Z,17Z))	1.24±0.37	ns	–1.32	–0.01
	1-16:0-2-18:1-phosphatidylcholine	1.25±0.16	0.41	17.52	ns
	1-18:3-2-18:1-phosphatidylcholine	1.25±0.18	0.34	18.55	0.16
	PE-NMe2(16:0/16:0)	1.25±0.32	0.15	–1.01	ns
Heteroaromatic compounds	5-(2-Furanyl)-3,4-dihydro-2H-pyrrole	1.22±0.32	17.96	1.23	–0.31
	3,4-Dihydro-4-[(5-methyl-2-furanyl)methylene]-2H-pyrrole	1.25±0.19	20.92	0.18	0.09
Indoles and derivatives	3-(1H-Indol-3-yl)-2-propenoic acid	1.22±0.36	0.55	1.10	–0.30
	5-Methoxyindoleacetate/ Indolelactic acid/ Methyl 1-methoxy-1H-indole-3-carboxylate/ Methyl oxindole-3-acetate	1.22±0.36	0.57	1.09	–0.30
	Indole-3-ethanol/ Tryptophol	1.25±0.19	20.91	0.18	0.09
Prenol lipids	Hovenidulcigenin A	1.24±0.38	ns	–18.55	0.06
	Hydroxysintaxanthin 5,6-epoxide	1.25±0.34	ns	–1.32	–0.006
Pyrrolizines	2,3-Dihydro-1H-pyrrolizine-5-carboxaldehyde	1.22±0.32	17.96	1.23	–0.31

	1-(2,3-Dihydro-1H-pyrrolizin-5-yl)-2-propen-1-one	1.25±0.19	20.92	0.18	0.09
Quinolines and derivatives	Edulitine	1.22±0.36	0.57	1.08	–0.30
	Graveolinine/ Graveoline	1.24±0.40	ns	1.32	0.19
	6-Methylquinoline	1.25±0.19	20.91	0.18	0.09
Steroids and derivatives	Taurochenodesoxycholic acid	1.22±0.45	ns	18.64	0.26
	Withaphysacarpin/14alpha-Hydroxyixocarpanolide/2,3-Dihydrowithanolide E/ Perulactone B	1.22±0.23	ns	–0.28	–17.4
	Lithocholic acid glycine conjugate	1.24±0.37	21.31	ns	0.19
	Cucurbitacide E	1.24±0.38	ns	–1.32	–0.07
	2-Hydroxyestrone sulfate	1.24±0.32	–18.01	–0.36	0.04
	3b-Hydroxy-5-cholenoic acid	1.24±0.35	ns	–1.32	–0.04
Tetrahydrofurans	Tetrahydrofurfuryl acetate/ Botryodiplodin	1.23±0.19	ns	0.17	20.27
Other compounds	2-Aminoacetophenone	1.22±0.32	17.96	1.23	–0.31
	L-Menthone 1,2-glycerol ketal	1.24±0.39	–16.87	ns	0.19
	Avenalumin II	1.24±0.40	ns	1.32	0.19
	Canavaninosuccinate	1.23±0.28	0.21	0.65	19.61
	4-hydroxysphinganine	1.24±0.39	ns	1.32	0.13
	4-Hydroxycyclohexylcarboxylic acid	1.22±0.19	ns	0.17	20.27
	Dihydro-2,4-dimethyl-6-(2-methylpropyl)-4H-1,3,5-dithiazine	1.23±0.33	9.27	9.30	–0.33
	Ethyl levulinate	1.22±0.19	ns	0.17	20.27
	AS 1-5	1.01±0.53	19.91	ns	ns
	N-(2,5-Dihydroxyphenyl)pyridinium(1+)	1.22±0.35	0.52	1.11	–0.31
	(S)-Pterosin K	1.22±0.29	0.20	0.63	–10.32
	6-Chloro-N-(1-methylethyl)-1,3,5-triazine-2,4-diamine	1.22±0.36	0.56	1.08	–0.30

	Dictyoquinazol C	1.24±0.32	ns	–1.32	0.03
	3-[(5-Methyl-2-furanyl)methyl]-1H-pyrrole	1.25±0.19	20.91	0.18	0.09
	(R)-Boschniakine	1.25±0.19	20.91	0.18	0.09
	Acetyl-methylpyridine derivatives	1.22±0.32	17.96	1.23	–0.32

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Figure(s)

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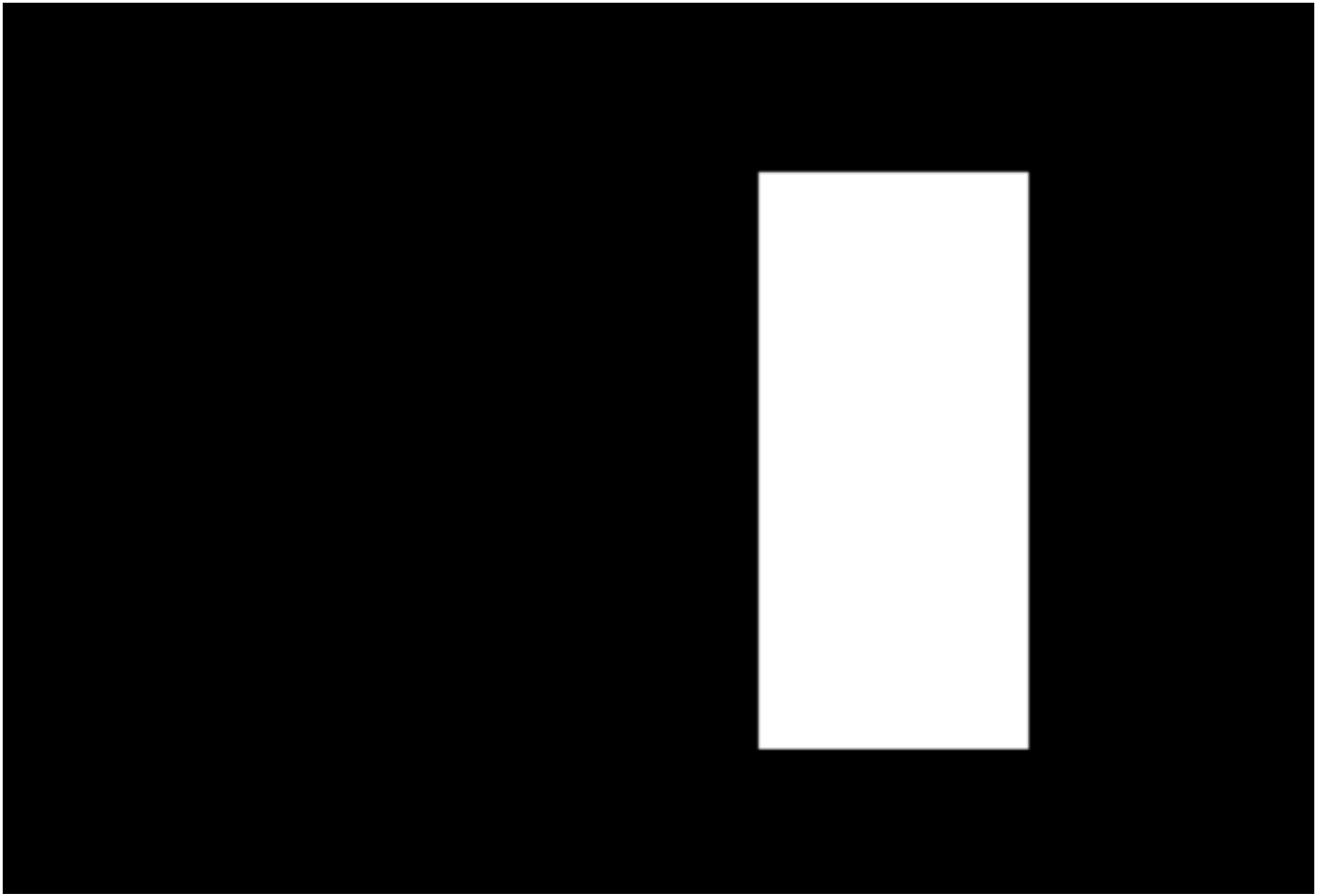

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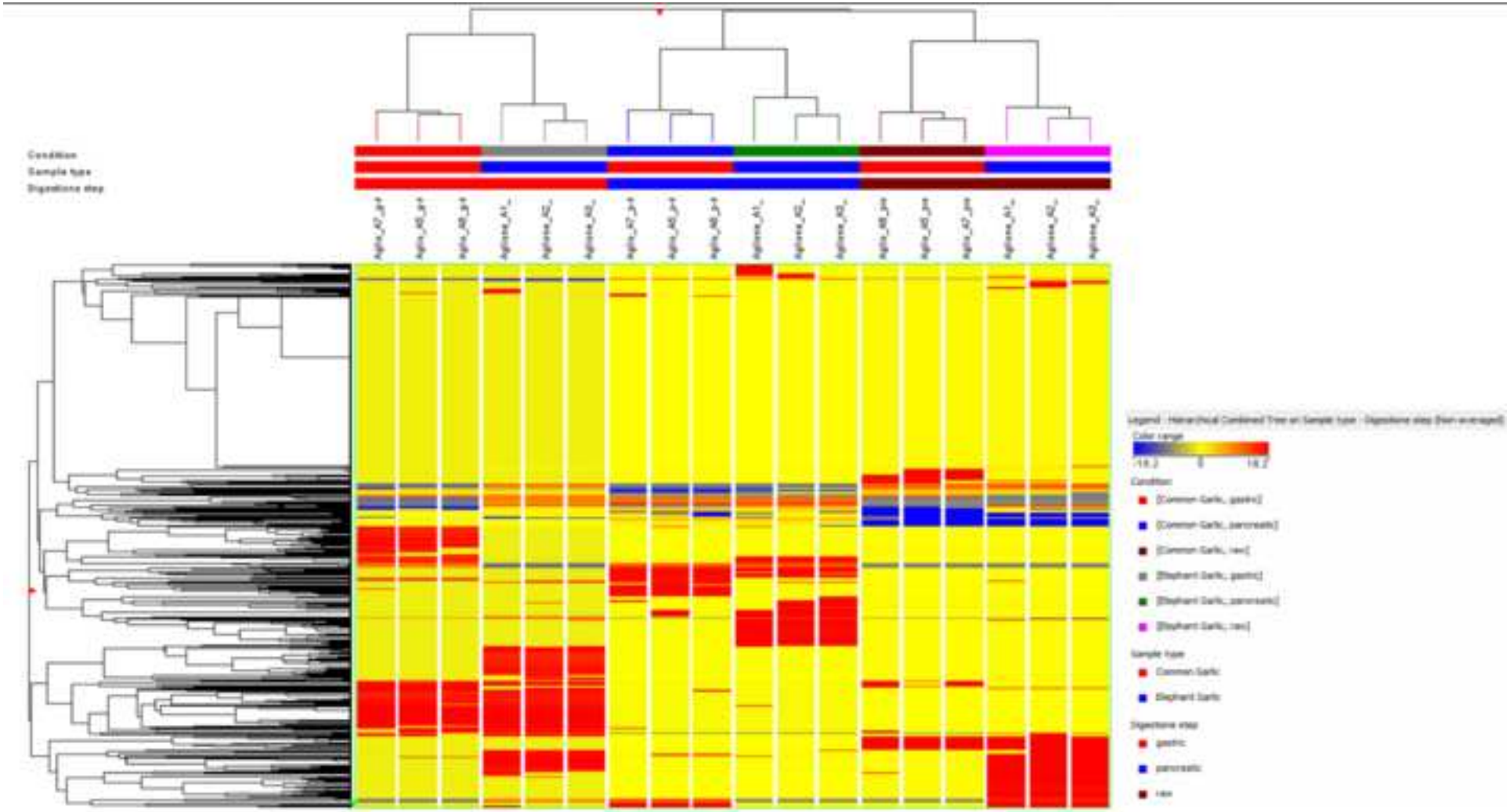

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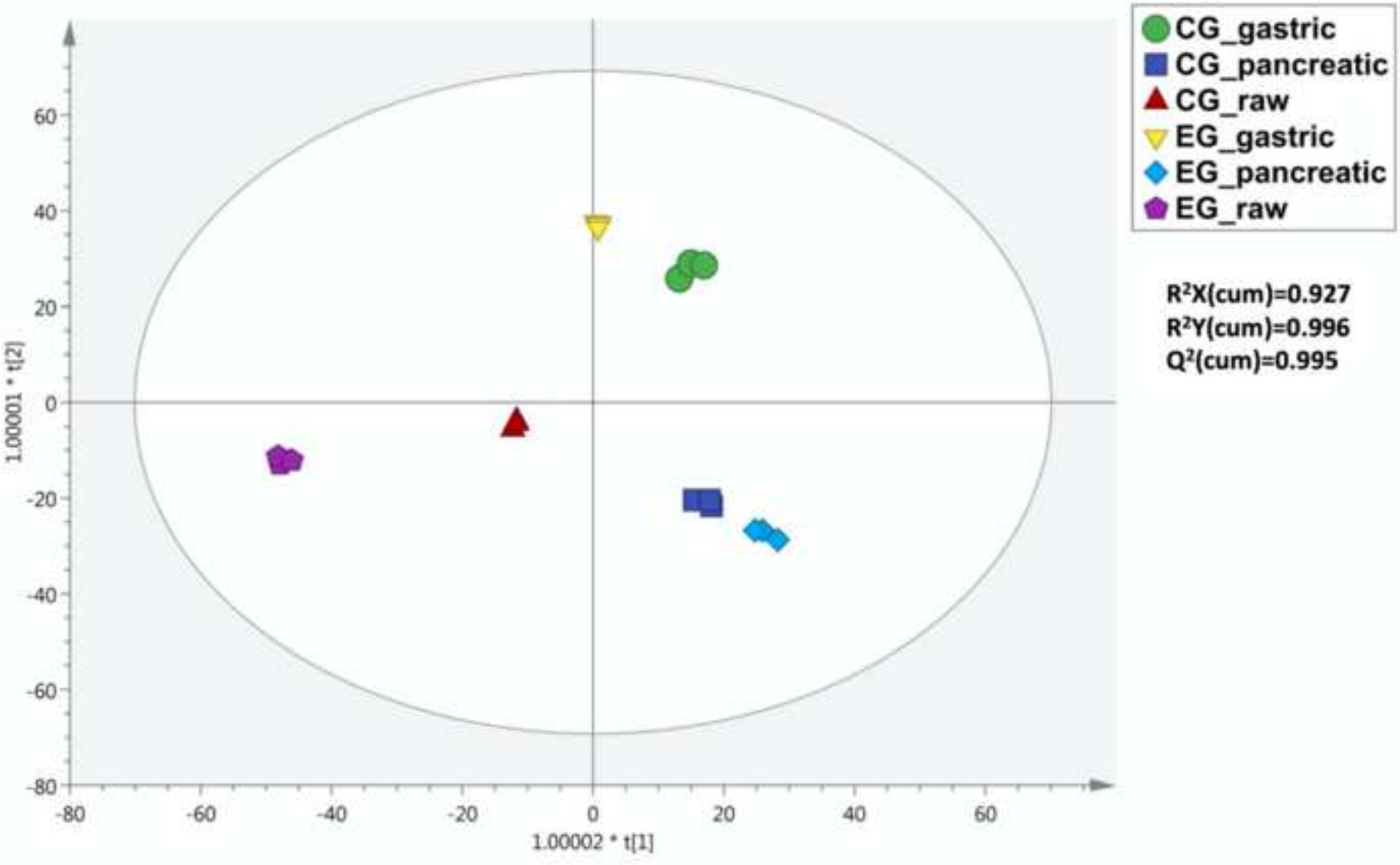

Supplementary Material

Click here to download Supplementary Material: Table S1.xlsx

Table S1. Classification of 2745 mass features according to the database FoodDB together with in

Compound	CG gastric	CG gastric
CDP-DG(18:1(11Z)/20:4(5Z,8Z,11Z,14Z))	1	1
CDP-DG(18:1(9Z)/20:4(8Z,11Z,14Z,17Z))	1	1
CDP-DG(18:1(11Z)/20:4(8Z,11Z,14Z,17Z))	1	1
CDP-DG(18:1(9Z)/20:4(5Z,8Z,11Z,14Z))	1	1
Neurine	1.37E+07	1.32E+07
Choline	1.40E+07	1.35E+07
Angiotensin II	1	1
Trigofoenoside E1	1	1
Parillin	1	1
Tuberoside B (Allium tuberosum)	1	1
Asparasaponin I	1	1
Protodioscin	1	1
Tuberoside F	1	1
1-Desulfoyessotoxin	1	1
Deltoside	1	1
Avenacoside A	1	1
26-Desglucoavenacoside B	1	1
Dulcin	1	1
Tomatoside A	1	1
Asparagoside G	1	1
Trigofoenoside D	1	1
Trigofoenoside F	1	1
Tuberoside C (Allium tuberosum)	1	1
Balanitoside	1	1
Tuberoside L	1	1
Matesaponin 2	1	1
Helianthoside A	1	1
Medicoside I	1	1
Pitheduloside K	1	1
Pacific Ciguatoxin 4A	1	1
Cyclolinopeptide D	1	1
Trigofoenoside C	1	1
Melongoside P	1	1
Soyasaponin bg	1	1
Bradykinin hydroxyproline	1	1
Eruboside B	1	1
Isoeruboside B	1	1
Cistocardin	1	1
Bradykinin	1	1
Yayoisaponin C	1	1
Tragopogonsaponin Q	1	1
Camellidin II	1	1
Capsianoside III	1	1

Supplementary Material

Click here to download Supplementary Material: Table S2.xlsx

Table S2. Volcano plot comparing raw elephant garlic *vs* common garlic by coupling ANOVA (

Moderated T-Test [Elephant Garlic, raw] *vs* [Common Garlic, raw] P ≤ 0.01 FC ≥ 5.0

Compound
L-Proline
5-Acetyl-2,3-dihydro-1,4-thiazine
L-2-Amino-3-(1-pyrazolyl)propanoic acid
trans-Carvyl acetate
Rhubafuran
(R)C(S)S-Alliin
Polyvidone
6-Acetylfuranofukinol
Urodiolenone
Citflavanone
Cyclocalopin F
Sterebin A
(1R*,3S*,3'R*)-1,2,3,4-Tetrahydro-1-(2-thio-3-pyrrolidinyl)-beta-carboline-3-carboxylic acid
L-Menthone 1,2-glycerol ketal
N-gamma-Glutamyl-S-allylcysteine
N-gamma-Glutamyl-S-cis-(1-propenyl)cysteine
Rosmarinic acid
Isotriglochinin
Irigenin
Gerberinol
2-Hydroxyestrone sulfate
Artonin K
4'-phosphopantetheine
Gonyautoxin V
Gingerenone B
Dihydrofukinolide
Myristicanol B
Boviquinone 4
2-(2-Methylbutanoyl)-9-(3-methyl-2E-pentenoyl)-2b,9a-dihydroxy-4Z,10(14)-oplopadien-3-or
Clausarinol
Lusitanicoside
Erinacine D
ent-Epiafzelechin-(2alpha-7,4alpha-8)-catechin
DG(18:3(9Z,12Z,15Z)/18:0/0:0)
Ramontoside
Epiafzelechin-(4alpha-8)-pelargonidin 3'-glucoside

Compound
N-Methylcalystegine C1
Xylopine
Alkaloid A6
Cyclolinopeptide D
Bradykinin hydroxyproline

***Declaration of Interest Statement**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: