A fingerprinting metabolomic approach reveals deregulation of endogenous metabolites after the intake of a bioactive garlic supplement.

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Abstract

Garlic (Allium sativum) has been described as containing phytonutrients with healthy properties. In this study, the effect of a bioactive garlic food supplement intake on human plasma metabolome was examined with the aim of understanding the mechanisms of action and involved pathways responsible for beneficial effects. With this purpose, a dietary intervention assay was performed in thirty healthy volunteers collecting plasma samples before intake and after one month of daily supplement consumption. Plasma samples were analysed by a fingerprinting metabolomic strategy based on HPLC-ESI-QTOF-MS. Our results revealed a total of 26 metabolites affected by supplement intake. In general, alterations in phospholipid metabolism were shown, detecting an increase in lysophosphatidylcholines, lysophosphatidylethanolamines and acylcarnitines. It is also remarkable that the level of four fructosamines decreased after the assay. These results are according with the antioxidant and antiglycation properties that have been previously associated with garlic extracts.

Keywords: food supplement; fructosamines; garlic; HPLC-ESI-QTOF-MS; lysophosphatidylcholines; metabolomics.

1. Introduction

Metabolomics is an 'omics' technology that aims to study all low molecular weight molecules present in biological systems, which are known as metabolites. In this way, this tool allows to find alterations and interactions in the organism due to different conditions or causes (Agin et al., 2016). Currently, the main analytical techniques able to detect the greatest number of metabolites used in metabolomics studies are ^{1}H nuclear magnetic resonance spectroscopy (^{1}H-NMR) and mass spectrometry (MS) (Mumtaz et al., 2017).

Most metabolomics studies have been focused on human diseases, in order to know the pathways involved in their development and also to find biomarkers that allow the improvement of their diagnosis, prognosis and treatments (Johnson, Ivanisevic, & Siuzdak, 2016; X. Wang, Chen, & Jia, 2016). On the other hand, metabolomics studies have also been reported in other areas with different aims such as, classifying species, studying toxicity (Farag, Fekry, et al., 2017), or in the field of nutrition, mainly distinguished into three types of studies: dietary biomarker discovery, relation of diet and diseases and dietary intervention studies (Brennan, 2013; Gibbons, O'Gorman, & Brennan, 2015).

The last ones try to understand how certain foods or diets impact in the metabolic pathways focusing on both endogenous and exogenous metabolites. In this way, metabolomics has been widely applied to dietary intervention studies performed with foods highly consumed daily in the human diet such as butter, milk, cheese, tea, chocolate, cocoa, vitamins or fish oils, among others (Brennan, 2013; Zheng, Clausen, Dalsgaard, & Bertram, 2015).

Nevertheless, due to consumer concerns and demands, other types of food have appeared in the market whose effects in metabolome deserve further attention. In recent years there is a great interest in new nutritional products such as nutraceuticals, functional foods and food supplements. This kind of product has beneficial properties in the human health due to their high content in bioactive compounds, as the case of polyphenols. The dietary intake of phenolic compounds has presented beneficial properties in several diseases such as neurodegenerative diseases, cancer, hypertension or cardiovascular diseases (Del Rio et al., 2013; Rodriguez-Mateos et al., 2014). One example of supplement food containing these type of compounds has been detailed by Letizia Bresciani et al. who characterized 119 phenolic compounds in three food supplements which contained 36 different vegetables, fruits and berries (Bresciani et al., 2015).

Some dietary intervention studies have been also found in literature regarding specific compounds or food supplements. For instance, the effects of vitamin E supplementation (Wong & Lodge, 2012), intake of a functional beverage based on a grape skin extract (Khymenets et al., 2015) or grape extracts or wine supplementation (Jacobs et al., 2012) on human metabolism have been studied.

Among different products with bioactive compounds, garlic (*Allium sativum*) is one of the most famous since antiquity that has gained a great interest due to its varied composition including vitamins, phenolic acids, dipeptides, fatty acids, flavonoids and organosulfur compounds. The combination of these compounds makes this matrix has excellent properties such as anticancer, antioxidant, antibacterial, antimutagenic, antiplatelet, antimicrobial, antiaging and antihyperlipidemic activities, as well as immunomodulatory capacity and being able to modulate glucose and insulin levels. In this way, *Allium* present health properties for treatment of hypercholesterolemia, cancer

hypertension, diabetes type 2, cataract, obesity and disturbances of the gastrointestinal tract (Amagase, Petesch, Matsuura, Kasuga, & Itakura, 2001; Farag, Ali, et al., 2017; Kopec, Piatkowska, Leszczynska, & Sikora, 2013).

Despite the number of dietary intervention studies has recently increased, there is still a lack of information on how food matrices, mainly new nutritional products, affect human metabolism. In this way, there is an urgent need to study the effect of these products in the metabolism due to their bioactive properties, which may help to understand their beneficial effects and the mechanisms of action and involved pathways in the human organism. Due to its composition in bioactive compounds and health benefits, garlic extracts are currently being used as nutraceutical or dietary supplement despite their impact in the human metabolome has not been deeply studied.

In this context the present study aims to examine the human metabolism changes due to a prolonged intake of a bioactive garlic supplement by means of a dietary intervention assay. The importance of this study is that it allows knowing what metabolic pathways are mainly altered in healthy individuals due to garlic consumption. The expected results can be related to the health benefits of garlic.

2. Material and Methods.

2.1.Garlic supplement

Aliocare ®, a product containing 14.5% of organosulfur compounds, was provided by DOMCA S.A. (Granada, Spain).

2.2.Chemicals.

All chemicals were of analytical reagent grade and used as received. Formic acid and LC-MS grade methanol for mobile phases were purchased from Fluka, Sigma-Aldrich (Steinheim, Germany) and Fisher Scientific (Madrid, Spain), respectively. Water was

purified by a Milli-Q system from Millipore (Bedford, MA, USA). For plasma treatment, ethanol and methanol (Fisher Scientific Madrid, Spain) were used.

2.3.Dietary intervention nutritional assay

Thirty healthy volunteers (15 men and 15 women), age range of 20-40 years, were recruited in the city of Granada (Spain) to participate in the intervention nutritional assay. Each volunteer signed a consent form after receiving a detailed explanation of the study.

Exclusion criteria was based on current physical status and history of conditions including chronic severe diseases, current infection and antibiotic treatment or anti-inflammatory drugs within the previous two months, and any diseases or medications that could interfere with study outcome measures. Participants were withdrawn if they ingested food containing alliaceae or if they suffered diseases that require treatment with antibiotics or anti-inflammatory drugs during the study period.

Participants were informed to abstain from the intake of garlic, onion, leek and nutritional supplements (prebiotics, fitobiotics, vitamins or minerals) within the previous three weeks. The ethic committee of the University of Granada approved the study. During the study, the volunteers ingested one gelatin capsule contained 70 mg of garlic supplement per day. At the beginning and at the end of the study, blood samples were collected from participants into citrate containers. Plasma samples were obtained by centrifugation of containers for 15 min at 2000 *g* at 4 °C, then rapidly frozen and stored at −80 °C until further treatment and analysis.

2.4.Sample treatment.

Plasma samples, which were stored at -80 °C, were thawed on ice. A plasma aliquot of 100 µl was mixed with 200 µl methanol:ethanol (50:50, v/v) in order to remove the protein content (Bruce et al., 2009). Afterwards, the mixture was vortex-mixed and then

was kept at -20 °C during 30 min in order to achieve an efficient protein precipitation and avoid possible degradations. Next, the sample was centrifuged during 10 min at 14800 r.p.m. and 4 °C, and the supernatant was evaporated to dryness under vacuum in a centrifugal evaporator (Concentrator Plus, Eppendorf, Hamburg, Germany) during 2 h. Afterwards, the dry residue was reconstituted in 100 µl of initial mobile phase conditions (0.1% aqueous formic acid:methanol, 95:5, v/v) and centrifuged as mentioned above in order to remove solid particles. Finally, a 40 µl aliquot was transferred into HPLC vials and stored at -80 °C prior to analysis. A quality control sample (QC) was prepared by mixing equal volumes (20 µl) from each sample and treated as described above (Dettmer, Aronov, & Hammock, 2007).

2.5.HPLC-ESI-QTOF-MS analysis.

Analyses were performed using an Agilent 1260 HPLC instrument (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 6540 Ultra High Definition (UHD) Accurate Mass Q-TOF equipped with a Jet Stream dual ESI interface.

The compounds were separated using a reversed-phase C18 analytical column (Agilent Zorbax Eclipse Plus, 1.8 µm, 4.6×150 mm) protected by a guard cartridge of the same packing. The mobile phases were water containing 0.1% of formic acid and methanol as solvent A and B, respectively. The following gradient of these mobile phases was used in order to obtain an efficient separation: 0 min [A:B 95/5], 5 min [A:B 90/10], 15 min [A:B 15/85], 30 min [A:B 0/100], and 35 min [A:B 95/5]. Finally, initial conditions were kept for 5 min at the end of each analysis to equilibrate the analytical column before the next run. The autosampler and column compartment temperatures were set at 4 and 25 ºC, respectively, whereas the flow rate and the injection volume were 0.4 mL/min and 5 µl.

Detection was performed in positive-ion mode over a range from 50 to 1700 m/z. All spectra were corrected by means of continuous infusion of two reference masses: purine (m/z 121.050873) and hexakis (^{1}H, ^{1}H, ^{3}H-tetrafluoropropoxy) phosphazine or HP-921 (m/z 922.009798). Both reference ions provided accurate mass measurement typically better than 2 ppm.

Ultrahigh pure nitrogen was used as drying and nebulizer gas at temperatures of 200 and 350 ºC and flows of 10 and 12 L/min, respectively. Other optimized parameters were as follows: capillary voltage, +4000V; nebuliser, 20 psi; fragmentor, 130 V; nozzle voltage, 500 V; skimmer, 45 V and octopole 1 RF Vpp, 750 V.

The analytical sequence of the samples consisted in: 2 blanks, 5 QCs, 5 randomized samples, 1 blank, 2 QCs, 5 randomized samples, etc. Finally, a MS/MS analysis of the QC sample was performed in order to facilitate the identification of potential biomarkers. This experiment was performed using nitrogen as the collision gas with the following collision energy values: 10 eV, 20 eV and 40 eV.

2.6.Data processing.

Recursive Feature Extraction for small molecules was performed by means of MassHunter Profinder software (B.06.00, Agilent Technologies) to generate a list of the representative features present in plasma samples with their integrated areas. This algorithm combines "Molecular Feature Extraction" with "Find by Ion" algorithms (Kitawa et al., 2013). Therefore, the first algorithm finds features which are defined as the combination of co-eluted species that are related by isotopic distribution, presence of adducts, loss of molecules and/or charge-state envelop. Secondly, the features found in the samples are aligned by mass and retention time. Finally, a list with the resulting features is created and used to find them in the same samples more accurately.

Peaks were filtered by intensity threshold of 1250 counts. $[M+H]^+$, $[M+Na]^+$ and $[M-H_2O]$ were the considered species with a maximum charge of 2. Feature alignment parameters were ± 0.25 minutes and 40 ppm ± 4 mDa for retention time and mass windows, respectively. The integration method was Agile2 carrying out an average of spectra at peak start and end to subtract a background spectrum. Nevertheless, integration results were manually supervised to correct defaults.

2.7.Statistical analysis.

Initially, the data were explored by unsupervised Principal Component Analysis (PCA) to check the reproducibility according to the distribution of QC samples and to identify any outliers. For multivariate analysis, data were transformed by means of log transformation to get a Gaussian distribution of the data and were set to Pareto scaling to make each variable comparable to each other (van den Berg, Hoefsloot, Westerhuis, Smilde, & van der Werf, 2006).

Features were normalized according to the QC samples (more details are described in the results section), and afterwards the features with high variability (RSD>30 %) in the QC samples were removed (Dunn et al., 2011).

After these steps, a supervised Partial Least Squares Discriminant Analysis (PLS-DA), a hierarchical clustering via heatmap and univariate statistical tests (paired t-test and paired fold change analysis) were performed in order to find metabolic differences due to nutritional supplementation. Both univariate and multivariate statistical tests were carried out in Metaboanalyst 3.0 software (Xia et al., 2015; Xia & Wishart, 2016).

3. Results

*3.1.*Data quality assessment.

The data processing described in material and methods section allowed obtaining a total of 306 molecular features. Firstly, PCA was performed for overall data in order to check the analytical reproducibility according to QC samples distribution. An analytical drift was detected due to the dispersion of the QC samples in the PCA scores plot (**Figure 1a**) according to their injection order.

This bias is often present in large-scaled non-targeted metabolomic studies and is usually related to fluctuations in the ionization efficiency of the electrospray interface (ESI) throughout the analytical sequence. In order to correct this variability, different strategies have been described in bibliography (Mizuno et al., 2017). Some of them are based on the use of the QC samples to monitor the drift and correct it (Dunn, Wilson, Nicholls, & Broadhurst, 2012; Kamleh, Ebbels, Spagou, Masson, & Want, 2012).

In this case, the integrated areas obtained for each feature in each sample were normalized by the sum of the total useful signal from the nearest QC in order to correct the aforementioned drifts and to get the areas of the samples comparable between them (Gika, Macpherson, Theodoridis, & Wilson, 2008). The improvement of data quality after applying this normalization procedure is shown in **Figure 1b,** where there is a clear clustering of QCs in PCA scores plot. Outliers were not detected and a slight grouping between two groups can be appreciated.

*3.2.*PLS-DA model and univariate statistical analysis.

3.2.1. PLS-DA model.

A PLS-DA model was built to discriminate the samples according to the supplementation. **Figure 2a** shows the scores plot of the PLS-DA model where the samples are clearly grouped according to their conditions

The model was established with two components obtaining the following performance parameters by 10-fold cross validation: accuracy, 0.9808; R2, 0.8899 and Q2, 0.7805. In

order to test for possible overfitting, a permutation test was performed with 2000 permutations and using the prediction accuracy during training and the separation distance (B/W) as statistics tests. The results of these tests are showed in **Figures 2b** and **2c** resulting p-values under 5 E-4 which means that there is no overfitting in the model (Xia & Wishart, 2011). The PLS-DA model was also validated by means of the Receiver Operating Characteristic (ROC) curve (Steyerberg et al., 2010; Worley & Powers, 2013) (**Figure 2d**), obtaining an area under the curve (AUROC) value of 0.995 (95% CI: 0.954-1), showing a perfect discrimination between both groups.

A total of 76 molecular features, whose VIP (variable importance in projection) values were higher than 1.0, were selected as responsible for the sample discrimination.

3.2.2. Univariate statistical tests.

Univariate analyses were performed on the 76 selected features from PLS-DA model. Significant metabolites between the pre and post supplementation were estimated by a paired t-test (p-value ≤ 0.05) and paired fold change analysis (FC>1.5 in at least 75% of pairs). As a result of both tests, 39 significant features were obtained.

ROC curves were also constructed for the significant metabolites. AUROC values were used to evaluate the discriminatory power of each metabolite. A good curve is considered when the AUROC is higher than 0.7-0.8 (Xia, Broadhurst, Wilson, & Wishart, 2013). The AUROC of the selected significant features were higher than 0.75, which means that these metabolites could be considered biomarkers of the garlic extract intake. The top six metabolites with the higher AUROC values (**Figure 3**) were L-palmitoylcarnitine, 3-OH-cis-5-octenoylcarnitine, LysoPC(18:0), N-1-Deoxy-1fructosylTryptophan, Threonine-Methionine-Tryptophan (Thr-Met-Trp) and N-1-Deoxy-1fructosylTryptophan.

*3.3.*Altered metabolites after garlic supplement intake.

The 39 statistically significant features were attempted to identify. This identification was carried out through the comparison of the accurate mass, isotopic distribution and fragmentation patterns obtained in MS/MS analysis with the online available metabolomic databases such as METLIN (http://metlin.scripps.edu), LipidMaps (http://lipidmaps.org), and Human Metabolome Database (http://hmdb.ca), as well as MS/MS fragmentation resources such as MetFrag (http://msbi.ipb-halle.de/MetFrag/).

As a result, 26 metabolites of the 39 candidates could be identified. Within the identified metabolites, four lysophosphatidylcholines, namely LysoPC(14:0), LysoPC(16:0), LysoPC(17:0) and LysoPC(18:0) were identified as two isomeric species. **Table 1** lists the significant metabolites which were identified together with their retention times, vip-values, molecular formulas, scores, p-values, in addition to the AUROC values. Regarding unknown features, their corresponding parameters are located in the **Table S1.**

3.4. Hierarchical clustering analysis.

Hierarchical clustering analysis was applied to twenty-six metabolites that were identified using a Pearson distance measure and Ward clustering algorithm. **Figure 4** shows the resulting heatmap where the metabolites clustering indicates two separate groups depending on whether the concentration increase or decrease after supplementation. In this way, N-palmitoyl tryphophan and four fructosamines compounds (Valine, Trypthophan, Leucine and Isoleucine) were the metabolites whose concentration decreased after garlic supplementation. On the opposite, the rest of significant identified metabolites concentrations increased after the intervention assay. On the other hand, the sample clustering shows also two groups according to the class. All samples were correctly classified with the exception of the one collected from the male volunteer number 10 after the supplementation (PH10-2). The cause of the wrong

cluster of this sample could be that the initial levels of the fructosamines in this person (PH10-1) were very high in comparison with the rest of volunteers as can be observed in the intensity colors of these compounds in the heatmap.

4. Discussion.

Among the identified significant metabolites, it should be highlighted the number of lysophosphatidylcholines (LysoPC(14:0), LysoPC(15:0), LysoPC(16:0), LysoPC(17:0), LysoPC(18:0), LysoPC(P18:0), LysoPC(20:3)), which were detected in higher concentration after the nutritional supplementation. Among them, LysoPC(17:0) was the most increased (Fold Change: +2.29). Thus, this is clear evidence that the consumption of the garlic food supplement altered the phospholipid metabolism. In this sense, Lysophosphatidylcholines (LPC) are bioactive phospholipids which are originated by the hydrolysis of phosphatidylcholines (PC) mediated by the phospholipase A_2 in living cells (Jackson, Abate, & Tonks, 2008). Another way of producing LysoPC, which is highly related to blood concentration, is derived from the reaction of PC and cholesterol in liver by means of lecithin:cholesterol acyltranferase (LCAT) enzyme (Rousset, Vaisman, Amar, Sethi, & Remaley, 2009).

The relationship of lysophosphatidylcholines with inflammatory processes and with the modulation of the immune response is well-known (J. H. S. Kabarowski, Xu, & Witte, 2002). The deregulation of LysoPC concentration in plasma has been reported in bibliography in numerous studies mainly focused on diseases. In this sense, it has been found that lower concentrations of LysoPCs are related to a higher risk of several types of cancer, such as prostate, breast or colorectal cancer (Kühn et al., 2016; Zhao et al., 2007). Moreover, same trends have also been reported for other types of diseases such as Alzheimer (Y. et al., 2014), obesity or Type 2 Diabetes (Barber et al., 2012).

On the other hand, there are studies which have related the increase of LysoPC in plasma to the consumption of nutritional supplement in humans, as the case of vitamin E (Wong & Lodge, 2012). In fact, vitamin E has been described as a component of garlic, which has showed bioactive properties with beneficial effects on human health. Among them, it could be highlighted its antioxidant effect and, its functions as cardioprotective agent, regulator of specific gene expression and reducer of inflammation and oxidative stress (Rizvi et al., 2014).

In addition, LysoPCs have been implicated in the immune response as an immunoregulation factor. It has been studied how concentration of LysoPCs changes during the immune response, and in these cases a decrease of concentration has been found as the general tendency (Wikoff, Kalisak, Trauger, Manchester, & Siuzdak, 2009). According to that, the observed decrease of this family of compounds has been observed in autoimmune diseases such as multiple sclerosis (Del Boccio et al., 2011). However, there is a bit of controversy due to other studies have shown the opposite trend for several autoimmune diseases such as atherosclerosis and systemic lupus erythematous (J. H. Kabarowski, 2009). Therefore, nowadays there is no clear relationship between these compounds and the immune response. Nevertheless, it seems that the consumption of the garlic supplement could probably cause immunomodulatory effects because of its content in organosulfur compounds, specially enriched in propyl propane thiosulfate (PTSO), which has previously showed properties for modulating the immune response in humans (PCT/ES2014/070928, 2014). In this way, the observed deregulation of LysoPCs could be a metabolic consequence caused by the immunomodulatory capacity of the PTSO derivatives in the organism.

Moreover, other significant metabolites are also related to phospholipid metabolism, as the case of acylcarnitines (L-palmitoylcarnitine, 3-OH-cis-5-octenoylcarnitine, L-

oleoylcarnitine), 1-O-Hexadecyl-sn-glycero-3-phosphocholine and lysophospatidylethanolamines (LysoPE(16:0), LysoPE(20:4), LysoPE(22:6)). Similarly, the level of these metabolites also increases after the supplementation.

Acylcarnitines are metabolites whose function is the transport of the fatty acids into the mitochondria, and therefore, they are associated with the metabolism of fatty acids and amino acids oxidation. The increase of this kind of compounds in plasma has been associated to deregulation in fatty acid oxidation and with higher risk of certain diseases, such as cardiovascular diseases and diabetes (Stephanie J. Mihalik et al., 2010) although this risk depends on the length of the chain, showing no significant differences in long-chain acylcarnitines (S. J. Mihalik et al., 2012). In addition, previous studies have shown that garlic extracts decrease the risk of cardiovascular diseases due to the inhibition of lipid oxidation and oxidation of low density lipoprotein (Iciek, Kwiecień, & Włodek, 2009; Lau, 2006). Nevertheless, in other illnesses, like adult celiac disease, it has been found a decrease of these metabolites with respect to the controls (Bene et al., 2005). In spite of that, it is important to highlight that under normal conditions the up-regulation of long-chain acylcarnitines can be caused by the fatty acid composition of the dietary intake. Therefore, the observed increase could be associated with the fatty acid composition of the food supplement. In fact, palmitic acid has been found as the major fatty acid in garlic (Tsiaganis, Laskari, & Melissari, 2006) and, in addition, studies have showed that the higher concentration of acylcarnitines due to palmitic acid intake is not associated to deregulation of β-oxidation (Kien et al., 2015). On the other hand, studies have revealed bioactive properties of palmitoylcarnitine, which has been described as a local immunomodulatory and antibacterial molecule (Hulme et al., 2017; Wenderska, Chong, McNulty, Wright, & Burrows, 2011). Therefore, it is not clear the

370 impact that the increase of these molecules could have in the human metabolism of
371 healthy people.
372 On the other hand, it is also remarkable the decrease in the concentration of four
373 fructosamines (1-amino-1-deoxy-D-fructose) metabolites with Valine, Trypthophan,
374 Leucine and Isoleucine as the aminoacid part. These compounds are called Amadori
375 products, which are made up by the early stage of the Maillard reaction. The formation
376 of fructosamines occur both enzymatically and non-enzymatically and is commonly
377 produced in foods and *in-vivo* (Mossine & Mawhinney, 2010). The decrease of
378 concentration of these metabolites is in accordance with the antiglycation properties
379 which have been found for garlic extracts. Thus, these extracts are able to inhibit the
380 formation of early glycation products like fructosamines (Elosta, Slevin, Rahman, &
381 Ahmed, 2017). The concentration of fructosamine derivatives is directly related to the
382 advanced glycation end products (AGE), which have attracted a great interest in recent
383 years due to the relationship between their high concentration and diseases related to
384 aging and diabetes (Gkogkolou & Böhm, 2012). In this way, previous studies have
385 showed the positive effect of garlic supplement in the management of type 2 diabetes
386 mellitus. (Padiya & K. Banerjee, 2013; J. Wang, Zhang, Lan, & Wang, 2017).
387 According to the obtained results, it can be concluded that the bioactive garlic food
388 supplement alters mainly the phospholipid metabolism in healthy people. Among the
389 deregulate compounds, it is remarkable the concentration increase of acylcarnitines,
390 lysophosphatidylcholines and lysophospathidylethanolamines, joined with the decrease
391 of fructosamines after the supplement intake. These observed alterations could be highly
392 correlated with the antioxidant and antiglycation properties that have been previously
393 described for garlic extracts. In addition, most of the observed tendencies are opposed to
394 disease states. Among them, it should be highlighted the increase in concentration of 7

lysoPCs, whose increase have demonstrated a relationship with the prevention of several diseases, especially cancer. The obtained results suggest that the prolonged intake of a garlic food supplement have an effect in several metabolic pathways in healthy humans. These findings have been related to the bioactive properties of garlic against several diseases. In this sense, further similar studies using patients of these diseases, should be conducted in order to increase the knowledge about assuring the beneficial effects of garlic.

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Conflict of interest.

All authors declare that they have no conflict of interest.

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Figure Captions.

Figure 1. PCA scores plot from Raw Data and Normalized Data (red, pre-treatment; green, post-treatment, blue, QC samples).

Figure 2. A supervised Partial Least Squares Discriminant Analysis (PLS-DA). **Fig2a.** PLS-DA scores plot (red and green points represent samples of pre and post-treatment, respectively, the circular areas represent the 95% confidence region of each group); **Fig2b-c.** Permutation test results (Statistical tests: 2c separation distance (B/W), 2d prediction accuracy during training); **Fig2d**. ROC curve for PLS-DA model validation.

Figure 3. ROC curves corresponding to the six metabolites that present the highest AUROC (L-palmitoylcarnitine, 3-OH-cis-5-octenoylcarnitine, LysoPC(18:0), N-1-Deoxy-1fructosylTryptophan, Thr Met Trp and N-1-Deoxy-1fructosylTryptophan).

Figure 4. Hierarchical clustering via heatmap (Pearson and Ward as distance measure and clustering algorithm) of the 25 significant identified metabolites. (0: pre-treatment, 1: post-treatment).

644 **Table 1.** Molecular and statistical details (Retention times, masses, molecular formulas with their scores, p-values, VIP-values and areas under

645 ROC curve) of identified metabolites that presented significant differences between before and after garlic supplement intake.

	RT (min)	Mass (Da)	p-value	VIP value	AUROC	Molecular Formula	Score	Proposed Metabolite
SIGNIFICANT METABOLITES	5.5	279.1311	1.35 E-10	2.1887	0.9260	$C_{11}H_{27}NO_7$	94.6	N-(1-Deoxy-1fructosyl)Valine
	10.0	293.1473	8.20 E-7	1.8322	0.8036	$C_{12}H_{23}NO_7$	99.7	N-(1-Deoxy-1fructosyl)Isoleucine
	10.6	293.1475	1.74 E-8	1.9199	0.8297	$C_{12}H_{23}NO_7$	98.1	N-(1-Deoxy-1fructosyl)Leucine
	13.9	301.1886	5.24 E-11	4.2654	0.9560	$C_{15}H_{27}NO_5$	83.8	3-OH-cis-5-octenoylcarnitine
	15.5	366.1427	1.46 E-10	2.1661	0.9200	$C_{17}H_{22}N_2O_7$	97.1	N-(1-Deoxy-1fructosyl)Tryptophan
	20.9	213.2434	4.41 E-6	2.2279	0.8956	$C_{14}H_{31}N$	85.6	Tetradecylamine
	22.5	436.1749	1.31 E-9	2.6085	0.9620	$C_{20}H_{28}N_4O_5S$	91.1	Thr Met Trp
	24.2	399.3282	5.08 E-13	2.6069	0.9840	$C_{23}H_{45}NO_4$	77.9	L-palmitoylcarnitine
	24.7	425.3444	1.14 E-7	1.6577	0.8571	$C_{25}H_{47}NO_4$	84.4	L-oleoylcarnitine C18:1
	26.6	467.2935	4.24 E-6	1.7287	0.8187	$C_{22}H_{46}NO_7P$	83.8	LysoPC(14:0) isomers
	27.3	467.2966	9.38 E-6	1.6591	0.8132		95.3	
	27.9	511.3213	9.29 E-9	2.0390	0.8819	$C_{24}H_{50}NO_8P$	95.0	PS(O-18:0/0:0)**
	28.7	481.3396	1.71 E-6	1.6793	0.8558	$C_{23}H_{48}NO_7P$	97.5	LysoPC(15:0)
	28.8	525.2681	6.33 E-7	1.7375	0.8517	$C_{27}H_{44}NO_7P$	98.9	LysoPE (22:6)
	28.9	501.2759	1.05 E-7	1.6691	0.8640	$C_{25}H_{44}NO_7P$	90.0	LysoPE (20:4)
	30.2	495.3265	2.03 E-11	1.1813	0.9148	$C_{24}H_{50}NO_7P$	81.9	LysoPC (16:0) isomers
	29.4	495.3188	2.01 E-11	1.9116	0.8665		96.5	
	30.1	453.2765	3.92 E-10	1.6357	0.8640	$C_{21}H_{44}NO_7P$	82.0	LysoPE(16:0)
	30.8	442.3133	1.61 E-6	1.1305	0.8764	$C_{27}H_{42}N_2O_3$	69.6	N-palmitoyl tryptophan
	31.2	509.3449	2.80 E-6	2.0982	0.8750	$C_{25}H_{52}NO_7P$	97.1	LysoPC (17:0) isomers
	31.7	509.3357	6.75 E-8	2.3073	0.9200		98.7	
	32.0	481.3396	5.86 E-11	2.3294	0.8956	$C_{24}H_{52}NO_6P$	99.3	1-O-Hexadecyl-sn-glycero-3-phosphocholine
	32.3	523.3463	2.90 E-11	2.5224	0.9360	$C_{26}H_{54}NO_7P$	95.6	LysoPC (18:0) isomers

	33.6	523.3463	1.50 E-10	2.2400	0.9320		96.7	
	32.6	507.3567	3.72 E-8	1.7478	0.7912	$C_{26}H_{54}NO_6P$	98.7	LysoPC (P18:0)
	33.0	545.3404	1.08 E-9	2.1003	0.9011	$C_{28}H_{52}NO_7P$	92.6	LysoPC (20:3)

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