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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.

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; 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 products 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

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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 14,800 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 °C, 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 °C and flows of 10 and 12 L/min, respectively. Other optimized parameters were as follows: capillary voltage, +4000 V; 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