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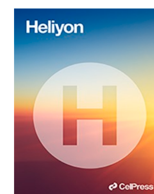

Research article

Helix aspersa aspersa flour: An evaluation for dietary supplementation

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A R T I C L E I N F O

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A B S T R A C T

This study assesses the nutritional composition and safety of lab-produced snail flour derived from *Helix aspersa aspersa*, an herbivorous pulmonated gastropod mollusc that occupies various trophic levels in food chains. Our analysis focused on key nutritional aspects, including moisture, ash, protein, and fat contents. Contaminant analysis on the powder showed levels below detectable limits for PAHs, PCBs, PBDEs. The heavy metal concentration was found to be either on par with or lower than values reported in existing literature, indicating the safety of these snail powders for human consumption. Our results revealed a notable presence of polyunsaturated fatty acids and essential amino acids and strongly support the idea that snail powders can serve as sustainable protein sources in both human and animal diets.

1. Introduction

The terrestrial snail is widely consumed in various countries around the world, including Spain, Morocco, France, Portugal, Italy, USA, Turkey, China, Indonesia, and several African countries [1]. Among these, Morocco, Lithuania, and Romania emerge as significant producers, while France, Spain, and Romania stand out as the largest importers [1]. In the Atlantic and Mediterranean regions, the Brown Garden Snail (*Cornu aspersum*, Müller, 1774) and the Roman Snail (*Helix pomatia*, Linnaeus, 1758) are the most consumed species. Other snail species, such as *Archachatina marginata*, *Achatina achatina*, *Achatina fulica*, and *Helix lucorum*, enjoy popularity in various parts of the world [1]. Snails, typically consumed as whole molluscs after a purging treatment, offer a high-quality meat source, rich in protein and low in fat, making them a healthy choice for human consumption [2–7].

Additionally, snail meat is low in carbohydrates, providing a low-calorie alternative, while being a rich source of essential minerals such as calcium, potassium, magnesium, copper, zinc, selenium, and B-group vitamins [8]. Recent research has also shed light on the potential of snail protein to yield bioactive peptides through enzyme hydrolysis, demonstrating protective effects against cardiovascular diseases and presenting a sustainable source of high-quality protein. Furthermore, probiotic lactic acid bacteria strains have been

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identified in the digestive tract, meat, and slime of edible snails, demonstrating inhibitory activity against various pathogenic bacteria, fungi, and yeast [9]. Thanks to these advantageous attributes, the consumption of both fresh and dried snails, as well as insects [10], is on the rise across multiple countries [1,6]. Recent years have seen a growing interest in snails, not only for their culinary appeal but also for their potential in mucus production for cosmetic purposes and the creation of cellulose derivatives like snail slime film [11]. Embracing a circular economy perspective, these versatile creatures, often considered waste after mucus extraction, find new life as a valuable source of fresh food, contributing to a broader bioeconomic profit. Furthermore, snails present an intriguing opportunity for farmers. Their ease of breeding, lower demand for human labour compared to traditional livestock production, and minimal financial investment required to initiate production make them a compelling and sustainable choice. This method of animal husbandry is characterized by its sustainability, requiring minimal space and water resources, while operating efficiently and producing negligible levels of greenhouse gas emissions and pollutants [1]. Consequently, snail farming can seamlessly integrate into organic farming practices, making it a viable option for several world regions in various stages of development. Whether in the form of fresh snail meat or snail powder, the production of snail-based food sources offers an alternative, sustainable solution worth exploring. This becomes particularly crucial for ensuring food security in a future marked by the ongoing challenges of population growth and climate change. It might also help against the condition known as 'hidden hunger', which refers to micronutrient deficiency and inadequate calorie intake in their diets. Von Grebmer et al. [12] support this finding, emphasizing the need for addressing food systems transformation and local governance to combat hunger. However, akin to other animal products [13–16], snails can accumulate various contaminants, including heavy metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs), originating from waste byproducts (gases, particles, sludge, liquid effluent) released into the environment, primarily from industrial activities. Many of these compounds are highly toxic and persist in the environment, posing significant risks to both human and ecosystem health [13,16] and having the potential to result in carcinogenic outcomes [17]. Upon release, these compounds can migrate into the soil, water, and plants, where they become part of the food chain, with a pronounced affinity for products rich in fat due to their high lipophilic nature [17–19]. Furthermore, terrestrial species have long been also employed as sentinel organisms for the surveillance of environmental pollution [20,21]. The presence of these contaminants in these animals arises from a series of processes, including absorption, assimilation, storage, transmission, and excretion, following exposure [22]. Gastropods demonstrate remarkable adaptability, thriving in diverse environments such as woodlands, rocky terrains, agricultural landscapes, and even anthropogenic environments. Their primary diet consists of plants, lichens, fungi, and soil, with a notable impact on their growth due to calcium content [23]. Similar to earthworms, snails display subterranean behavior, particularly during periods of rest, as well as warm (estivation) and cold (hibernation) phases. During these times, they have a propensity to burrow themselves into the soil, sometimes to depths of several centimeters. Numerous studies have investigated the accumulation of metals, persistent organic pollutants (POPs), and microplastics in snails, focusing on multiple anatomical regions, including the viscera (comprising the kidney, hepatopancreas, heart, and a section of the genital system extending into the foot), the foot (encompassing the anterior part of the digestive tract and the nervous system), and the hemolymph [4,20,21,24]. These investigations have consistently revealed a dose-dependent increase in pollutant concentrations within these anatomical regions, influenced by varying environmental conditions and the organism's bioavailability [24].

Given the limited available information regarding the potential risks and benefits associated with the inclusion of snail powder as a dietary supplement in human and animal diets, a comprehensive nutritional assessment was undertaken. This assessment encompassed the entire snail body, comprising the meat, visceral mass (hepatopancreas), and operculum. Therefore, the aim of this study was the characterization of the *Helix aspersa aspersa* powder. These molluscs were raised using an external system. In this work were examined profiles of fatty acids (FAs), amino acid and protein content, potential trace metal presence (such as As, Cd, Co, Cr, Cu, Ni, Pb, V and Zn), and persistent organic pollutants (polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and polybrominated diphenyl ethers).

2. Materials and methods

2.1. Animal species and morphometric characterization

The study was conducted on snails belonging to the *Helix aspersa aspersa* species, which exhibit significant polymorphism in their shells' shape, size, and color. Within this species, different forms are recognized based on height/diameter ratios, and on this basis are differentiated into minor, normal, major, and maxima. The snail has a convex conoid shell (length: 25–35 mm; diameter: 20–40 mm) that expands obliquely at the top and consists of four to five coils; its buccal opening is oblique and oval, with a light-colored columellar edge; the shell color varies from yellow to greenish to grey, influenced by the soil composition in which the species resides; the operculum of this species is not very resistant and may become slightly calcareous in winter; the meat's color is greenish, tending to darken, but it lightens when the snail is in captivity [25]. The *Helix aspersa aspersa* species is well adapted to humid environments and is found in gardens, between hedges, on tree trunks, among walls and rubble, on cliffs, and in lagoon areas, even on sandy soils in the wild. It is well-suited for heliciculture and highly marketable; after trimming and purging, the snail generally weighs 10–12 g. The species lays 3–4 annual deposits, with an average of 80–85 eggs per nest, varying according to the regions and climate of the habitat [25].

2.2. Farming system

The gastropods were bred in an organic snail farm named LUMACA-RE, located in Nettuno (RM) in 2023. All the animals during

their life are stimulated for three times to produce the mucus employed in cosmetic products. At the end of this mucus production cycle the molluscs after purging and washing can be destined to human consumption. In this farm the snails were reared throughout their entire life cycle under a biological regime using an external system and were fed with fresh forage supplemented with a commercial mixture, certified as pesticide-free, containing calcium carbonate.

2.3. Sampling and cluster analysis

A total of 33 deceased Helix *aspersa aspersa* snails were received as a gift from "LUMACA-RE as a frozen foodstuff delivered in dry ice. The deceased whole molluscs bodies were hand washed, labelled, and their weight, with the shell, was recorded before removing their entire soft bodies.

Subsequently, the snails were measured for shell diameter, shell height, opening diameter, total weight, shell weight, and animal weight before being lyophilized and analyzed. The FASTCLUS procedure in SAS 9.2 software [26] was used to cluster the animals based on these six variables (shell diameter, shell height, opening diameter, total weight, shell weight, and animal weight). It is required that the number of clusters (k) be specified before analysis. In our study, the procedure was performed with pre-determined numbers of clusters (2–4 times) to assess the optimal number of groups representing the current sample. Finally, the three-cluster set was applied because it distinguished the most meaningful separated clusters.

In this study samples of soil and commercial feed were also taken to be analyzed.

2.4. Snails' flour characterization

The 3 snails clusters were placed into sterile urine containers, frozen at −80 °C, for two days, and then lyophilized using a freeze dryer Labconco (Kansas City, USA) Freezone 2.5 L for three days. The lyophilized snails were manually minced and finally pulverized using Retsch RM 200 agate milling system to obtain a homogenous powder. The particle size (mean size: 62.66 μm) of this lab-made flour was measured by means of laser scattering distribution analysis (Partica LA-950).

2.4.1. Proteins and amino acids determination

Moisture, ash, and crude protein measurements were performed in triplicate on the obtained powders, following the AOAC method [27]. The protein amount was determined applying the conversion factor of 6.25 to the corresponding nitrogen content [27].

Before determining the amino acid composition of snail flour, a series of standard solutions of amino acids were prepared to develop the instrument method and calibration curves. The amino acid mixture 79248 (Sigma Aldrich®) was appropriately diluted in Milli-Q water to freshly prepare standard solutions with concentrations of each amino acid at 0.5 mM, 0.25 mM, 0.125 mM, 0.05 mM, and 0.025 mM.

For the GC-MS determination of the amino acid composition of snail flour samples, ethyl chloroformate (ECF) was used as the derivatization reagent. About 10 mg of snail flours were hydrolyzed at 110 °C for 24 h with 200 μl of 6 M HCl in an autoclavable vial of 300 μl after deoxygenation of the solution and headspace of the vial using a gentle nitrogen flow. The following day, the vial was cooled to room temperature, and the solution was dried under a gentle N_2 flow, and the residues dissolved in 150 μl of Milli-Q water and 150 μl of chloroform. After agitation with a glass capillary directly in the vial, the chloroform phase was discarded, and 100 μl of the water solutions were recovered. After filtration using 0.45 μm PTFE filters, these solutions were transferred into a second micro-vial and treated to derivatize the amino acids.

In brief, 100 μl of the filtered aqueous extract of each snail flour sample or 100 μl of each amino acid standard solution were: i) mixed with 50 μL of a 4:1 solution of ethanol and pyridine and stirred using a glass capillary stick; ii) added of 10 μL of ECF and stirred slowly as this reaction releases CO_2 gas causing effervescence; iii) added of 50 μL of a 1 % ECF solution in chloroform; iv) the resulting solution neutralized by adding 50 μL of a saturated $NaHCO_3$ solution and stirred until the effervescence (CO_2) ceased. Following the derivatization step, each sample consisted of two layers: an aqueous top layer and an organic bottom layer.

The lower organic layer was recovered and dried by adding anhydrous sodium sulfate, and finally transferred to a fresh vial for the following GC-MS analysis. The amino acid GC/MS determination was performed using a Thermo Fisher TSQ 8000 (Waltham MA, USA) instrument equipped with a Thermo Trace 1300 GC. The GC column was a 20 m, 0.18 mm i.d., 0.18 μm film thickness TRACE Gold TG-XLBMS (Thermo Fisher). Helium was used as the carrier gas at a flow rate of 0.8 mL/min. The thermal gradient program was set as follows: starting at 100 °C and held for 2 min, then ramped at 10 °C/min to 230 °C, followed by a ramp from 230 °C to 280 °C at 30 $°C·min^{-1}$ and held for 5 min. A 1 μL injection with a 1:25 split ratio was made, and the sample inlet temperature was set to 280 °C.

2.4.2. Lipids determination

Approximately 0.5 g of snail powders (two aliquots for each cluster) were accurately weighed to determine the fatty content and 2 ml of dichloromethane and 4 ml of methanol were added to each sample. The slurry placed in a 12 ml falcon tube was vortexed and then added with further 2 ml of dichloromethane and 2 ml of water, vortexing the tube for 1 min at each solvent addition step. After centrifugation for 5 min at 3500 rpm in a SLBR centrifuge, Thermo Scientific™ (Waltham, MA, USA) the organic phase settling at the bottom of the tube was collected. The residue was submitted to a second extraction step adding an aliquot of 2 ml of dichloromethane, and vortexing the tube for 1 min. After centrifugation for 5 min at 3500 rpm the organic phase settling at the bottom was collected and combined with the first aliquot. To remove any residue, the reunited aliquots were further centrifuged for 5 min at 3500 rpm and the resulting clear liquid was recovered in a pre-weighed vial. The samples were dried by means of GeneVac EZ-2 (Genevac), and the mass of the lipids weighed.

2.4.3. Fatty acids profile

For Fatty Acid Methyl Esters (FAMEs) determination, approximately 1 g of each powder was accurately weighed to extract the lipidic fraction [28]. The procedure implied the following steps: each weighed sample was placed in a glass screw cap tube with 10 ml of a chloroform/methanol solution (2:1 v/v); the tube was capped and placed in an ultrasound bath for 30 min; after the ultrasound treatment, the entire content of the tube was carefully transferred into a glass funnel fitted with fast filter paper to separate the lipid-containing solution from any solid tissue fragment, the filtrate was collected in a second glass tube, and the filter was washed with 5 ml of chloroform, collecting the wash into the same tube.

To remove impurities the clean-up procedure prevised the addition of 2 ml of 1 % saline water to the lipid extract to separate polar compounds from the lipids. The mixture was gently agitated using a vortex mixer for about 1 min to prevent pressure buildup. After centrifugation at 3000 rpm for 5 min, the lower chloroform phase, containing the desired lipids, was collected, and the aqueous phase was discarded (in some cases, between the two liquid phases, a solid tissue fragment in the middle was present). The lower chloroform phase was collected, which contains the desired lipid fraction, and the aqueous phase was discarded. The chloroform solution was poured into a third glass tube containing a spoonful (about 0.5 g) of anhydrous sodium sulfate (Na_2SO_4) to remove traces of water from the chloroform solution. The clear solution was then collected into a 25 ml balloon and dried using a rotary evaporator. Finally, 1 ml of hexane was added to the dried lipid extract. The clear hexane solution recovered into a 1.5 ml vial was vortexed for 30 s before the *trans*-esterification step.

This last phase was performed directly into the injection vial, by adding 0.2 ml of 2 M alcoholic potassium hydroxide (KOH) solution to the hexane extract. The mixture was agitated by vortex mixing for 30 s, and KOH was allowed to react with the lipids for at least 30 min before FAMEs determination. The upper portion of the solution was injected using a Thermo Fisher (Waltham MA, USA) TriPlus autosampler. GC/MS analysis was performed by means of a Thermo Fisher (Waltham MA, USA) TSQ 8000 instrument equipped with a Thermo Trace 1300 GC. The experimental parameters included a 20 m, 0.18 mm i.d., 0.18 μm film thickness TRACE Gold TG-XLBMS (Thermo Fisher) column, with He used as the carrier gas at a flow rate of 0.8 mL/min. The temperature gradient was set as follows: starting at 50 °C and held for 4 min, then ramped at 25 °C/min to 185 °C and held for 4 min, followed by a ramp from 185 °C to 210 °C at 3 °C/min and held for 3 min, and finally from 210 °C to 220 °C at 3 °C/min. The temperature was then ramped at 15 °C/min to reach 310 °C, resulting in a total run time of 36 min. A 1 μL injection with a 1:25 split ratio was made, and the sample inlet temperature was set to 300 °C.

The identification of FAMEs in the snail flours was accomplished using the Supelco 37 Component FAMEs mix standard (Supelco, Bellefonte, PA, USA). The mass spectrometer, equipped with an Electron Ionization (EI) source set at an ionization potential of 70 eV, operated in Selected Ion Monitoring (SIM) mode, monitoring ions at m/z 55, m/z 57, m/z 69, m/z 74, and m/z 87, and in full scan mode with a scan range of m/z 50–400. The transfer line temperature and ion source temperature were both set at 290 °C.

The FAMEs were determined based on their retention times and by comparing their spectra with the NIST 2015 Mass Spectral Library. The quantities of fatty acids (FAs) were expressed as relative percentages with respect to the total fatty acid content. Peak integration was performed using Xcalibur™ software (Thermo Scientific™, Waltham, MA, USA).

2.4.4. Lipids index

Based on literature data [29,30], the health-promoting index (HPI), atherogenicity (IA) and thrombogenicity (IT) indices were proposed as a dietary risk indicator of lipids for cardiovascular diseases. These indexes were determined according to the following equations:

$$HPI = (MUFA + PUFA) / [(C12{:}0 + 4 \times C14{:}0) + C16{:}0]$$

$$IA = [(4 \times C14{:}0) + C16{:}0 + C18{:}0] / [MUFA + PUFA\text{-}n6 + PUFA\text{-}n3]$$

$$IT = (C14{:}0 + C16{:}0 + C18{:}0) / (0.5MUFA + 0.5PUFA\text{-}n6 + 3PUFAn3 + PUFA\text{-}n3/PUFA\text{-}n6)$$

HPI scores have been used to assess the general nutritional value of dietary fat while the IA represents the relationship between the sum of the main saturated fatty acids and that of the main classes of monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids. Saturated fatty acids are considered pro-atherogenic as they ease the adhesion of lipids to cells of the immunological and circulatory systems, which can contribute to atherosclerosis. On the other hand, unsaturated fatty acids are considered anti-atherogenic as they inhibit the aggregation of plaque, diminish the levels of cholesterol, esterified fatty acids, and phospholipids, thus helping to prevent the development of coronary diseases.

The IT indicates the tendency to form clots in the blood vessels and considers the relationship between the pro-thrombogenic (saturated) and the anti-thrombogenic fatty acids (MUFAs, PUFAs – n6, and PUFAs – n3).

2.5. Statistical analysis

The data regarding the fatty acids, amino acids and heavy metals were statistically analyzed by the SAS 9.2 software (2010), using a generalized linear model (GLM) that included the effects of cluster (3 levels). Results are reported as LSM and differences between means were tested by Student's "t" test. Statistical significance was attributed to p values < 0.05.

2.6. Flour of snails' environmental contaminants

2.6.1. Contaminants materials

Methanol, dichloromethane, chloroform, and hexane solvents pesticide grade were purchased from VWR (Milano, Italy). SPE (solid phase extract) Florisil tubes 1 g/6 ml, NaCl, Na_2SO_4, and KOH pellets were from Supelco (Bellefonte PA, USA).

The standard mixes used to calibrate the instrument were: (a) poly-chlorinate biphenyl mix (PCB mix contained PCB28, PCB52, PCB101, PCB81, PCB77, PCB123, PCB114, PCB118, PCB105, PCB153, PCB138, PCB126, PCB128, PCB156, PCB157, PCB167, PCB180, PCB169, PCB170, PCB189, PCB209, each component at 20 μg/g in hexane), (b) polycyclic aromatics hydrocarbon (PAH—Mix9 contained Naphthalene, Acenaphthylene, Acenaphthene, Phenanthrene, Anthracene, Fluoranthene, Fluorene, Pyrene, Benzo(a) Anthracene, Chrysene, Benzo(b)Fluoranthene, Benzo(k)Fluoranthene, Benzo(a)Pyrene, Indeno(123cd)Pyrene, Dibenzo(ah)Anthracene, Benzo(ghi)Perylene, each component at 10 μg/g in hexane) obtained from Dr. Ehrenstorfer GmbH, (c) poly-bromurate diphenyl ethers (PBDE mix containing BDE28, BDE47, BDE66, BDE85, BDE99, BDE100, BDE153, BDE154, BDE183, each component at 10 μg/g in acetone), all mixes purchased from Dr Ehrenstorfer GmbH. The standard mixtures used as an internal standard or to syringe standard were respectively: PAH deuterated Mix 77 (containing Acenaphthylene D8, Benzo(a)pyrene D12, Pyrene D10) and PAH deuterated Mix 25 (containing Acenaphtene D10, Chrysende D12, Perylene D12, Phenanthrene D10).

2.6.2. Contaminants extraction

To obtain suitable samples for detecting potential organic contaminants such as PAHs, PCBs, and PBDEs in lab-made snail flours, we developed and optimized an extraction procedure based on the previously described fat residue extraction method. Initially, the snail fat sample was treated with 1 ml of 2 M sodium hydroxide, vortexed, and centrifuged to saponify the lipids. Subsequently, 2 ml of an acetone:hexane mixture (20:80) was added to extract the lipophilic fraction of the contaminants.

After vortexing and centrifugation, the upper organic phase was collected. This organic solvent extraction was performed twice, and the resulting organic phases were combined into a single vial. Next, 0.5 g of anhydrous sodium sulfate was added to the combined phases to remove any remaining water, followed by vortexing for 30 s. Then, 200 mg of dispersed Florisil SPE was added to the vial containing the organic phase, vortexed for 30 s, and centrifuged for 5 min at 3500 rpm using a Thermo SLBR centrifuge (Thermo Scientific™, Waltham, MA, USA). After centrifugation, the supernatant was collected in an Eppendorf tube, dried, and reconstituted with 475 μl of hexane and 25 μl of a 1 ppm syringe standard solution (PAH deuterated Mix 25). Finally, the sample was transferred to GC/MS vials for analysis.

For PAHs, PCBs, and PBDEs determination, we utilized a Thermo Trace GC 1300 coupled with a Thermofisher TSQ 8000 triple quadrupole mass spectrometer (Thermo Scientific™, Waltham, MA, USA). The experimental conditions for PAHs analysis were as follows: a GC column TraceGOLD™ TG-XLBMS (30 m, 0.18 mm i.d., 0.18 μm film thickness) was used, and the PTV injector parameters included PTV mode: CT Splitless, split flow: 40 ml/min, Split ratio: 6.7, Splitless time:2 min, Constant flow: 0.80 mL/min, sample volume: 2.0 μl, Max Temp: 340 °C. Additionally, the GC oven program parameters were set with an initial temperature of 40 °C held for 2 min, followed by a thermal gradient at 10 °C/min up to 320 °C, and then held for 5 min. The MS transfer line temperature was 280 °C, the ion source temperature was 300 °C, and the ionization mode was EI. The Limit of detection (LOD) and Limit of quantitation (LOQ) for PAH were 2 and 5 μg/Kg^{-}, respectively.

For PCBs and PBDEs determination, the experimental conditions were the following: GC column: TraceGOLD™ TG-XLBMS (30 m, 0.18 mm i.d., 0.18 μm film thickness). PTV injector parameters: PTV mode: large volume, split flow: 30 ml/min, Split ratio: 6.7, Splitless time: 2 min, Constant flow: 1.200 mL/min, sample volume: 5.0 μl/L, Max Temp: 350 °C. The MS transfer line temperature was 280 °C, the ion source temperature 300 °C, and the ionization mode was EI. The thermal program developed for GC/MS analysis of pollutants in *Helix aspersa aspersa* flours started with an initial isothermal step at 40 °C held for 2 min, followed by a thermal gradient at 4 °C/min up to 300 °C, and then a thermal gradient at 30 °C/min up to 320 °C held for 4 min.

In Tables 1S and 2S, the experimental conditions for MS analyses of pollutants in lab-made snail flour samples were reported. The LOD and LOQ for PCBs and PBDEs were 0.1 and 0.5 μg/kg, respectively.

2.7. Contaminants in soil and feed

To assess the potential presence of contaminants, we applied the same optimized extraction procedure used for lab-made flours to soil and feed samples collected from the organic snail farm.

2.8. Metal investigation

About 0.25 g of the lyophilized and pulverized snails, feed and soil were previously digested and mineralized using a microwave oven MARS-5 Xpress (CEM, World Headquarters, Matthews, NC, USA) with a high-pressure rotor. In particular, the carefully weighed sample was treated with 2 ml of HNO_3 (65 %) and 2 ml of H_2O_2 (30 %) and then mineralized at a temperature of 200 °C. After mineralization, the samples of snails and feed were diluted to 10 ml using 1 % HNO_3 solution.

The contents of arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb) and vanadium (V) were determined (with subsequent and appropriate dilutions) by ICP-MS (Icap Q- Thermo-Icap) in digested samples. Analyses were carried out by external calibration using standards in the same acid matrix of samples prepared by dilution of ICP-MS High-Purity Standard Solutions. The accuracy of the method was validated by the analysis of Certified Reference Materials for lobster hepatopancreas (TORT-3, National Research Council Canada). The accuracy was between 0.4 and 15 %. Reagent blanks and duplicate samples of feed

were used to monitor the appropriateness and reproducibility of the preparation and analytical procedures. The analytical precision, based on triplicated runs (RSD%, n = 3) of each sample (snails, soil, and feed) was <10 %, and the reproducibility was better than 7 %.

The LOD, expressed as a concentration, represents the smallest measure that can be reliably detected using this analytical procedure. In this work, the mean blank signal was employed for the calculation of the signal LOD:

$$X_L = X_B + KS_B$$

where X_B is the mean of the blank measures (4 measures in this procedure), S_B is the standard deviation of the blank measures, K factor (equal to 3) chosen according to the confidence level desired. LODs (μg/g) for each metal were: As 0.000284; Cd 0.00024; Co 0.000225; Cr 0.0175; Cu 0.0528; Ni 0.003278; Pb 0.00096; V 0.000105.

Samples were prepared and analyzed with great caution to minimize contamination from air, glassware, and reagents, all of which were of Suprapur quality.

3. Results and discussions

3.1. Snails flour characteristic, amino acids, and fatty acid profiles

To fully understand the dietary benefits of breeding and consuming snails, it is essential to explore their composition, enabling a comprehensive evaluation of the nutritional content provided by different edible snail species. In our lab-made snail flours, we used the entire soft body of the animal as it is also usually consumed. Based on biometric parameters (shell diameter, shell height, opening diameter, total weight, shell weight, and animal weight), subjects were well-distributed among three clusters (A, B and C; Table 1), presenting a high F ratio. In synthesis, all clusters presented dimensions comparable with those indicated for *H. aspersa aspersa* [25], with an animal size in A < B < C.

The three lab-made snail flours revealed the following chemical composition (as g/100 g of fresh weight snail meat): moisture content between 83.76 % and 83.82 % (B > A > C; average 83.79 ± 0.03 % RSD), ash content between 1.04 % and 1.29 % (C > A > B; average 1.14 ± 0.13 % RSD), protein content between 8.47 % and 8.98 % (A > C > B; average 8.66 ± 5 % RSD), and fat content between 0.86 % and 1.55 % (B > C > A; average 1.21 ± 20 % RSD). It's worth noting that the nutritional composition of snails can vary due to factors such as feeding habits, body condition, environmental factors, and species [7,31]. The moisture, ash, protein, and fat content values observed in this study are comparable to those obtained from cage-grown *H. aspersa aspersa* under optimal environmental conditions (Table 2). Across various species of snails (land, freshwater, or marine), the fat content in snail meat, ranges between 0.4 % and 8.5 % [7; Table 2], which generally falls below levels found in traditional animal-derived foods.

Fatty acids play crucial roles in the human body, serving as structural components of membrane lipids, precursors to molecules like eicosanoids and prostaglandins, and essential components of metabolism. The consumption of saturated fatty acids is generally deemed undesirable due to their association with elevated cholesterol levels and the risk of cardiovascular disease [32]. Dodecanoic (lauric), tetradecanoic (myristic), and hexadecanoic (palmitic) acids have been associated with elevated cholesterol levels, whereas octadecanoic (stearic) acid has been suggested to potentially lower cholesterol levels [30]. Fatty acids belonging to the n-3 and n-6 groups cannot be synthesized by the human body and must be acquired through the diet to mitigate the risk of cardiovascular diseases [29]. The 3 clusters showed no significant differences in the fatty acids profiles and the results were reported as means ± SD of the 3 samples in Table 3. The fatty acid profile of snail flour, primarily consists of polyunsaturated fatty acids (PUFAs), followed by saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs). The dominant saturated fatty acids are hexadecenoic (palmitic) acid (16:0) and octadecanoic (stearic) acid (18:0), while *cis*-9-octadecanoic (oleic) acid (18:1 n-9), *cis*-9,12-octadecadienoic (linoleic) acid (18:2 n-6), and *cis*-11,14-eicosadienoic acid (20:2) are the predominant polyunsaturated fatty acids. These findings align with previous studies (Table 2), although minor discrepancies may be attributed to variations in sample preparation. Notably, our lab-made flours include the hepatopancreas, which exhibits a higher fatty acid content compared to snail meat. The HPI, IA, and IT calculated for the snail powders were 4.19, 0.47, and 0.7, respectively. These results suggest that the consumption of these flours may contribute to promoting human health by potentially reducing plasma cholesterol and phospholipid concentrations. This effect could potentially aid in preventing micro- and macro-coronary diseases while also exhibiting anti-atherogenic activity.

Considering the amino acid composition, the statistical analysis showed significant differences between the clusters, but the overall contents (Table 4) were comparable with those reported by other authors (Table 3S). It is crucial to consider the composition of essential amino acids in fresh tissue, which in total showed higher value in cluster B (average 46.54 mg/g) than in C (average 41.61

Table 1
Snails biometric parameters (means ± SD) of each cluster.

Cluster	A	B	C
Number of individuals	7	16	10
Total weight g	10.89 ± 1.54	13.27 ± 1.40	16.49 ± 1.69
Animal weight g	7.19 ± 2.65	8.17 ± 1.45	12.78 ± 1.88
Shell weight g	3.99 ± 1.26	5.20 ± 2.57	3.76 ± 0.93
Opening diameter mm	17.43 ± 1.27	19.69 ± 1.62	20.60 ± 1.35
Shell height mm	20.71 ± 1.11	27.13 ± 3.26	27.80 ± 2.82
Shell diameter mm	32.71 ± 2.98	34.63 ± 1.45	37.70 ± 1.64

Table 2
Proteins, lipids, and fatty acids composition (relative % of total fatty acids) on fresh meat compared with literature data. The symbol * in table is referred to dry matter samples.

	Protein (g/100 g)	Lipids (g/100 g)	SFA	MUFA	PUFA	Reference
Helix aspersa aspersa	*8.7*	*1.2*	*32.8*	*28.4*	*38.9*	*This work*
Helix aspersa	8.1–16.4	0.4–0.7	22.2–28.8	20.7–23.8	34.4–58.4	[7]
Helix pomatia	10.7–15.4	1.1–1.2	32.4–39.3	18.0–20.2	25.8–39.7	[1,3,7]
Helix pomatia	80.17*	4.24*				[1]
Helix lucorum	10.8	1.5	21.7	26.5	51.8	[2,7]
Assyriella escheriana			23.1	15.0	62.4	[7]
Assyriella guttata			23.1	16.1	56.8	[7]
Eobania vermiculata			22.7–25.6	21.8–32.2	44.4–55.6	[2,7]
Cornu aspersum	59.5–67.4*	4.8-7.30*	23.0–33.9	22.8–35.6	31.5–52.4	[1,3,7]
Theba pisana			20.7–25.3	30.5–31.3	44.2–48.1	[7]
Helix sp.	8.64	0.57				[7]
Achatina sp.	9.9	1.4				[7]
Achatina achatina	17.2	2.21				[7]
Achatina fulica	10.0–13.7	0.91–1.6				[7]
Limicolaria sp.	5.86–17.5	0.8–4.3				[7]
Limicolaria aurora	14.8	2.8				[7]
Archachatina marginata	15.0–20.8	0.79–2.4				[7]
Archatina sp.	14.5	4.2				[7]

Table 3
Relative percentages of FAs (determined as methyl esters) in laboratory-produced snail flours.

Fatty acid (as methyl ester)		g/100 g FA		g/100 g FA
Tetradecanoic (Myristic) acid	C14:0	0.663	±	0.030
Pentadecanoic acid	C15:0	0.159	±	0.002
Hexadecanoic (Palmitic) acid	C16:0	13.393	±	2.069
Heptadecanoic acid	C17:0	1.715	±	0.716
Octadecanoic (Stearic) acid	C18:0	15.574	±	1.492
Eicosanoic (Arachidic) acid	C20:0	1.276	±	0.482
SFA		**32.782**	**±**	**2.693**
cis-10-Heptadecenoic acid	C17:1	1.049	±	0.462
cis-9-octadecanoic (Oleic) acid	C18:1	27.311	±	1.795
MUFA		**28.360**	**±**	**1.853**
cis-9,12-Octadecadienoic (Linoleic) acid	C18:2 ω6	16.639	±	5.755
cis-11,14-Eicosadienoic acid	C20:2 ω6	10.496	±	2.724
cis-8,11,14-Eicosatrienoic acid	C20:3 ω6	3.437	±	0.793
cis-5,8,11,14-Eicosatetraenoic acid	C20:4 ω6	5.031	±	1.816
Total ω6		**35.603**	**±**	**6.668**
cis-5,8,11,14,17-Eicosapentaenoic acid	C20:5 **ω3**	0.655	±	0.265
cis-4,7,10,13,16,19-Docosahexaenoic acid	C20:6 **ω3**	2.599	±	1.871
Total ω3		**3.254**	**±**	**1.889**
Ratio Sum ω6/Sum ω3		10.943		
PUFA		**38.857**		**6.931**

mg/g) and in A (average 36.47 mg/g) (Table 4). Notably, our lab-made flours did not evidence presence of tryptophan, possibly due to the acid hydrolysis process [7]. Interestingly, land snails demonstrate relatively high concentrations of flavor-enhancing amino acids, particularly aspartic and glutamic acids (Table 4, Table 3S). Aspartic acid is a nucleotide constituent, and glutamic acid is involved in the synthesis of gamma-aminobutyric acid, a neurotransmitter. Glycine and alanine are also present in high concentrations, contributing to a pleasant sweet–umami taste [33]. The highest content of glutamic acid has also been reported in the literature for other land snail's species (Table 3S). Furthermore, protein and essential amino acid content in snail flour ranges from 52 to 59 and 23–25 g/100 g, respectively. This suggests that the average essential amino acid content in snail flour is approximately ten times higher than that found in maize flour [34], around eight times higher than that in rice flour, and six times higher than that in wheat flour [35]. Consequently, even in small quantities, snail flour can effectively supplement protein-deficient cereal flours and compensate for diets lacking in essential amino acids.

3.2. Organic contaminants in snails' flour

The quality of snail flour was assessed with regard to occurrence of potential environmental organic contaminants, such as PAHs,

Table 4
Amino acids composition of the 3 lab-made snail flours from Italian *Helix aspersa aspersa* expressed in mg/g of fresh tissue (mean ± SD).

	Cluster		
	A	B	C
Essential			
Arginine	$1.59^{b} \pm 0.14$	$2.17^{a} \pm 0.03$	$2.00^{a} \pm 0.09$
Histidine	1.73 ± 0.00	1.91 ± 0.08	1.46 ± 0.33
Isoleucine	$5.11^{c} \pm 0.06$	$6.75^{a} \pm 0.15$	$6.41^{b} \pm 0.20$
Leucine	$7.43^{c} \pm 0.11$	$9.71^{a} \pm 0.04$	$8.05^{b} \pm 0.12$
Lysine	$5.00^{c} \pm 0.06$	$7.42^{a} \pm 0.12$	$6.08^{b} \pm 0.01$
Phenylalanine	$6.50^{b} \pm 0.02$	$8.79^{a} \pm 0.03$	$6.60^{b} \pm 0.02$
Threonine	$1.35^{b} \pm 0.12$	$1.71^{a} \pm 0.00$	$1.83^{a} \pm 0.03$
Valine	$6.64^{b} \pm 0.14$	$7.15^{b} \pm 0.65$	$8.14^{a} \pm 0.19$
Methionine	$1.11^{a} \pm 0.02$	$0.92^{c} \pm 0.02$	$1.04^{b} \pm 0.00$
Total essential	$36.47^{c} \pm 0.58$	$46.54^{a} \pm 0.37$	$41.61^{b} \pm 0.97$
Not essential			
Alanine	$8.03^{b} \pm 0.20$	$8.65^{a} \pm 0.06$	$7.88^{b} \pm 0.21$
Aspartic acid	$13.07^{c} \pm 0.09$	$17.61^{a} \pm 0.90$	$14.92^{b} \pm 0.12$
Glutamic acid	$5.55^{b} \pm 0.49$	$6.47^{a} \pm 0.08$	$5.72^{b} \pm 0.22$
Glycine	$7.54^{ab} \pm 1.22$	$8.69^{a} \pm 1.60$	$5.94^{b} \pm 0.03$
Proline	$4.73^{a} \pm 0.55$	$4.87^{a} \pm 0.59$	$3.54^{b} \pm 0.04$
Serine	0.15 ± 0.00	0.16 ± 0.00	0.14 ± 0.00
Tyrosine	$3.41^{b} \pm 0.52$	$4.07^{a} \pm 0.01$	$3.00^{b} \pm 0.00$
Cysteine	$0.62^{b} \pm 0.01$	$0.82^{a} \pm 0.01$	$0.88^{a} \pm 0.01$
Glutamine	$0.24^{ab} \pm 0.00$	$0.27^{a} \pm 0.02$	$0.21^{b} \pm 0.01$
Total not essential	$43.35^{b} \pm 3.08$	$51.62^{a} \pm 1.14$	$42.23^{b} \pm 0.37$

The results indicate mean values of three measurements performed on each sample. On rows: ^{a}, ^{b} and ^{c} = $p \leq 0.05$.

PBDEs, and PCBs, which can spread over long distances from their emission sources [16]. This risk cannot be excluded *a priori*.

For this scope, we analyzed the fat residue in snail meat obtained from lipid extraction due to their affinity to lipophilic tissue and can be accumulated in the adipose tissue of animals. Subsequently, these could be transferred to humans when eating up, representing a potential risk for health.

Moreover, samples of feed and soil collected from the organic snail farm were analyzed by GC-MS/MS technique determining 16 PAHs, 21 PCBs, and 8 PBDEs in two separate chromatographic runs. It is worth noting that there are currently no specific regulations in place governing the microbiological and chemical requirements for the consumption of molluscs such as H. *aspersa*. In our study, we compared the levels of each contaminant found with the established lowest limits set for "meat" by European Regulation [16].

The analyses of snail flour, feed and soil samples revealed concentrations of PAHs, PCBs, and PBDEs that were all below the LOD thresholds (LODs: PAHs 2 μg/kg fresh weight; PCBs and PBDEs 0.1 μg/kg fat). These levels were also lower than the lowest European limits established for PAHs in smoked meat (benzo(a)pyrene 2 μg/kg; sum of benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene, and chrysene: 12 μg/kg) and non-dioxin-like PCBs in meat from ruminants, pigs, and poultry (sum of PCB28, PCB52, PCB101, PCB138, PCB153, and PCB180: 40 ng/g fat). Chlorinated pesticides were not investigated, as the commercial mixture used for supplemental was certified as pesticide-free. For risk assessment evaluation, it is also important to consider the *pro-capita* ingestion rate, that for snails is in general low, and in Italy on average equal to 180 g in a year [1]. Therefore, these results obtained for organic contaminants showed no potential risks to human and animal health by consumption (as integration or sustenance) of flour of snails, that are bred in an organic farm under good environmental conditions and fed with certified feed.

Despite this, it should be necessary to implement the EU legislation on contaminants in food including the snails and their derived products, also considering the growing consumption in different countries.

3.3. Metals in snails' flour

Edible snails offer comparable, if not superior, nutritional value to conventional animal-origin foods. However, it is crucial to address safety concerns when using snails as food, particularly regarding the potential accumulation of heavy metals, such as cadmium (Cd), lead (Pb), arsenic (As), nickel (Ni), chromium (Cr), copper (Cu), vanadium (V), zinc (Zn) and cobalt (Co), in their tissues. As previously reported for organic contaminants, EU limits for heavy metals in snails have not been established. Therefore, we compared the metals' concentration with the lowest limits reported in European regulation for "meat" [16].

Incidentally, the statistical analysis revealed significant differences between the heavy metals' contents in 3 clusters, distinguishing the lab-made snail flours (Table 4S). Concerning the toxicants, Cd levels were lowest in cluster B (average 1.90 μg/g), higher in cluster C (average 2.34 μg/g) and highest in cluster A (average 2.67 μg/g), while Pb levels were higher in cluster B (average 0.79 μg/g) than in cluster A (average 0.36 μg/g) and in cluster C (average 0.27 μg/g). Additionally, the highest concentration of Cu in snail flour was detected in cluster B (average 219.13 μg/g). The differences found may be related to the health statuses of the clustered gastropods, although there is insufficient data to adequately explain these findings. The range of values of heavy metals' contents found in the 3 clusters of lab-made flours and in fresh meat were reported in Table 5.

The concentrations of Cd and Pb in snail powder were found to be lower than those detected in molluscs collected in various

Table 5
Heavy metals in snail tissue are reported as µg/g of fresh meat. The symbol * in table is referred to dry matter samples.

	Cd	Pb	Ar	Cr	Ni	V	Co	Cu	Author
Helix *aspersa aspersa* fresh meat	*0.35–0.49*	*0.05–0.14*	*0.018–0.022*	*0.05–0.08*	*0.10–0.14*	*0.09–0.11*	*0.04–0.07*	*14.1–39.8*	*This work*
Helix *aspersa aspersa* flour	*1.9–2.67*	*0.27–0.79*	*0.10–0.12*	*0.28–0.47*	*0.59–0.81*	*0.53–0.63*	*0.23–0.38*	*77.46–219.13*	*This work*
Helix *aspersa*								21–252	[7]
Helix *aperta*	0.004–0.03	0.003	0.01–0.08	0.03–0.07	0.08–0.14	0.003	0.003	23.13–48.20	[24]
Helix *lucorum*								34	[7]
Helix *pomatia*	0.04–0.10							4.82–300	[4,7,8]
Helix *vladika*	1.57*	0.86*							[22]
Helix *secernenda*	1.49*	0.11*							[22]
Archatina sp.								6.2	[7]
Archachatina marginata								6.4–10.3	[7]
Limicolaria sp.								1.6–2.9	[7]
Limicolaria flammea								25.3	[7]
Achatina fulica								5.8	[7]
Achatina achatina								7.7–33	[7]
Cornu aspersum								3.99–6.22	[8]
Eobania vermiculata		0.32–2.25	0.08–1.00	0.99–15.87	0.044–10.92				[21]
Theba pisana	0.003–0.004	0.003–0.07	0.02	0.02–0.03	0.09			22.11–24.88	[24]
Cernuella virgata	0.01	0.003	0.03	0.04	0.10		0.003	26.23–29.34	[24]

contaminated sites across Italy [36] and were comparable to levels found in diverse European and non-European countries (Table 5). However, the values for Pb and Cd were slightly higher compared to the EU limits for other meats (Pb: 0.10 μg/g fresh weight, Cd: 0.05 μg/g fresh weight; [16]). The concentrations of these elements in snails are influenced by their environmental exposure and dietary habits (Table 5). Previous studies have reported absorption efficiencies of approximately 68–72 % for Cd and 43 % for Pb, respectively (Baroudi et al., 2020). In the case of Cd, snails exhibit less control over its absorption, leading to the accumulation of higher levels without causing severe effects. This phenomenon is likely attributed to the presence of metallothionein-like proteins in the snail's digestive gland, which bind to Cd [20]. Although Cd levels in snail tissue exceed current regulations for meats [16], the consumption of snail meat is significantly lower compared to other types of meats. For instance, in countries with high snail consumption like France [1], the average annual consumption is estimated to be around 600 g per capita, whereas other types of meat are consumed at an average of approximately 70 kg per capita. This translates to a consumption ratio of approximately 1:100. As a result, even in the worst-case scenario and in a long-term exposure, the cadmium intake from snail meats would amount to less than 1/10 of the intake of these toxic metals from other meats.

Considering this aspect, even a significant increase in snail meat consumption should not lead to a notable increase in the risk of chronic heavy metal poisoning. A similar argument applies to Pb, which, according to our studies, was at the legal limit for meats [16]. The main sources of exposure to heavy metals for the snails were feed and soil, and our molluscs presented only Cd concentration that could be related to those found in the investigated matrices (Table 6). The As concentration in the snail's flour was notably lower compared to previous investigations on H. *aspersa*, a species known for its tendency to accumulate this element in the hepatopancreas (Table 5). Regarding Cr and Ni (Table 5), our findings were comparable [1] and lower [36] than those found on the same species in contaminated areas of Italy. Previous studies on *Helix aspersa* indicated a preference for accumulating Ni primarily in the hepatopancreas, potentially contributing to increased levels of oxidative stress parameters. In contrast, the Cr levels were more evenly distributed across the entire snail's body [36]. Lower concentrations were also detected for V and Co (Table 5), two elements that have received limited attention in studies involving this species [36]. Finally, in this study Cu levels were found to be higher or within the range of values observed in other studies on the same species (Table 5). This element, together with Zn, is required for snail physiological functioning. Particularly, Cu is required in large amounts as an element of hemocyanin, that is converted and absorbed after accumulation. The absorption of Cu is controlled until it exceeds the threshold rate [20]. Therefore, the concentration of Cu found in snails in our study may reflect the high level of this element found in soil (Table 6), confirming the high absorption efficiency that the snails exhibit for this element (90 %; [20]). Even for human species, Cu represents a micro essential element, with a recommended daily intake ranging from 300 up to 1300 μg per capita [37].

According to our measurements the average Cu content in snail meat is 39.8 mg/kg and considering the annual consumption of snails (600 g per capita) [1], it results in an annual copper intake of 23.88 mg per year or 65 μg per capita per day. To significantly affect daily Cu intake, the annual consumption of snail meats would need to be substantially increased (at least 5 folds). Considering the use of snail flours and its impact on human health in the long term it follows that the administration of just 3–4 g of snail flour (either alone or mixed with other substrates) would fulfill the recommended daily copper intake for children or adults. This would keep Cd exposure within safe ranges.

Zinc levels in Helix *A*. flours are in the same range of the Cu levels (Table 4S), and therefore about 3 folds the levels found in beef. Taken into consideration the requirements of Zn in the human diet [38], these flours could be also considered for its supplementation.

Further research is however still warranted to investigate the mechanisms influencing the transfer of all elements from both the feed and the environment into the soft tissues and in the shells of the snails [39].

4. Conclusions

This study provides valuable insights into the nutritional composition and potential contaminant levels in lab-made snail flours from Helix *aspersa aspersa*.

Regarding pollutants and heavy metal concentration, this study highlights the importance of monitoring and understanding organic and inorganic contaminant accumulation in edible snails, such as H. *aspersa aspersa*, as their consumption represents a potential route of exposure. The elevated Cu and Zn levels in snail flours suggest their suitability for supplementing copper and zinc in the diet. However, supplementation with snail flour should be accompanied by a preliminary evaluation of associated Cd and Pb levels, or at least in appropriate substrate selection during snails breeding. Notably, considering the amino acid composition and the FA profile, snail flours can be considered valuable for human and animal consumption for formulating nutraceuticals and food supplements.

On the other hand, further research and appropriate regulatory measures are necessary to ensure the safety and quality of snails and snail-derived products, determining the limits for a specific set of contaminants following appropriate methods for human risk assessment by dietary intake.

As the demand for novel and environmentally friendly foods continues to grow, snail farming could represent a viable and eco-conscious solution, simultaneously addressing food security, nutritional diversity, and waste recovery within the agricultural sector. Some limitations of our study have to be considered, though. Indeed, our study was conducted on animals that were biologically bred and had a supplemented diet, that could lead to an accumulation of specific substances. Long-term studies should be warranted to fully explore the potential benefits and commercial applications of these snail flours, fostering innovation and progress in the field of alternative protein and fat sources.

Table 6
Trace metal concentrations (μg/g) in feed and soil in our study as means values (±% RSD).

Metal	Feed	Soil
Arsenic	0.60 (±11)	11.9 (±3)
Cadmium	0.73 (±12)	0.51(±5)
Chromium	10.2 (±15)	74.7(±5)
Cobalt	0.53 (±8)	9.99(±4)
Copper	62.7 (±16)	25.6(±5)
Lead	0.23 (±14)	51.1(±1)
Nickel	2.61 (±10)	20.9 (±3)
Vanadium	15.6 (±7)	111.1(±5)

Data availability statement

Data and code availability into a publicly available repository: No, raw data will be made available on request, refined data have been entirely reported in this article.

CRediT authorship contribution statement

David Bongiorno: Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Cristina Giosuè:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Serena Indelicato:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Data curation. **Giuseppe Avellone:** Writing – review & editing, Data curation. **Giuseppe Maniaci:** Formal analysis, Data curation. **Marianna Del Core:** Formal analysis, Data curation. **Fabio D'Agostino:** Writing – review & editing, Data curation, Conceptualization.

Declaration of competing interest

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33373.

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