Evaluation of the microbiological and chemical aspects of autochthonous wild snails in Sardinia

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Abstract

This study was conducted to acquire knowledge on the epidemiology and ecology of some zoonotic agents in snails. Chemical and microbiological analysis was carried out on 46 samples of snails belonging to the species of *Helix aspersa* and *Helix (Eobania) vermiculata*. The association between heavy metals and wild snails, a native consumer product in the Region of Sardinia, was determined.

The molecular characterisation of *Listeria monocytogenes* virulence genes has shown a genetic profile that deserves more attention for the improvement of surveillance and risk prevention. Specimens of *H. vermiculata* showed higher concentrations of cadmium (M=0.80±0.56 mg/kg) than *H. aspersa* (M=0.61±0.17 mg /kg).

A further objective was to determine whether the samples showed significant differences from the point of view of secretion characterisation, in terms of protein content, and to identify species-specific correlations and possible relationships with the environment. The presence of *Salmonella enterica* sub.sp *houtenae* (6,14: z4, z23) and *Salmonella enterica* subsp *diarizonae* (47: k: e, n, z15) (1 sample), *Listeria monocytogenes* (2 samples) with Molecular characterization of virulence genes together with the measurement of heavy metals in samples of wild snails has shown a health and hygiene profile that would deserve greater attention for the improvement of the surveillance and prevention of microbiological and chemical risk in such products which currently show a tendency towards increase in consumption.

Introduction

Sardinia is the region of Italy that traditionally has the highest consumption of snails with a total quantity of about 9,000 tons, representing about 22% of the total amount consumed annually in Italy, and with an average of 5.38 kg per inhabitant. This is 8 times higher than the national average although it has not reached the levels found in France (1 kg per person per year) (Corda *et al*., 2014). The considerable consumption of snails and, consequently the marked demand, is mainly met by importation from abroad, from the farms present in the region and from spontaneous harvesting, which represents a widespread traditional activity. Snails are attracted to mammalian faeces from which contamination by pathogenic microorganisms can occur if the faeces are polluted (Barker *et al*., 2001). In a study, in which samples of snails from the regional, national and extra-community markets were examined in the search for *Salmonella spp.* (29, 20 and 25 specimens respectively), its presence was only recorded in one sample (3.4%) of non-EU origin. The presence of *L. monocytogenes* (12%) was found in samples from Tunisia and Greece and from some regional companies (Corda *et al*., 2014). *Salmonella zanzibar* was detected in a sample from Tunisia, while *Salmonella arapahoe* in one from a local market in the province of Cagliari.

Pathogenic bacteria and fungi have been detected in species of African origin such as *Achatina fulica, Limicolaria* spp. and *Helix pomatia*. The latter, in particular, presented microbial concentrations of up to 10 CFU/g, with the *presence of pathogenic E. coli, Staphylococcus aureus*, *Salmonella* spp. and mushrooms belonging to the *Aspergillus* spp. Genus *Salmonella* spp. submitted concentrations of 8.6-1,8×10^{5}CFU/g highlighting a level of risk. (Radzki *et al.,* 2018).

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Numerous studies have also used snails as bioindicators of environmental pollution, highlighting how they accumulate various heavy metals (Boshoff *et al.,* 2013; Cicero *et al.,* 2015; Massadeh *et al*., 2016). The levels of contamination from residues of their meat, however, have always been assessed from the point of view of environmental pollution and almost never from the point of view of health and hygiene as a food in itself (Huang *et al*., 2018). Even though these products represent a productive and economic reality with prospects of growth, there is a lack of standardisation and sector specification that defines the health hygiene requirements of a microbiological, chemical, virological and parasitological nature (Jesse *et al*., 2011).

The aim of the work was to evaluate some hygienic and sanitary aspects by means of microbiological, virological and environmental chemical characterisation (contamination by heavy metals) of the

meat of native snails of the *H. aspersa* and *H. vermiculata* species. Another objective was to characterise the mucus deriving from wild snails, both in terms of quantity and quality of protein content and to understand whether the species and the territory can be considered variables. Finally, in order to carry out a comparative study in relation to the matrix, two methods of microbiological research were compared: classical culture and automated methods based on the ELFA immunoenzymatic method (Enzyme Linked Fluorescense Assay). Considering the significant and widespread frequency of spontaneous harvesting activity in Sardinia, the purpose of the work was to evaluate some sanitary aspects through the microbiological, virological and environmental chemistry (heavy metal contamination). Another objective was to characterize the mucus both in terms of quantity and quality of the protein content, and to understand if the species and the territory can be considered variable in relation to the place of origin. For greater accuracy of the results, a study in relation to the matrix compared the results obtained from the application of two microbiological research methods: classical and automated culture with ELFA (Enzyme Linked Fluorescense Assay) immunoenzymatics.

Materials and methods

Sampling

46 wild snail samples were collected from September 2014 to the end of 2015 in 12 areas of the Sardinian countryside (Figure 1).

The selection of sampling sites envisaged criteria attributable to indiscriminate wild collection areas, areas presumably contaminated by heavy metals, areas at high risk of extinction of the species and of greater consumption of wild snails. Thanks to the collaboration of workers from the Sardinian Forestry Service, qualified personnel were able to travel within the forest area to carry out sampling. The site harvested 600g per sample of the two species under study: *Helix aspersa* and *Helix (Eobania) vermiculata*. The samples (shells, adults and some operculates) were placed in a net with the collection coordinates indicated on the sampling sheet and transported to the laboratory. Each sample was assigned an acceptance number and a progressive identifier from 1 to 16 for the *Helix aspersa* species and from ‘’a” to”j” for the *Helix vermiculata* species. The samples were placed in the appropriately disinfected baskets where intestinal content was eliminated for 15 days in order to simulate a product suitable for sale and then for consumption. The secretion was deburred and collected.

Classical microbiological investigations

Microbiological research, microbiological aimed at the isolation of pathogens of major importance in the food sector *L. monocytogenes, Salmonella* spp., *Escherichia coli* O157: H7, Norovirus and hepatitis A virus, was carried out on 46 samples represented *by Helix aspersa* (No. 16) *and Helix (Eobania) vermiculata* (No.30) (Table 1).

The determination of microbiological parameters was carried out at the Laboratory of Food Microbiology 02-LA of Cagliari’s Complex Structure of Experimental Zooprophylactic Institute of Sardinia, which operates in accordance with the UNI EN CEI ISO 17025:2005 standard for “General requirements for the compe-

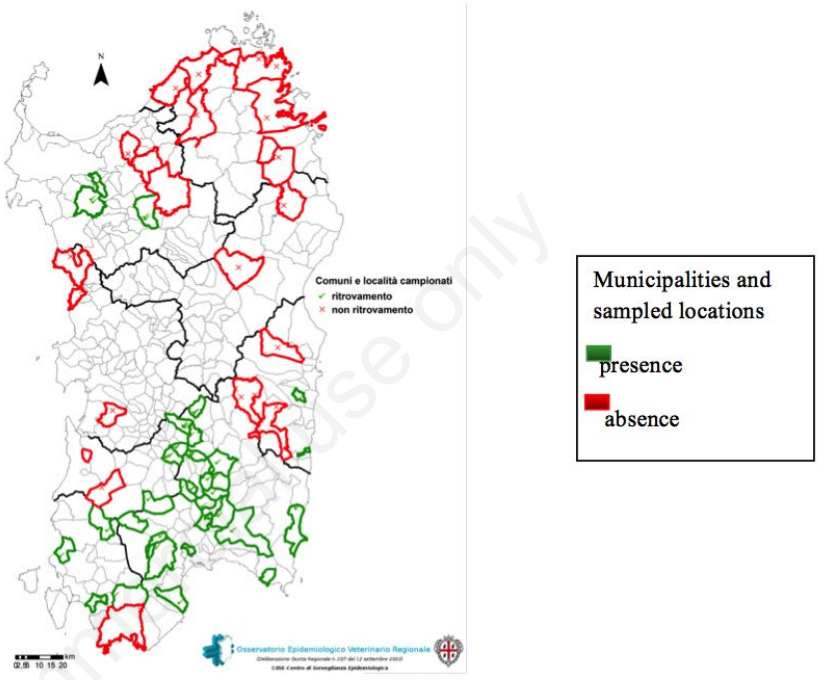

Figure 1. Area under study: sampled and unsased sites.

Table 1. Identification (Id) and location of sampling collection *Helix vermiculata* and *Helix aspersa*.

Id	*Helix vermiculata*	Id	*Helix vermiculata*	Id	*Helix aspersa*
b	Villanovafranca	ee	Pimmentel	1	Villanovafranca
c	Nurallao	gg	Serdiana	2	Nurallao
f	Santadi	mm	Gesturi	3	Santadi
g	Uta	mm	Mores	4	Samatzai
l	Sarroch	oo	Mores	5	Dolianova
m	Gonnesa	pp	Serramanna	6	Sinnai
o	Giba	qq	Ossi	7	Armungia
p	Gesico	ss	Vallermosa	8	Muravera
q	Gesico	uu	Villaspeciosa	9	Gergei
r	Gesico	yy	Ittiri	10	Pimmentel
s	Senorbì	x	Donori	11	Serdiana
t	Armungia	y	Elmas	12	S.Andrea frius
z	San Basilio	a	Villamar	13	Gesturi
aa	Domusnovas	j	Villamar	14	Ossi
bb	Siurgus Donigala			15	Villamar
cc	Gergei			16	Ittiri

tence of test and calibration laboratories". The following reference strains were used for positive controls: *L. monocytogenes* NCTC 7973, *L. harmless* ATCC 33090, *L. ivanovii* NCTC 11846, *Escherichia coli* O157:H7 (VT-) NCTC 12900 and *Salmonella Typhimurium* ATCC 14028. As regards the treatment of the samples, we proceeded in accordance with the UNI EN ISO 6887-2 standard ("Microbiology of the food chain - Preparation of test samples, initial suspension and decimal dilutions for microbiological analysis - Part 2: Specific rules for the preparation of meat and meat products") and detected *L. monocytogenes* (UNI EN ISO 11290-1: 2005 - Microbiology of animal feed and food. Horizontal method for searching and counting *L. monocytogenes*. Part 1: Method for research. Amendment 1: 2004 "Modification of isolation media and the haemolysis test, and inclusion of precision data" with the primary enrichment of the sample, which was performed in Fraser Broth Half Concentration, with a sample / liquid soil ratio of 1:10 (25g sample + 225ml Fraser Broth Half Concentration). Samples were incubated at 30°C for 24±3 hours. A loop of broth culture was inoculated onto a plate of ALOA medium and on a plate of OXFORD. A 0.1 mL subculture was also prepared from the broth culture in a tube containing 10 mL of Fraser Broth. Incubation was carried out at 37°C for 48±3 hours. Subsequently, a loop of broth was inoculated onto an ALOA soil plate and an OXFORD plate. The plates were examined after incubation at 37°C for 24±3 hours and, in the absence of typical colonies, they were re-incubated for another 24±3 hours. The search for *Salmonella* spp. (ISO 6579:2008 – Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella* spp) required a pre-enrichment phase in which 25 g of sample was added to 225 ml of Buffer Pepton Water. An enrichment phase in which 1 ml of the previous solution incubated at 37°C for 18-20h was added to 10 ml of MKttn (Mueller Kauffmann Tetrathionate added with novobiocin and iodine iodide) and 0.1 ml was added to 10 ml by Rappaport Vassiliadis. The MKttn was then incubated at 37°C and the Rappaport Vassiliadis at 41.5°C. After 24h the two broths were sown in the first-choice soil, that is XLD (Xylose Lesine Desoxycholate agar) and in the second-choice soil, called SM 2. The search for *E. coli* O157 (ISO 16654:2001 - Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Escherichia coli* O157) involved an enrichment phase in which 25 g of sample were homogenised in 225 ml of Tryptone Soy Broth with novobiocin. The suspension was incubated at 41.5°C for 18h; the immunocapture and 1 ml of supernatant of the enrichment culture were then transferred to a tube containing 20 µl of immunomagnetic particles and everything was mixed for 10 minutes. The tube was then placed in the magnetic plate to allow the particles to aggregate. After several washes, the resuspended particles in sterile phosphate buffer were seeded in a CT-SMAC plate (Sorbitol Mac Conkey agar supplemented with Cefixime and potassium tellurite) and in an MCS plate (McConkey Sorbitol). Both plates were incubated at 37°C for 18-24h. The search for *Clostridium perfringens* involved sowing by inclusion with 10-15 ml of SC (Sulfite-Cycloserine agar) and a subsequent 10 ml layer of the same medium. After solidification, the plate was incubated at 37°C for 22 hours in anaerobiosis. Characteristic colonies were confirmed using LS Medium. Each selected colony was inoculated in thioglycolate and incubated anaerobically at 37°C for 18-24h. After incubation, 5 drops of the thioglycolate culture were found to be gas producing and the iron sulfite precipitate was considered to be *C. perfringens*.

Virological research of Norovirus (NoV) and hepatitis virus (HAV) was carried out using conventional Reverse Transcription-Polimerase Chain Reaction (RT-PCR) and REAL-TIME PCR according to the analytical method for food.

Immunoenzyamatic method for the detection of *L. monocytogenes* and *Salmonella* spp.

On 46 represented samples of *Helix aspersa* (n.16) and *Helix (Eobania) vermiculata* (n.30) the immunoenzymatic method was applied with the mini-Vidas LMO2 system (ELFA: Enzyme Linked Fluorescent Assay) with the help of the instrument VIDAS (BioMérieux). For *Listeria monocytogenes*, 0.5 mL was withdrawn from the Fraser enrichment broth incubated for 24 hours and transferred to the microorganism test strip.

The mini-Vidas LMO2 system was able to perform immunoenzymatic fluorescence analysis (ELFA: Enzyme Linked Fluorescent Assay) with the help of the VIDAS (bioMérieux) instrument. L. *monocytogenes* was identified from Fraser enrichment broth incubated for 24 hours, by taking 0.5 mL which were then transferred to the strip for the determination of the microorganism. The result was expressed as "positive" or "negative" based on the comparison between the Relative Fluorescent Value v relative fluorescence value measured at 450 nm of the sample and the fluorescence value of a standard. For *Salmonella,* 25g of the sample were homogenised with 225 bpw supplemented with 1 ml of *Salmonella* supplement and incubated at 37°Cfor 18-24h. 10 ml of the broth were subsequently heated for 5 minutes to 100°C. The samples were cooled to room temperature for 10 minutes, 0.5 ml were then transferred to the strip and inserted into the instrument. The result calculated and interpreted automatically by the instrument is expressed as "positive" or "negative". The VIDAS test up *Salmonella* test uses a cocktail of highly specific capture antibodies, directed against *Salmonella* O and H antigens, and allows the identification of motile and immobile strains. A proportion of the enrichment broth comes into contact with the antigens present which bind to *the anti-Salmonella* antibodies fixed on the inner walls of a cone. Unattached components are eliminated by washing.

Subsequently, conjugated antibodies, labelled with alkaline phosphate, are sucked in/released by the cone and are fixed to *Salmonella* antigens, which in turn are bound to antibodies attached to the cone wall.

The intensity of the emitted fluorescence is measured at 450 nm. The value is compared with internal reference values, and each result is interpreted as positive or negative.

Serotyping of *Salmonella* spp.

The colony was isolated from a sample of *Helix aspersa*. For the determination of the H flagellar antigens, the isolated colony in pure culture on an agar blood plate was placed in a tube containing 2° agar mobility and allowed to incubate at 25°C for 24 hours. This was carried out by the cross-analysis of the results relating to flagellar antigens H and somatic O. For the determination of flagellar antigens H, an isolated colony in pure culture on an agar blood plate was placed in a tube containing agar mobility 2° and left to incubate at 25°C for 24 hours. A loop of the lateral portion of the bacterial colony developed with the characteristic "umbrella" shape and was transferred to 2 ml of liquid BHI and incubated at 30°C for 18 hours.

Finally, 2 ml of saline solution with 1% formalin were added to the broth culture and left to rest for about 2.5 hours. 50 µl of the bacterial suspension were added to 7.5 µl of each of the flagellar H anti-ships, indicated by the capital letters A, AB, C and D, in a 96-well microplate with a round bottom. This plate was then incubated at 52°C for 1 hour, after which the first optical reading was carried out to check for the presence of an agglutination lattice indicating a

positive reaction, or a precipitation button indicating negative reaction. The results were noted, and a second reading was carried out after 18 hours of incubation of the microplate at room temperature. For the determination of somatic antigens rr, an abundant bacterial patina taken from pure culture on a blood-agar plate was resuspended in 1 ml of NaCl 0,2%. The bacterial suspension, autoclaved at 121°C and cooled in water, was centrifuged at 3,000 rpm for 20 minutes. The pellet obtained was resuspended in 200 µl of 0.2% NaCl. 10 µl of bacterial suspension were mixed with 15 µl of each O I/II and O V/VI multipurpose antiserum on an object slide. The presence of agglutination was detected after 1-1.5 minutes in one or the other of the two multipurpose serums. The monovalent antiserum corresponding to the positive polyvalent serum was subsequently tested individually with the same bacterial suspension and in the same way.

The strains identified by biochemical confirmation as *Salmonella* spp. were subjected to serotyping which allowed the respective antigenic formula to be attributed to each stem through the determination of the somatic antigen (O), flagellar antigen (H) and capsular antigen (V) in accordance with the antigenic formulas listed in the Kauffann-White scheme.

Determination of *L. monocytogenes* virulence factors

L. monocytogenes was isolated on 2 samples of *Helix aspersa* and the isolated colonies were treated for the extraction of bacterial DNA from pure culture. For the extraction of bacterial DNA from pure culture, the bacterial strains biochemically identified as *L. monocytogenes* were subjected to molecular characterisation by PCR for the identification of the main genes responsible for virulence. Pure bacterial cultures, resulting from overnight incubation at 37°C in Nutrient Agar, were used for the preparation of the bacterial suspension in 1ml of sterile PCR water (Roth). The culture was centrifuged at 15000xg (~13000 rpm) for 5 minutes; the supernatant was removed, and the bacterial pellet resuspended in 1 ml of sterile PCR water. The tubes containing the suspension were placed in a thermoblock at 100°C for 10 minutes and subsequently used for the PCR reactions.

The primers used to search for the virulence genes of *L. monocytogenes* (rrn, hlyA, actA, inlA, inlB, iap, plcA, plcB and prfA) were provided by the Sigma-Aldrich corporation (Table 2); "illustra RTG PCR Beads" (GE Healthcare) was used as the reaction mix. *L. monocytogenes* ATCC 35152 was used as the reference strain (positive control) while sterile water for PCR was the negative control.

The virulence genes were detected through three different multiplex PCRs (Table 3): one for the genes rrn, hlyA and

Table 2. Primers for the research of *Listeria monocytogenes* virulence genes.

Target	Name	Primer sequence 5'-3'	Dimensions (bp)	Bibliography
Rrn	rrn-F rrn-R	CAG CAG CCG CGG TAA TAC CTC CAT AAA GGT GAC CCT	938	Tell M., *et al.* 2007
hlyA	hlyA-F hlyA-R	CCT AAG ACG CCA ATC GAA AAG CGC TTG CAA CTG CTC	702	
Act	actA-F actA-R	GAC GAA AAT CCC GAA GTC AA CTA GCG AAG GTG CTG TTT CC	268 the 385	
inlA	inlA-F inlA-R	CCT AGC AGG TCT AAC CGC AC TCG CTA ATT TGG TTA TGC CC	255	
inlB	inlB-F inlB-R	AAA GCA CGA TTT CAT GGG AG ACA TAG CCT TGT TTG GTC GG	146	
plcA	plcA-F plcA-R	CCG CGG ACA TCT TTT AAT GT CGA GCA AAA CAG CAA CGA TA	192	
plcB	plcB- F plcB-R	ATT TTC GGG TAG TCC GCT TT GGG AAA TTT GAC ACA GCG TT	261	
iap	iap-F iap-R	ACA AGC TGC ACC TGT TGC AG TGA CAG CGT GTG TAG TAG CA	131	Yadav *et al.* 2010
prfA	prfA PrfB lip1 lip2	CTG TTG GAG CTC TTC TTG GTG AAG CAA TCG AGC AAC CTC GGT ACC ATA TAC TAA CTC GAT ACA GAA ACA TCG GTT GGC GTG TAA CTT GAT GCC ATC AGG	274	Wernars *et al.* 1992 Jofrè *et al.* 2005

Table 3. Multiplex PCR: reaction mixture.

Multiplex 1		Multiplex 2		Multiplex 3	
rrn - F	0.5 µl	inlA - F	0.5 µl	plcA –F	0.5 µl
rrn- R	0.5 µl	inlA – R	0.5 µl	plcA - R	0.5 µl
hlyA - F	0.5 µl	inlB – F	0.5 µl	plcB - F	0.5 µl
hlyA - R	0.5 µl	inlB - R	0.5 µl	plcB - R	0.5 µl
actA - F	0.5 µl	iap - F	0.5 µl	H2O PCR	20 µ
actA - R	0.5 µl	iap - R	0.5 µl	DNA template	2 µ
H_2O PCR	20 µ	H_2O PCR	20 µ	Total volume	25 µ
DNA template	2 µ	DNA template	2 µ		
Total volume	25 µ	Total volume	25 µ		

actA (Multiplex 1), one for the genes inlA, inlB and iap (Multiplex 2) and the last for the plcA and plcB genes (Multiplex 3). In all cases, 2µl of extracted DNA and 0.5µl of each primer were added to the reaction mix (GE Healthcare). The thermic profiles used for the PCR reactions are shown in Table 4. The PCR products were visualised in 2% agarose gel, after staining with ethidium bromide.

Determination of heavy metals

For the determination of heavy metals, 38 samples of purged and frozen snails were used, belonging to the species *Helix aspersa* (n. 14), *Helix vermiculata* (n. 24). Each sample in turn was composed of a certain number of samples (5-10 on average). The examination was carried out on the entire edible part of the snail, consisting of the pedal muscle, the head and the internal organs. The analytical determination of the metals was performed using inductively coupled plasma optical emission spectrometry (ICP-OES). To start the digestion of the sample and chemically promote the action of the mineralizer, 6 ml of nitric acid and 2 ml of hydrogen peroxide were added. The examination was carried out on the entire edible part of the snail, consisting of the pedal muscle, head and internal organs. The analytical determination of metals was carried out using inductively coupled plasma optical emission spectrometry (ICP-OES). The preparation of the sample involved the withdrawal of 1.5-2 g, and transfer into the corresponding vessel. To start digestion of the sample and chemically assist the action of the mineralizer, 6 mL of nitric acid and 2 mL of hydrogen peroxide were added.

Each vessel was closed with the corresponding lid and locked, in order to apply a force of 12 kN, to avoid possible bleeding and loss of the sample during the irradiation process.

The containers were then placed in the appropriate cavities of the microwave mineralizer. After starting the software program from the specific display, it was possible to monitor the real parameters of the mineralization and the curve of the reached temperatures.

The process was completed in two stages: the first, lasting 15 minutes, in which the maximum temperature of 200°C was reached, the second, with the same duration as the previous one, in which the temperature plateau was maintained.

At the end of irradiation, the vessels were cooled by forced ventilation, until room temperature was reached. At this point, the containers were removed from the mineralizer and opened with the appropriate key under chemical fume hood.

The sample obtained was filtered to eliminate any trace of impurities and then brought to a volume of 50 mL, through the addition of demineralized water with a high degree of purity (conductivity < 0.1 µS/cm).

The sample solution was analysed by the ICP-OES system, which aspirate and nebulizes the solution vial positioned in the autosampler coupled to the instrument (ICP-OES Spectro Ametek).

Table 5 shows the measuring ranges used during the tests.

The average concentrations of the two metals in the different species of snails were calculated and used for the evaluation of the weekly Intake (EWI - Evaluation Weekly Intake) associated with the consumption of that food and calculated per kilogram of standard body weight of an adult conventionally fixed at 70 kg. Assuming an average consumption of snails of about 150 g per week, a risk assessment was made by comparing the EWI with the safety threshold dose (PTWI - Provisional Tolerable Weekly Intake) established by the Joint FAO / WHO Expert Committee on Food Additives (JEFCA). Snails are not included in the EC Regulation 1881/2006, which defines the maximum levels of some environmental contaminants in food products, and therefore in order to have a regulatory reference with which to compare the results obtained, the maximum limits allowed for bivalve molluscs were considered, given that the characteristics of gastropods are similar to this class of food.

Determination of proteins in secretions

Mucus from 46 represented samples of *Helix aspersa* (n.16) and *Helix* (Eobania) *vermiculata* (n. 30) has been filtered in order to eliminate the coarse impurities and then lyophilized, using a Lio-5P (5Pascal S.r.l., Milan, Italy). Freeze-drying or cryo-drying is based on the sublimation of the aqueous component of the sample. In fact, it is first frozen in liquid nitrogen and then attached to the instrument at -45 °, overnight. Once the liquid part has been sublimated, which in the mucus corresponds to 95-97% of the total, the dry substance is recovered, and FT-IR analysis and the Bradford assay are carried out.

Using an FT-IR spectrum 100 spectrometer (Perkin Elmer Inc., Waltham, Massachusetts, USA) a first analysis was carried out on the quality of the various samples, through the study of the characteristic transmittance peaks. Once a minimum quantity of lyophilizate had been placed on the diamond in Zinc Selenide (ZnSe) and the program started, set at a wavelength of 4000-650 nm and 4 scans, it was possible to acquire the spectrum with the peaks due to the oscillatory motion of the bonds of molecules dissolved in the sample excited by a light source.

After selecting the spectra of the most promising samples, that is the most defined ones and with the highest transmittance peaks, we proceeded with the Bradford assay. This spectroscopic analytical technique is performed using Bradford's solution, that is Coomassie Brilliant Blue G-250 reagent.

Therefore, several plastic cuvettes for spectrophotometry were prepared, one "blank", three with increasing concentrations of the standard (albumin), to provide the calibration line and the others with the samples. 800µl of MilliQ water and 200µl of reagent were added to all the cuvettes, to reach a volume of 1 ml; the "blank" was then left as it was, 3.6 and 9µl of BSA (albumin) were added to those providing the calibration line and 1µl of lyophilized and resuspended sample was added to the other. At this point the cuvettes were inserted into a spectropho-

Table 4. Thermal profiles Multiplex 1, 2, 3.

Step	Multiplex 1			Multiplex 2,3		
	Temperature	Minutes	Cycles	Temperature	Minutes	Cycles
Initial denaturing	95°C	5:00	1	95°C	5:00	1
Denaturation	94C°	1:20	24	94°C	1:00	35
Annealing	55C°	1:30		55°C	2:00	
Extension	72C°	2:00		72°C	1:00	
Final extension	72C°	10:00		72°C	5:00	

tometer with a wavelength set at 595nm, and the absorbance was measured.

Statistical tests

The baseline distribution of variables considered was evaluated by mean (standard deviation) and median (I-III quartile). Any possible differences between the two species and by type of element, were highlighted by means of T-student test for variables with normal distribution, or with the Wilcoxon rank test for variables with non-Gaussian distribution. p-value <0.05 was identified as statistically significant for all analyses except for the multiple comparisons for which the Bonferroni correction was applied (p-value <0.01). Statistical analyses were conducted with the use of the STATA / SE software for Windows version 13 (StataCorp, College Station, TX, USA).

Results

Microbiological and crop analyses

Out of 46 samples of purged snails belonging to the *species Helix aspersa* (No.16) *and H. (Eobania) vermiculata* (No.30), weighing about 600 g total for each sample, No. 2 samples showed the presence of *L. monocytogenes. L. ivanovii* was found in samples with identification no. 3 and ''f''.

In the sample with identification no. 7 *Salmonella* spp. was isolated and, following serotyping, 2 sub species of *Salmonella enterica* sub.sp *houtenae* (6,14: z4, z23) and *Salmonella enterica* subsp were identified *diarizonae* (47: k: e, n, z15). All the samples were negative for *Clostridium perfringens*, but in the samples identified with nos. 14 and 16 the presence of *Clostridium butyrricum* was found; both *Clostridium bifermentans* (id. no. vv, j, f) and *Clostridium baratii* (id. no. 16) were present. No *E. coli* O157, Norovirus, HAV was found in any sample.

Serotyping

Table 6 reports the results of serotyping, genetic line, ST genes and virulence following the characterization of the 2 strains of *L. monocytogenes*.

Immunoenzyme investigations

The 2 samples shown to be positive for *L. monocytogenes* by culture analysis were both confirmed by immunoenzymatic analysis. The sample resulting as positive for *Salmonella* spp. in the culture analysis was confirmed with immunoenzymatic tests.

Characterization of virulence genes

The two strains *of L. monocytogenes* isolated in wild snails belonging to the *species Helix aspersa* have been shown to belong to two main genetic lines: line I and II. Genetic line I is characterised by the absence of the coding gene for the prfA protein regulating the expression of virulence genes*(prf*A) and includes the strain with serotype 4b/4e and with an allelic profile represented by ST 54. Genetic line II includes strains expressing *prf*A and hlyA virulence genes *coding* for listeriolysin synthesis (O) identified with serotype 1/2a and with an allelic profile represented by ST 155 (Table 6).

Molecular characterisation of the virulence profile of isolated strains showed the presence of *rrn, inlB, plcA* genes, while *hly*A, *actA inl*B, *plcB* and *prfA* genes were present exclusively in the first strain, and *iap* and *inlA* genes were absent in both (Coroneo *et al*. 2016, Marras *et al*. 2019).

Chemical analytical investigations

On average, specimens of *Helix vermiculata* showed higher concentrations for cadmium (M=0.80 mg/kg) than *H. aspersa* (M= 0.61 mg/kg) (Table 7). In 29% of the *H. vermiculata* samples tested, the cadmium content was higher than 1 mg / kg, the reference value established for bivalve molluscs, used in this work for the assessment of compliance with the legal limits.

Lead is more present in the *species H. aspersa* (M=0.23) than in *H. vermiculata* (M=0.14) but this difference was not statistically significant.

Mercury was found in all samples below the quantification limit of the test method. A quantity of 0.316 mg/kg of nickel was detected in a single sample. The concentration of iron is comparable in both species under study. Chromium in the *H. aspersa species* showed an average concentration of 0.04mg/kg while in *H. vermiculata* the value was slightly lower (M= 0.03mg/kg).

Copper was found in similar concentrations in both species: in *H. aspersa* (M=18.093mg/kg) and *H. vermiculata* (M= 20mg/kg).

Zinc detected in *H. aspersa* (20.12 mg/kg) was found to be present in a higher dose than in the other species (15.4 mg/kg). Table 8 show the average concentrations of lead and cadmium per species, the average weekly consumption of snails, EWI, PTWI, and the percentage of intake per week, resulting from snail consumption, calculated against the safety threshold dose (% PTWI).

Table 5. Measuring range for the determination of heavy metals (*).

Element	Measuring range, mg/kg
Lead	0.00-5
Cadmium	0.001-1
Mercury	0.005-0.5
Iron	0.005-10
Total chromium	0.001-2
Nickel	0.050-10
Calcium	0.2-400
Copper	0.010-20
Zinc	0.010-20

*The lower limit of the measuring field corresponds to the limit of quantification of the test method (LQ).

Table 6. Characterization of virulence genes in wild snail *listeria monocytogenes* strains.

Id	Sample	Serotype	Genetic	ST	Genes of virulence								
					rrn	*hlyA*	*actA*	*inlA*	*inlB*	*iap*	*plcA*	*plcB*	*prfA*
6	Sinnai	1/2a	II	ST155	+	+	+	-	+	-	+	+	+
8	Muravera	4b/4e	I	ST54	++	-	-	-	+	-	+	-	-

Protein determination in the secret

As can be seen in Table 9, the data relating to the protein concentration expressed in µg / ml are reported together with the relative judgment on the quality obtained by IR analysis. As can be seen, in FT-IR spectrophotometry, the sample with id. no. 2 shows the bands between 1650 and 1400, the exact values being 1637.39 and 1536.12 (Figure 2).

The quality of the secretion, in the samples analysed in this study, is species-specific, in fact there are differences in the secretion of the *Helix aspersa* spp. compared to that of *H. vermicultata* (Figure 3).

Discussion

This study made it possible to acquire knowledge on the microbiological and chemical risk profile of wild snails used for food in Sardinia.

The presence of a strain of *L. monocyto-*

Table 7. Average, SD, median, quartile (I and III) and p-value for species of snails under study.

Element	*Helix aspersa* (mean (sd) median [I-III quartile]	*Helix vermiculata* (mean (sd) median [I-III quartile]	Overall	P –value*
Cadmium	0.61 (0.17) 0.66 [0.47 – 0.71]	0.80 (0.56) 0.59 [0.38 – 1.2]	0.73 (0.45) 0.65 [0.40 – 0.85]	0.228
Lead	0.23 (0.24) 0.11 [0.082 – 0.39]	0.14 (0.15) 0.98 [0.056 – 0.13]	0.18 (0.20) 0.099 [0.067 – 0.2]	0.078
Mercury	All < 0.005	All < 0.005	All < 0.005	-
Iron	23.9 (11.5) 21.7 [18.1 – 28.7]	24.2 (8.2) 24.1 [17.3 – 28.2]	24.1 (9.4) 22.9 [18.1 – 28.2]	0.605
Chrome	0.04 (0.023) 0.038 [0.025 – 0.05]	0.029 (0.016) 0.025 [0.014 – 0.04]	0.033 (0.2) 0.032 [0.02 – 0.04]	0.099
Nickel	All < 0.005	All < 0.005	All < 0.005	-
Calcium	3016 (1085) 3036 [2172 – 4193]	5131 (1878) 5335 [3900 – 6301]	4330 (1914) 4200 [2903 – 5389]	0.0005
Copper	18.1 (6.3) 18 (13.1 – 21.3)	20.1 (35.2) 13.3 [8.9 – 17.3]	19.3 (27.9) 14.1 [10.4 – 19]	0.026
Zinc	20.1 (8.1) 17.1 [13.8 – 26.9]	15.4 (7.6) 13.4 [10.8 – 17.2]	17.2 (7.9) 14.6 [11.6 – 18.8]	0.0387

Reported values in mg/kg. *Values performed with T-student tests and Wilcoxon-rank tests

Table 8. Average concentrations of lead and cadmium found in *Helix vermicultata* and *Helix aspersa*, average weekly consumption of snails, EWI*, PTWI, percentage of weekly intake with respect to the safety threshold dose (%PTWI).

	Helix aspersa		*Helix vermiculata*	
	Lead	Cadmium	Lead	Cadmium
Average concentration (µg/Kg)	238	613	140	764
Average weekly consumption (kg)	0,150	0,150	0,150	0,150
EWI (µg/kg bw/week)*	0,51	1,3	0,3	1,64
PTWI (µg/kg bw/week)	25	2,5	25	2,5
%PTWI	2,04	52	1,2	65

*The estimated weekly intake level EWI (Evaluation Weekly Intake) was calculated by multiplying the average concentration value by the amount by weight of the weekly portion divided by the standard weight of an adult conventionally fixed at 70kg.

Table 9. Protein concentration and quality assessment of the secretion.

Id.	Liquid weight (g)	Tare (g)	Weight (g)	Realized (%)	Locality	*Helix*	Locality (µg/ml)	Quality*
2	22,7	110,88	0,99	2,94	Nurallao	aspersa	313	O
9			2,00		Gergei/Gesturi	aspersa	231	O
7			2,13		Armungia	aspersa	141	B
5	16,52	107,17	1,78	10,75	Dolianova	aspersa	117	B
s	37,11	106,74	1,61	4,33	Senorbì	vermiculata	99	B
q	41,22	106,59	1,94	4,71	Gesico	vermiculata	61	M
nn	45,50	114,56	2,07	3,5	Mores	vermiculata	0,11	S
xy	41,92	102,93	2,11	5,02	Ittiri	vermiculata	0	S

Protein concentration expressed in µg/ml. *Quality rating: O (excellent), B (good), M (average), S (poor).

genes belonging to serotype 1/2a and the expression of virulence genes such as hlyA, actA, inlB, plcA, plcB, and prfA demonstrate the circulation of a virulent stem, carried by wild snails in the territory of the Sardinia Region. All this was also confirmed in a previous study on farm snails in which the profile referred to virulence was superimposable (Corda *et al*. 2014).

Salmonella subsp *enterica* was identified in a sample after serotyping houtenae and *Salmonella enterica* subsp. *diarizonae* and there have been some cases of the association of this serotype with exposure to reptiles or in HIV positive patients in contact with birds and dogs.

From the results obtained in this work and from the comparison with other studies carried out on farmed snails, it can be said that these types of samples are a favourable substrate for the development of the genus *Listeria*. In all these works, the circulating serotypes belong to the genetic line I (4b/4e) and II (1/2a) and there is a constant presence of some of the virulence genes.

The traditional consumption of both wild and farmed snails presupposes a long-lasting heat treatment, but there is a possibility of on-site cross-contamination with foods that do not require heat treatments prior to consumption or contamination of humans both in handling and during harvesting.

It would be desirable to pay particular attention to the circulation in the regional territory of particular bacterial strains of *L. monocytogenes* in a limited population such as the one studied here. It can be hypothesised that this food matrix could represent a form of potential reservoir for the cross-transmission of *L. monocytogenes* to the consumer, even if the product undergoes heat treatment before consumption (Parisi *et al*. 2007). For food businesses in which processing phases are carried out, it is appropriate that the risk attribution is carried out specifically through the adoption of specific assessment tools in order to ensure that the food is handled, packaged, and transported in adequate hygiene conditions. This situation highlights the importance of improving the surveillance of listeriosis through molecular typing (work currently being developed by the ECDC and EFSA), and the importance of strengthening control measures for *Salmonella* spp. (EFSA 2013). As to the chemical risk assessment, the samples of wild snails presenting concentrations of Pb and Cd in their meat were exposed to heavy metal contamination differently from what is reported by the estimates on per capita consumption / year equal to 180g which would not pose a risk to human health.

It is known that human activity increases the concentration of these metals compared to those normally present in the environment and this raises concern for the food safety of these products. Lead and cadmium are in fact associated with industrial activities, urban waste, car exhaust gases. Greater concern is addressed to collection in the vicinity of roads, where levels above the limits permitted by current legislation for similar food products could be reached (EC Regulation 1881/2006), underlining that the maximum limits permitted by European legislation are not defined for snails (Scheifler *et al*. 2020)

Snails live in close contact with the environment (soil, water) and given their feeding (plants) and evolutionary strategies, they can accumulate substances of anthropogenic origin and heavy metals.

The results obtained show that in the sites closest to the sources of possible pollutants, a greater number of samples belonging to the *H. vermiculata* species were collected which, of the two species, appears to be the one with the highest concentration of heavy metals ($P<0.05$). The evaluation of the intake of the two toxic metals resulting from the consumption of the snail food was evaluated with reference to the PTWI (Provisional Tolerable Weekly Intake) values established by the Joint Committee of FAO/WHO Experts on Food Additives.

The data obtained show a high diversity depending on the metal considered. For lead we observe an EWI value corresponding to 2.04% (*H. aspersa*) and 1.2% (*H. vermiculata)* with respect to the weekly exposure threshold dose, while for cadmium the percentage of exposure is higher ($p<0.05$). In this case, the consumption of a single portion of snails of the species *H. aspersa* and *H. vermiculata* would respectively lead to a level of cadmium intake equal to 52% and 65% of the safety threshold value. If the

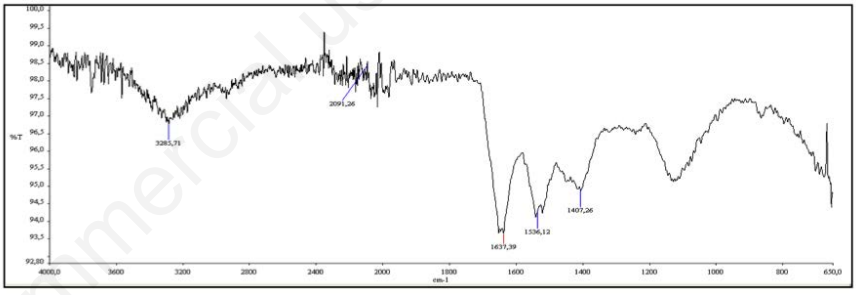

Figure 2. IR spectrum of a secretion defined as "Excellent Quality".

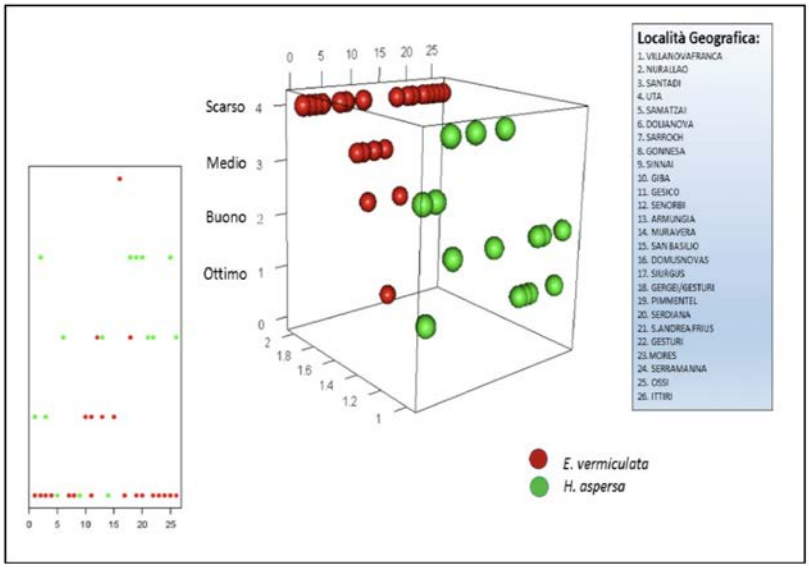

Figure 3. Graphic representation of the quality of the secret in relation to the species.

average cadmium concentration in the *vermicultated Helix* samples is evaluated with a TWI equal to 2.5 µg/kg, approximately 0.230 kg of snails are required to exceed the threshold dose. If the consumption of snails continues to increase compared to current consumption, a condition of considerable risk for human health could arise. The risk deriving from consumption is high and not negligible, also in consideration of the fact that not only snails but other food products are the main source of cadmium intake for humans.

The variability of the quality of the secretion can be observed both in terms of quantity and quality of the protein fraction and this could be attributable to the species and area of origin of the snails.

Conclusions

Snails can accumulate substances of anthropogenic origin and heavy metals. The traditional activity of spontaneous harvesting in Sardinia of snail meat and in particular of the species *H. aspersa* and *H. vermiculata* could lead to a condition of risk for human health. This is particularly relevant with a view to consumption that continues to show an increasing trend. The consumers' collection activity does not pay due attention to aspects of environmental contamination of various origins which greatly affects the food safety characteristics of the product. Looking at our results, citizens should be better informed about the potential risks associated with collection areas. Furthermore, institutions for public health should orient the traditional habit of collection towards previously identified protected areas. This should be done on the basis of a risk assessment which our study has intended to contribute. The results highlight the need to formulate and apply reference legislation to protect the consumers' health with a view to future increase of consumption.

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