Microbiological and chemical evaluation of *Helix* spp. snails from local and non-EU markets, utilised as food in Sardinia

Arianna Corda,[1] Laura Mara,[2] Sebastiano Virgilio,[2] Margherita Pisanu,[2] Giannina Chessa,[2] Antonio Parisi,[3] Maria Paola Cogoni[1]

[1]Istituto Zooprofilattico Sperimentale della Sardegna-Cagliari; [2]Istituto Zooprofilattico Sperimentale della Sardegna-Sassari; [3]Istituto Zooprofilattico Sperimentale della Puglia e Basilicata-Putignano, Italy

Abstract

With this study, 28 pools of snails of the genus *Helix*, respectively *Helix aspersa* (n=24) and *Helix vermiculata* (n=4) were analysed. They were taken from snail farming and stores. The snails were from Sardinia, other regions of Italy, and from abroad. All the samples were examined as pool looking for these microbiological target: *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli* O157, *Clostridium perfringens*, Norovirus and Hepatitis A Virus (HAV). In the same pools, the concentration of cadmium and lead by inductively coupled plasma mass spectrometry was also determined. The levels of these heavy metals were quite high, especially for cadmium. Two samples were positive for *Salmonella* spp., while no sample was positive for *Escherichia coli* O157, HAV and Norovirus. Two samples were positive for *Clostridium perfringens* and 8 for *Listeria monocytogenes*. The microrganisms related to *Listeria monocytogenes* were identified using biochemical techniques, then serotyped and gene sequenced by multiple loci sequence typing technique. Furthermore, antimicrobial resistence was tested on the same samples.

Introduction

In Sardinia, the snail farming has its own important meaning related to the massive consume of the mollusk in the island and to the current limited presence of farms. Indeed, Sardinia has a high per-capita consumption, almost 8 times the average national one. In 2011 commercial data stated a consumption of 43,500 quintals. The 90% of this product (especially *Helix aspersa* and *Helix aperta*) is imported from North-Africa. Even though snails represent an economic and productive resource with interesting commercial and nutritive outlook, the need for specific microbiological and chemical requirements for these products is highlighted. From the data of European Food Safety Authority (EFSA) and of the European Centre for Disease Prevention and Control (ECDC) implementing the Directive 2003/99/CE about the monitoring of zoonoses and zoonotic agents, it is evident how infections caused by zoonotic bacteria mostly associated to human pathologies are caused by microorganisms conveyed by food, especially from microorganisms such as *Salmonella* spp., *Campylobacter*, *Listeria monocytogenes*, *E.coli* verocitotoxigenes (European Food Safety Authority, 2013). In snail farming it is good practice to take measures, both during breeding and during the phase of purging. In farming, the continuous control of the structure is needed to avoid problems related to the introduction of other animals. The rats are an obvious problem to be monitored especially in wintertime as these animals become intrusive and dangerous. Within the enclosure it is necessary to maintain a high level of hygiene and the removal of excreta, cleaning of the cages and the general disinfection of the structure are fundamental to control cross-contamination.

The present study was designed to obtain microbiological and chemical data with an impact on hygienic and toxicological risk of the snails eaten in Sardinia.

Correspondence: Maria Paola Cogoni, Istituto Zooprofilattico Sperimentale della Sardegna-Sezione di Cagliari, Via dell'Acquedotto Romano, 09132 Elmas (CA), Italy.
Tel. +39.070.21914213 - Fax: +39.070.2135121.
E-mail: paola.cogoni@izs-sardegna.it

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Materials and Methods

The snails were collected in n=7 different farms located in the provinces of Cagliari and Sassari. Furthermore, other n=10 samples of different origin were collected in the provinces of Cagliari and Sassari. Two of them were from the wild, one from continental Italy, two from Greece, one from Algeria and four from Tunisia. Information regarding the farms subject of sampling are indicated in Table 1. In none of snail farming the co-presence of other animal species was detected. For farms located in the drainage basin of the *riu Montevecchio* (Gonnosfanadiga, Guspini and Arbus), it is highlighted the risk of heavy metal pollution of surface water bodies and ground water subterranean due to the activities of mine. No other sources of pollution that could be linked to the other farms were aware.

In total, 28 pool of samples of non-operculate snails were examined, 24 belonged to *Helix aspersa* and 4 to *Helix vermiculata*. For the microbiological, virological and chemical analyses the following methods were applied: *Listeria monocytogenes* UNI EN ISO 11290-1:2005; *Salmonella* spp ISO 6579:2002; *Escherichia coli* O157 ISO 16654:2001; *Clostridium perfringens* ISO 7937:2004. The determination of Norovirus GGI and GGII was performed according to Protocol of the National Reference Laboratory for Viral contamination in bivalve molluscs at the Istituto Superiore di Sanità, Rome - ISS, Determination of Norovirus and HAV in bivalve molluscs by RT - conventional PCR and real-time PCR.

For the detection of *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* O157 and *Clostridium perfringens*, 25 g of sample (pooled specimens of snails) consisting of hepato-pancreas and pedal muscle were analysed. For the detection of Norovirus and HAV, the hepato-pancreas was pulled out from 20 specimens, in order to have a pool of hepato-pancreas equal to 4 g. For the determination of cadmium and lead, 1 g of pooled sample was analysed.

The analysis of Norovirus and HAV consisted of three phases: i) preparation of the sample and the concentration of viral particles; ii) extraction of the viral genome; iii) real-time polymerase chain reaction (RRT-PCR): a mix for the determination of each target (NoV GI, GII NoV, HAV) was prepared. The serotyping of the isolated *Listeria monocytogenes* has been done using commercial antisera distributed by Denka Seiken Co. (Tokyo, Japan) for the determination of the flagellar's agents H and somatic O (Parisi *et al*., 2010).

The antimicrobial susceptibility of the *Listeria monocytogenes* strains was determined in a semiquantitative way according to the disc-plate technique by Kirby-Bauer. The followings are the antibiotics used: ampicillin (10 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), erythromicyn (15 μg), gentamicin (10 μg), penicillin (10 μg), rifampicin (30 μg), streptomycin (10 μg), sulfamethoxazol/trimethoprim (1.25/23.75

μg), tetracycline (30 μg) e vancomycin (30 μg). The genes *prfA*, coding for the protein prfA which regulates the expression of the virulence genes and hlyA coding for the synthesis of Listeriolisina O (Aznar and Alarcon, 2002) were tested by multiplex-PCR technique. The multilocus sequence typing (MLST) scheme, used for the characterisation of the strains of *Listeria monocytogenes*, was based on the sequence of the following housekeeping genes: ABC transporter (acbZ), beta-glucosidase (bglA), catalase (cat), succinyl diaminopimelate desuccinylase (dapE), D-amino acido aminotransferase (dat), lattato deidrogenase (ldh), and istidina chinase (lhkA). The primers described by Salcedo *et al*. (2003) were used with the exception of the *ldh* gene for which the primers suggested by an amended MLST scheme (Ragon *et al*., 2008) were used. The allocation of the sequence type (ST) has been done by the database Listeria MLST of the Pasteur Institute of Paris. The analytic determination of heavy metals was performed according to the EPA 3052 method for the sample's treatment and to the EPA 6020A for the instrumental measurement.

Results and Discussion

The samples from farms n. 3 and 6, resulted positive for *Listeria monocytogenes* (Table 2).

Table 1. Typology of farms sampled in Sardinia.

Farm	Species	Location	Altitude (m asl)	Surface (m^2)	Feed resource for snail	Farming operation	Breeding management
1	*H. aspersa*	Gonnosfanadiga (South/South-west)	180	200	Beet, sunflower, thistle	Collection, purging, drying	1 breed from continental Italy and 1 breed from Sardinia
2	*H. aspersa* and *H. pomatia*	Guspini (South/South-west)	137	1300	Rape, beet, radish	Collection, purging, drying	Breeds from Italian and Sardinian company
3	*H. aspersa* and *H. aperta*	Serrenti (Center/South)	114	5000	Rape, beet, radish, grasses	Collection, purging drying, packaging	Breeds from Italian company
4	*H. aspersa*	Gonnosfanadiga (South/South-west)	180	400	Cabbage, kale, chard, chicory	Collection, purging, drying, packaging	-
5	*H. aspersa*	Osilo (Northwest)	672	2500	Beet, flours of corn and barley	-	Breeds from Sicily
6	*H. aspersa*	Sassari (Apparreddu)	225	5000	Beets, corn, bran, barley, vitamins, carbonates and sulphates of calcium	-	Breeds from Sicily
7	*H. aspersa*	Sassari (S. Maria La Palma)	225	1500	Beet, feed and corn flours	-	Breeds from Sicily

Table 2. Results of sampling of snails made in snail farmings.

N. snail farming	Species	Salmonella° *spp.*	Listeria° *spp.*	*Cl. perfringens*° (ufc/gr)	Norovirus#	HAV#	Cadmium§ (mg/kg)	Lead§ (mg/kg)
1	*Helix aspersa*	nd	nd	<10	nd	nd	1.61	0.30
		nd	nd	<10	nd	nd	nr	nr
2	*Helix aspersa*	nd	nd	<10	nd	nd	1.73	0.12
		nd	nd	<10	nd	nd	2.54	0.29
3	*Helix aspersa*	nd	nd	80	nd	nd	0.55	0.25
		nd	d	30	nd	nd	0.44	0.24
		nd	d	<10	nd	nd	0.69	0.37
		nd	d	<10	nd	nd	0.80	0.55
4	*Helix aspersa*	nd	nd	<10	nd	nd	3.75	0.70
		nd	nd	<10	nd	nd	3.92	0.25
		nd	nd	<10	nd	nd	4.01	0.52
		nd	nd	<10	nd	nd	3.40	1.12
		nd	nd	<10	nd	nd	3.83	0.71
5	*Helix aspersa*	nd	nd	<10	nd	nd	0.08	0.05
6	*Helix aspersa*	nd	d	<10	nd	nd	1.06	0.06
		nd	d	<10	nd	nd	nr	nr
		nd	d	<10	nd	nd	nr	nr
7	*Helix aspersa*	nd	nd	<10	nd	nd	nr	nr

HAV, hepatitis a virus; nd, not detected; d, detected; nr, not received. °25 g of sample (pooled specimens of snails) consisting of hepatopancreas more muscle pedal were analysed; #20 specimens from which hepatopancreas was derived were used to have a pool of hepatopancreas equal to 4 g; §these metals were used in a pool of snails so that 1 g of sample was analysed.

The same results were also found in the samples of snails from Greece and Tunisia (Table 3). In two samples from farm n. 3, apart from the presence of *Listeria monocytogenes*, also *Clostridium perfringens* was found in amount of 80 and 30 ufc/g respectively. In two samples, one from Tunisia e one from the wild collection in Sardinia, a strain of *Salmonella Zanzibar* and one of *Salmonella Arapahoe* were found. Similar results have been reported by other authors (Marongiu *et al*., 1993; Tedde *et al*., 2009). No one of the tested sample was found with positive analytical result for *Escherichia coli* O157, Norovirus and HAV.

Most of the strains of *Listeria monocytogenes* were responsive to almost all of the antimicrobial tested. All of the strains (n=8) were sensible to the tetracycline, gentamicin, erythromycin, vancomycin and to the combination sulfamethoxazol-trimethoprim. Various reactions were highlighted for the ciprofloxacin. Furthermore, six strains out of eight showed an intermediate resistance. Only one strain manifested an intermediate resistance to streptomycin.

In each of the isolated bacterial strains of *Listeria monocytogenes* the *hlyA* gene was found, while the *prfA* gene was found in five of them. All of the isolated strains of *Listeria monocytogenes* that were analysed belong to two serotypes: 1/2a and 4b/4e. The serotype 1/2a belongs to the II genetic line while the serotype 4b/4e belongs to the I genetic line.

The MLST allowed to find 6 STs: ST217, ST37, ST1, ST2, ST204, ST7. Two strains had ST7 and two ST204. The isolated belonged to two main genetic lines (I, II). The I genetic line did not have the coding gene for the prfA protein and included three strains with serotype 4b/4e, two from the farm n.3 and one from the farm n.6. The II genetic line included strains that involve the virulence genes prfA and hlyA with serotype 1/2a, one from the farm n. 3, two from the farm n.6, one from Tunisia and another from Greece (Table 4).

Regarding heavy metals, the maximum level of contamination for both the examined metals were noticed in farm n.4 located in the municipality of Gonnosfanadiga. This finding has been confirmed by the analysis of several samples of the same breeding in different periods of the year, hence in different growth and feed conditions. For the other sampling zones, lead and cadmium showed medium-high values in farms n.2 and 3 and in one sample collected in the market. Minimum levels were registered in the sample of *Helix aspersa* took in farm n. 5.

Conclusions

This study allowed to acquire information on the microbiological and chemical contamination in locally-sourced and and imported-from-outside snails for alimentary purpose in Sardinia. Related to the contamination of *Salmonella Zanzibar*, *Salmonella Arapahoe* and *Listeria monocytogenes* it must be underlined that, even though they represent a risk, the type of treatment that snails usually are submitted to must be considered because correct cooking reduces the risk. The traditional cooking presupposes a long thermal treatment before consumption and considered that the whole body of the snail is consumed, this opens up the field to possible cross contamination with foods which do not require any ther-

Table 3. Results of sampling of snails from local and non-EU countries made in shops.

Country of origin	Species	*Salmonella*° spp.°	*Listeria*° m.°	*Cl. perfringens*° (ufc/g)	Norovirus#	HAV#	Cadmium§ (mg/kg)	Lead§ (mg/kg)
Algeria	*Helix aspersa*	nd	nd	<10	nd	nd	0.44	0.22
Tunisia	*Helix aspersa*	nd	nd	<10	nd	nd	nr	nr
Tunisia	*Helix aspersa*	nd	nd	<10	nd	nd	nr	nr
Tunisia	*Helix aspersa*	nd	nd	<10	nd	nd	1.88	0.78
Tunisia	*Helix vermiculata*	nd	nd	<10	nd	nd	1.26	0.08
Greece	*Helix vermiculata*	nd	d	<10	nd	nd	nr	nr
Greece	*Helix aspersa*	nd	nd	<10	nd	nd	nr	nr
Italy	*Helix vermiculata*	nd	nd	<10	nd	nd	1.76	0.27
Sardinia	*Helix vermiculata*	d	nd	<10	nd	nd	0.69	0.05
Sardinia	*Helix aspersa*	nd	nd	<10	nd	nd	nr	nr

HAV, hepatitis a virus; nd, not detected; d, detected; nr, not received.
°25 g of sample (pooled specimens of snails) consisting of hepatopancreas more muscle pedal were analysed; #20 specimens from which hepato-pancreas was derived were used to have a pool of hepatopancreas equal to 4 g; §these metals were used in a pool of snails so that 1 g of sample was analysed.

Table 4. Results of serotyping, analysis of virulence factors by multilocus sequence typing research on strains of *Listeria monocytogenes* isolates.

Origin	Species	*Listeria* m.	ST	Serotype	Genetic line	Genes presence *prfA*	*hlyA*
Snail farming n. 3	*Helix aspersa*	d	ST217	4b/4e	I	nd	d
		d	ST37	1/2a	II	d	d
		d	ST1	4b/4e	I	nd	d
Snail farming n. 6	*Helix aspersa*	d	ST2	4b/4e	I	nd	d
		d	ST204	1/2a	II	d	d
		d	ST7	1/2a	II	d	d
Tunisia	*Helix aspersa*	d	ST204	1/2a	II	d	d
Greece	*Helix vermiculata*	d	ST7	1/2a	II	d	d

ST, sequence type; nd, not detected; d, detected.

mal treatment before the consumption or also the possibility to contaminate humans during the manipulating phase and the harvest. *Clostridium perfringens*, due to the thermostable spores which can germinate and proliferate (Sartory *et al*., 1998), differs from the strictly vegetative bacteria. Indeed, some strains of *Clostridium perfringens* are the cause of the third most common foodborne disease in the United States. Our data suggests that snails are a favourable substrate for the growth of the Listeria genus, including the *Listeria monocytogenes* species and some serotypes of Salmonella.

The evidence that most of the strains of *Listeria monocytogenes* were sensible to almost all the tested antimicrobial, suggests that the total resistance to the antibiotics of *Listeria monocytogenes* isolated in commercialised snails in Sardinia is low, and this data is apparently caused by the low employment of antimicrobial in snails farms, in contrast to what frequently happens in human medicine and veterinary. These substances are used for animals therapy, prophylaxis or growth promotion. Also, the analysis performed with MLST that underlined the result of clones often involved in clinic cases, seemed to suggest that snails are a potential reservoir for the cross-contamination of *Listeria monocytogenes*. The data obtained suggest the adoption of appropriate control measures, yet not sufficient.

The Reg. EC n. 853/2004 (European Commission, 2004) states specific rules about the hygiene of the food of animal origin and reserves to snails (and frogs) only a few lines. Nowadays the need for a common rule to discipline the production and commercialisation of the snails and all others products of the snail farming together with the wellness of the animals is always more stringent. The absence or the limited presence of information for this specific sector does not guarantee the consumer and discourages the business for farming and marketing. Veterinary controls are the last part of the productive chain. Clear rules are needed for the labelling, traceability and the origin of the snails that reach the market. In Italy this issue is deeply felt because together with Spain and Greece, it is the country with the largest consuming rates.

For the chemical contamination the results are different zone by zone. Cadmium represents the highest risk, in the 63% of the analysed samples the amount of this metal is over 1 mg/kg, using this value as a reference for comparison to bivalve mollusks (Reg. EC n. 1881/2006; European Commission, 2006). The small number of samples does not allow us to make comparisons with the data in the literature (Ghidini *et al*., 2003; Scaffardi *et al*., 2007; Storelli and Marcotrigiano, 2001), this in relation also to the different species of snails analysed. However, it must be highlighted that the concentration of cadmium of our samples was higher than that reported by other authors.

Levels of heavy metals contamination in farmed products resulted to be particularly high, to confirm the fact that the quality of the environment of the production's zone has a significant impact on snail contamination. This makes it necessary to have a more strict regulation regime in order to authorise the snail farming.

The contamination level of cadmium is high and not negligible. Just a portion of snails is sufficient to cover the 40.3% of the tolerable weekly intake. However, it must be underlined that the consumption of snails is occasional and coincides with local food habits and has no place in the daily diet.

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