THE ABSTRACT OF THE DOCTORAL THESIS "RESEARCHES REGARDING THE BACTERIAL AND MYCOTICAL MICROFLORA OF THE FOOD SNAILS AND ITS SANITARY-VETERINARY SEMNIFICANCE"

The nutritive and dietetical special qualities of the snail meat has led to a growth of consumption , especially in countries like France, Italy, Spain and lately also in our country. This growth of consumption has determined the raising of snails in heliculture farms, farms in which their growth is controlled and oriented in the purpose of obtaining individuals that can be valorified for human consumption. The bigest part of the snails production in Romania is destined to export.

In this purpose, a serie of regulations concerning the snail meat higien and quality must be applied. This is why it is necessary to approach a research theme that would inventorize this product's microflora and its sanitary veterinary implications.

The research of the aspects regarding the snail meat's higien is necessary also because so far, no researches in this domain have been made in our country.

The objectives of this paper have been the following: to make some investigations regarding the snails morphology; to investigate some aspects concerning the structure and the composition of the snail's meat; to research and inventorize the bacterial and micotical microflora of the snail's fresh meat (from the shell, the foot and the meat); the identification of the genres and even of the bacterial species, using cultural, biochemical exams and other tests; researches regarding the identification of the bacterial microflora that is potential pathogen for humans (*L. monocytogenes, Salmonella spp., B. cereus, Yersinia spp., Clostridium spp*., *Pseudomonas spp*., etc); to research and inventorize the micotical and bacterial microflora of the fresh and snail meat products.

The biological material has been represented by food snails from belonging to 2 species: *Helix pomatia* and *Helix aspersa*.

To make the investigations, I used adult snails, collected from four different areas: a garden from Iasi county, a forest near the city and two farms (a raising farm of the specie *Helix*

pomatia from Harghita county and a raising farm of the specie *Helix aspersa* from Vaslui county).

In order to identify the mycotical and bacterial microflora, we have also used three types of snail meat products: fresh frozen meat, refrozen meat and adulterated snail meat sausages.

Due to the complexity of the activities suggested in the research project, the work methods have been histological, physico-chemical and microbiological.

The places where the researches took place are the following: the Faculty of Veterinary Medicine Iasi: into the Laboratory of Morphopathology, the Laboratory of Microbiology, the Laboratory of Food Control and the Laboratory of Micology; a private laboratory, where the physico-chemical investigations and a part of the bacteriological investigations took place; the Institute of Public Health, where the biochemical identifications of the isolated bacterial species have been made.

The researches have begun with an investigation on the new techniques used for the raising of snails. In this purpose, I have visited three heliciculture farms and a factory in Braşov county. It was noticed that the Teliu method used there is characterized by the conditions of growing and developing similar to the naturals ones and by using only echological techniques.

The researches have continued with a study of the interior structure of the snails belonging to the specie *Helix pomatia*, a local specie. These researches have represented a preparation for the study of the histological structure of the snails, which requires an exact acknowledgment of their morphology.

In this purpose I have made dissections and thus I have observed the snail's anatomy.

The dissection started on the ventral face of the foot, from the mouth, with two bucal lobes. Immediately after the opening I could notice the horn jaw, the crop, in the shape of a bulb and the esophagus with the salivary glands. The digestive system continues with the stomach and then with the intestine, which is surrounded by the snail's biggest organ, the digestive gland, wich has an essential role in digestion. The intestine opens to the exterior through the excretory pore.

At the level of the genital system, the following constitutive parts have been observed: the genital cloaca, the vagina, the spermoviduct, the dart sac, the mucus glands, the oviduct, vas deferens, bursa copulatrix, the hermaphroditic duct, the penis and the flagellum.

After the observation of the two major systems, I have detached the lung, which is a part of the mantle, thicker, musculous and very well vascularized. On its postero-upper side there is the heart, surrounded by the pericard and between the heart and the excretory chanel there is the only kidney of the snail, also called Bojanus' organ.

After the sectioning of the mantle, I could notice the collumelar muscle, which reuintes

all the snail's retractory muscles.

In order to identify the structure of the snail meat, I have collected tissue patterns from *Helix pomatia* snails. The patterns have been put through a histological analysis, with the following steps: fixation, embedding in parafine, setioning and coloration. Three methods of coloration have been used: Acid Schiff (PAS) staining, Hematoxyline-Eosin (HE) staining and Alcian Blue staining.

The histological exam of the foot, the mobile organ of the snail, revealed its muscular structure. The muscle is composed of smooth muscle cells intermingled with large spaces represented by haemocoelic capillary sinuses. Using the Alcian blue staining and the microscope with polarized light we could observe the shining collagen fibrils under the surface epithelium. Near the surface epithelium we could distinguish cells full of mucus, that go deep into the supepithelial tissue and are surrounded by smooth muscle cells and by connective tissue.

The ovotestis consists of four lobes, each of them represented by a large number of follicles intermingled with loose connective tissue. Inside the follicles we could notice: male germ cells that occupy the lumen of the follicles, female germ cells observed on the walls of the follicles, follicle cells and Sertoli cells. The hermaphroditic channel has in its structure a pseudostratificated cylindrical epithelium and loose connective tissue. The sperm channel, through which the sperm is transported to the penis, is a functional duct with a narrow lumen outlined by folded epithelial crests surrounded by a few muscle and connective fibres. Muscles with circular and longitudinal fibres delimitates the spermiduct. The penis is a muscular organ with circular, longitudinal, radial and oblique muscle fibres. It is outlined by a pseudostratificated cylindrical ciliated epithelium. The oviduct is a muscular duct with simple prismatic epithelium on a basement membrane. The cells of the epithelium are ciliated and secretory cells. The albumen gland is involved in the formation of the envelopes surrounding the fertilized oocytes and it opens in the oviduct. In the parenchymal glandular mass we could observe albumen secretory cells and labyrinthic cells.

The mantle's histological structure reveals its important functions for the snail: numerous big mucus-secreting and mucus-storing cells and also calcium deposits.

In order to determine the water percent of the meat I have used the classical method of meat dessication. The determination of the proteins was made indirectly, using the Kjeldahl method. To determine the raw fat I have used the Soxhlet method and for the determination of the mineral the meat calcination.

The purpose of the first analyses was to see if there are major differences between the composition of the active snails collected from the garden, of the snails collected from the forest and of the snails collected from a farm. It was noticed that the forest snails have a higher percent

of water than the garden snails. Also, the proteins percent and the minerals percent are higher in the meat of the forest snails. On the other side, the fat percentage is lower in the meat of the forest snails compared to the fat percentage in the meat of the garden snails. The meat with the highest nutritional value was the one of the farm snails. This is explained by the controlled raising of these individuals, in order to obtain a product of good quality, that can satisfy the consumers requests.

The second step was to establish the composition of the meat of the hibernating snails collected from the three areas and to compare the obtained data. I could notice in the case of all the three categories of snails a decrease of the water percentage and also a higher percentage of proteins, fat and minerals in the meat of the hibernating snails, comparatively to the meat of the active snails.

In order to identify the mycotical microflora, inoculations on the Sabouraud medium have been made.

On the inoculated medium with patterns collected from the shells surfaces, an average number of 9 CFU/cm^2 was obtained. Based on the macroscopical and microscopical examinations, three families of microfungi were identified. Best represented was the *Moniliaceae* family, with three genres: *Aspergillus, Chrysosporium* and *Fusarium*. Two genres belonging to the Mucoraceae family were identified: *Rhizopus* and *Mucor* and from the *Dematiaceae* family only one genre was identified: *Alternaria.* The predominant genre was *Aspergillus*, from which three species were identified: *A. fumigatus, A. flavus* and *A. niger*. The most frequent was isolated *Aspergillus fumigatus.*

The average number of CFU isolated from the foot surface was of 4 CFU/cm^2. Three families, respectively six micotical genres were identified: *Aspergillus, Penicillium, Mucor, Alternaria, Cladosporium* and *Fusarium*. Like in the case of the shell, the *Moniliaceae* family predominated, with three genres (Aspergillus, Penicillium and Fusarium), respectively five species: A. fumigatus, A. flavus, A. niger, Penicillium spp. and Fusarium spp.

The *Dematiaceae* family was represented by two genres, *Alternaria* and *Cladosporium*, amd from the *Mucoraceae* family the *Mucor* genre was isolated. Like in the case of the patterns collected from the shell surface, the *Aspergillus* genre was predominant. From this genre, three species were identified: *A. fumigatus. A. flavus and A. niger*. The most frequent isolated was *A. fumigatus*, like in the case of the patterns collected from the shell. From the *Mucor* genre the *Mucor racemosus* specie was identified and from the *Alternaria* genre, the *Alternaria alternata* specie was identified.

The mycotical microflora was also investigated in fresh meat, in fresh frozen meat, in adulterated frozen meat and in snail meat sausages.

In the case of the fresh meat, in most cases only one colony developed on the medium, colony represented by *Penicillium spp*. In 12 cases out of 30, no colony developed, while from the other patterns *Penicillium spp* (predominant) and *Fusarium solani* and *Alternaria alternata* were identified. In the case of the fresh frozen meat no colonies developed on the culture medium.

On the culture media inoculated with dilutions from the frozen meat and sausages, between two and three colonies belonging to the *Alternaria* and *Penicillium* genres developed. It was noticed the fact that the *Penicillium* genre, isolated from the foot surface, was also isolated from the snails meat and from adulterated products.

As regards the microbiological anaysis, the first phase was to isolate and identify the bacteria and fungi from the snails shell; to isolate and identify the bacteria and fungi from the foot surface; to isolate and identify the bacteria and fungi from the meat.

The work technique followed these steps: patterns collection; making the serial dilutions; the inoculation of the culture media; the microbiological staining; the biochemical tests. The results assembly shows small variations of the bacterial profile of the three snails categories: from the garden, from the forest and from the farm.

As regards the total germs number, this achieved the highest values in the case of the patterns collected from the shells surfaces of all the three categories of snails (from the garden, from the forest, from the farm). The average number obtained was between 54.4 and 70.9 $x10^6$ CFU/cm^2. The lowest value was from the patterns collected from the farm snails *H. pomatia* and the highest value was from the patterns collected from the forest snails. The average number of CFU isolated from the foot surface was between 45.1 and 62.9 $x10^6/cm^2$. The lowest number of CFU was isolated from the farm snails *H. aspersa* and the highest number of CFU was isolated from the forest snails. The lowest values of CFU were obtained from the patterns of snails meat, with an average number between 20.3 $x10^2/g$ in the case of the farm snails *H. aspersa* and 26.8 $x10^2/g$ in the case of the forest snails.

Regarding the identification of the isolated bacteria, it was noticed the predominance of a facultative anaerobic, Gram (-) bacterial microflora, belonging to the *Enterobacteriaceae* family.

This microflora varied accordingly to the snails origin, but predominant and present at all the three categories of snails were the genres *Citrobacter, Morganella, Klebsiella* and *Enterobacter. Citrobacter braakii, Citr. freundii* and *Citr. koserii* have been the predominant species in the case of the forest snails and the farm snails *H. pomatia,* while *Morganella morganii* predominated at the garden snails.

Two species belonging to the genre *Enterobacter* have been identified: *Ent. amnigenus* and *Ent. cloacae*, the last one having a higher frequency of isolation. *Klebsiella rhinoscleromatis*

have been mostly isolated from the meat of the farm snails *H. pomatia* and *Klebsiella pneumoniae* from the meat of the farm snails *Helix aspersa*.

The other species belonging to the *Enterobacteriaceae* family had a lower frequency and varied accordingly to the snails area. *Pantoea spp* and *Raoultella ornithinolytica* have been isolated only from the farm snails *H. aspersa*. *Proteus penneri* and *E. coli* have been most frequently isolated from *H. aspersa*, while *Hafnia alvei* and *Providencia rettgeri* have been isolated from the farm snails *H. pomatia* and from the forest snails. *Yersinia enterocolitica* have been isolated only from the meat of the farm snails *H. pomatia*.

The *Pseudomonadaceae* family predominated in the bacterial profile of the garden snails. Three species have been isolated: *Pseudomonas alcaligenes, Pseud. mendocina* and *Pseud. putida*.

As regards the Gram (+) bacterial population, this one has been represented by four families: *Listeriaceae, Aerococcaceae, Staphylococcaceae* and *Enterococcaceae.*

It could be noticed an obvious predominance of the *Listeriaceae* family, isolated from all the three categories of snails, both from the shell surface and from the meat. The highest number has been isolated from the shell surface and between the two present species, *Listeria monocytogenes* and *Listeria innocua, L. monocytogenes* has been predominant. This is of a special importance for the consumers health. It is a pathogen specie for human, which leads to one of the most severe food poisonings.

Only one specie has been identified from the *Aerococcaceae* family: *Aerococcus viridans*, isolated from the shell of the farm snails *H. aspersa*.

The bacteria belonging to the *Staphylococcacea*e have been identified only at the farm snails *H. aspersa*, from which three species have been isolated: *Staphylococcus aureus, Staph. sciuri* and *Staph. warneri*.

It was noticed a predominance of the facultative anaerobic bacteria, that can be often found in soil and water and some of them are even comensals of the human and animal intestine.

As regards the bacterial microflora existent in the snail meat products, the following objectives were: the determination of the mezofile aerobic microflora; the determination of *E. coli*; the isolation and identification of *Salmonella spp*; the isolation and identification of the coagulase-positive staphylococci; the identification of *Bacillus cereus*; the isolation of *Clostridium spp*; the isolation of other pathogen species, like *Listeria* and *Yersinia.*

Between the two types of meat, frozen and refrozen, no semnificative differences regarding the microbiological microflora were found. The same bacterial species were identified, with one exception represented by *Aeromonas hydrophila*, specie isolated only from refrozen meat. *Pseudomonas alcaligenes* and *Pseud. luteola, Pantoea, E. coli, Enterobacter amnigenus*

and *Listeria monocytogenes* were present in all the patterns.

In the frozen meat, the following bacteria were identified: *Enterobacter cloacae, Klebsiella oxytoca, Pantoea spp., Pseudomonas aeruginosa, Aeromonas hydrophila, Staphylococcus sciuri, Staph. capitis and Listeria monocytogenes*. The genres *Escherichia, Salmonella, Bacillus, Clostridium, Yersinia and Staph. aureus* were absent.

The bacterial species isolated from the experimentally adulterated sausages were:

As we can notice from the obtained results, the consumption of snail meat is not free of major risks for the consumers health. The main worrying factor is represented by the presence of *Listeria monocytogenes* both in the fresh meat and in the frozen meat and in the adulterated products. Also, a special importance has the maintaining of *E. coli* and *Pantoea spp*. in the snail meat after freezing. Regarding the adulterated products, it is semnificative the presence of the *Bacillus* and *Clostridium* genres, that produce severe food poisoning in human.