Advances in Microbiology, 2014, 4, 604-608
Published Online August 2014 in SciRes. http://www.scirp.org/journal/aim
http://dx.doi.org/10.4236/aim.2014.410066

Microbiota from *Helix aspersa* Müller in Barcelona Area (Spain)

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Received 11 June 2014; revised 15 July 2014; accepted 2 August 2014

Abstract

In recent decades snail consumption has been increasing throughout Europe. The Heliciculture meets this need and gets snail farms, in order to get consistent production in the shortest possible time. The research provided, has a main objective to establish the microbiota of *Helix aspersa* Müller, both from healthy animals, and individuals with gastrointestinal processes. The samples selected for the study were feces obtained from snails collected in both breeding farms as field snails. In the case, farm animals were selected apparently form healthy individuals and others were with signs of gastrointestinal process study. Aliquots of each sample were plated on a range of culture media and incubated under different conditions of temperature and respiration. From the results we can note that the healthy snails (farmed or free-living) have been isolated and identified mostly, as strains belonging to the genera *Pediococcus*, *Lactobacillus* and *Lactococcus* and five strains of yeasts of the genera *Candida* and *Cryptococcus*. Individuals suffering from gastrointestinal disturbances processes have been isolated in high proportion species of *Klebsiella*, *Pantoea*, *Citrobacter* and *Enterobacter*. The results obtained indicate that the snails with health problems reduce the presence of strains of *Lactobacillus* and *Lactococcus* in favor of increasing and/or establishing colonization by strains of Enterobacteriaceae.

Keywords

***Helix aspersa* Muller, Heliciculture, Lactic Acid Bacteria, Enterobacteriaceae**

1. Introduction

The snail is part of the general situation of agricultural activity, which is defined as "full cycle raising edible terrestrial snails." This is a word composed of "Helici", *Helix* derived from the name given to a genus of its shell

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How to cite this paper: Caullan, L.P., Vila, G.G., Angulo, E.A., Calvo, A., Marcelo, J.A. and Torras, M.A.C. (2014) Microbiota from *Helix aspersa* Müller in Barcelona Area (Spain). *Advances in Microbiology*, **4**, 604-608.
http://dx.doi.org/10.4236/aim.2014.410066

snails which have a *helix*, and "culture" derived from Latin that means growing Cultivare.

The snails have been part of the diet of mankind for thousands of years. Fossil remains of prehistoric shellfish found in caves prove it. Until recently activity was limited to simple search of snails, both for their own consumption or to be sold on the market. From the last century, the gastronomic qualities of the snail began to be so appreciated that it went on to become a highly sought-after food. In recent decades snail consumption has been increasing throughout Europe, particularly in Mediterranean countries, while there has been a gradual disappearance of these delicious shellfish in areas where they live in a free state, which contributes to predation of the species and the introduction of pathogens that harm the productivity of it [1] [2].

Helix aspersa Müller is also called common snail or Bovë, depending on the area of Europe. This variety is used on farms dedicated to raising snails for being the most popular in European countries where it is consumed by the snail, especially in France and Spain. Also this species is usually most familiar to consumers, speaking of a species native to these ecosystems and on the other hand this variety is best suited to rearing in captivity, thanks to its high prolificacy and high resistance. However, the development of this industry is limited by the presentation of bacterial gastrointestinal origin [3]. Wild snails with food and commercial interest, generate a whole agri-food production sector called "helicícola industry", which is linked to the rural implications that are majorly environmental, economic and cultural, including industries and downstream activities, such as preparation, processing, marketing and consumption of the product [4]-[8].

2. Materials and Methods

In order to assess the health status of snails have been sampled snail farms located in the province of Barcelona and parallel free-living snail, collected around the farm in order to compare the microbiota present in the thereof.

2.1. Processing the Samples

Samples from the farm were transferred to the laboratory under conditions of refrigeration, in order to carry out microbiological analysis.

For these analyzes were applied protocols established by the working group of the Laboratory of Applied Microbiology and Environ-mental Health Department and Anatomy, Faculty of Veterinary Medicine, Universidad Autonoma de Barcelona.

The samples were obtained from feces of healthy snails and snails that had a compromised health status, manifested by enteric problems. Additionally samples were obtained free-living snails.

These samples were stored in sterile containers and maintaining the cold chain is transferred to the laboratory for processing. Feces were weighed and resuspended in sterile 1/4 Ringer solution. Then, we proceeded to make a bank of decimal dilutions were plated on various culture media for microbiological assessment and establish broad microbiota in the faeces of the animals sampled.

The culture media used were Tryptic soy agar (TSA), general culture medium permits the growth of microorganisms demanding agar Man Rogosa Sharpe (MRS) to establish the lactic acid bacteria in general, and in particular for species of the genera *Lactobacillus, Leuconostoc* and *Pediococcus*, Mac Conkey agar (MK) in order to differentiate fermenting Gram-negative enteric bacilli and no lactose fermenters, agar Sabouraud (SAB) with antibiotic (Ab) allows the selective growth of filamentous fungi and yeast , Baird Parker agar (BP), which allows the development and differentiation of *Staphylococcus.*

2.2. Isolation and Identification

After incubation the stool samples was carried out snail isolation of the colonies grown on each of the culture media fields. Subsequently raised the strain identification, according to the methodologies established in our laboratory.

Once isolated colonies proceeded to differentiate according to their morphological characteristics such as shape, size, surface, edge lifting, internal structure, opacity, consistency.

By Gram staining microscopic characteristics were determined for strains such as: cocci, bacilli, coco-bacilli, etc., as well as the arrangement of bacterial cells (isolated, in chains, in clusters, tetrads, etc.) and classify in Gram-positive or Gram-negative according to the characteristics of the cell wall. Furthermore spore stains were performed that allowed us to demonstrate forms of resistance. Catalase test to check the presence or absence of

catalase enzyme found in most aerobic and facultative anaerobic bacteria. Hemolytic activity tests revealed possible pathogenicity.

2.3. Identification to Species Level by Biochemical Tests

The identification of these strains was carried out using biochemical tests which are used for miniaturized systems that contain different substrates which facilitate the specific identification of each microorganism.

For identification of *Lactobacillus* and *Bacillus* used API 50 CH®, for identification of yeasts API20 C AUX® and identification of bacteria belonging to the family Enterobacteriaceae and other non-fastidious Gram-negative bacilli was used API 20 E®.

2.4. Conservation of Isolates

In order to proceed to the preservation of the strains, we proceeded to the lyophilization thereof.

3. Results and Discussion

From MRS agar isolation were obtained 17 strains that showed morphologies and characteristics of lactic acid bacteria, of which 11 strains belong to the genera *Pediococcus*, *Lactococcus* and *Lactobacillus* (See **Table 1**).

The genera *Pediococcus* and *Lacobacillus* were isolate in a 27.27% corresponding to the specie *Pediococcus pentosaceus*, *Lactoccocus lactis* and Lactobacillus was isolate in a 45.45% corresponding to a species: *Lactobacillus plantarum*, *Lactobacillus brevis* and *Lactobacillus acidophilus.* Of these eleven strains, nine were isolated from farm and were identified as *Pediococcus pentosaceus*, *Lactoccocus lactis*, *Lactobacillus plantarum*, *Lactobacillus brevis* and *Lactobacillus acidophilus.* Free-living isolated two strains identified as: *Lactoccocus lactis*

Table 1. Strains isolated on MRS. Samples: Feces obtained from free-living snails and farm.

STRAIN	ORIGIN	CATALASE	HEMOLYTIC ACTIVY	IDENTIFICATION (API 50 CHL®)
Ca1	Farm	Negative	γ-Hemolysis	*Lactobacillus brevis*
Ca2	Farm	Negative	γ-Hemolysis	*Pediococcus pentosaceus*
Ca3	Farm	Negative	γ-Hemolysis	*Pediococcus pentosaceus*
Ca4	Farm	Negative	α-Hemolysis	*Lactococcus* sp.
Ca5	Farm	Negative	γ-Hemolysis	*Lactococcus lactis*
Ca6	Farm	Negative	γ-Hemolysis	*Lactococcus* sp.
Ca7	Free living	Negative	γ-Hemolysis	*Lactobacillus plantarum*
Ca8	Farm	Negative	γ-Hemolysis	*Lactobacillus plantarum*
Ca9	Free living	Negative	γ-Hemolysis	*Lactoccocus lactis*
Ca10	Farm	Negative	γ-Hemolysis	*Lactobacillus acidophilus*
Ca11	Farm	Negative	γ-Hemolysis	*Pediococcus pentosaceus*
Ca12	Farm	Negative	γ-Hemolysis	*Lactoccocus lactis*
Ca13	Farm	Negative	α-Hemolysis	*Lactococcus* sp.
Ca14	Farm	Negative	α-Hemolysis	*Lactococcus* sp.
Ca15	Farm	Negative	α-Hemolysis	*Lactococcus* sp.
Ca16	Farm	Negative	γ-Hemolysis	*Lactobacillus plantarum*
Ca17	Farm	Negative	γ-Hemolysis	*Lactobacillus* sp.

(Ca9) and *Lactobacillus plantarum* (Ca7).

Strains of *Lactobacillus plantarum* and *Lactococcus lactis* were isolated from stool specimens from snails, both as free life farms, which may show that these strains are typical species of snails, because it is part of the normal microbiota free-living snails.

In the bibliography, it is described that the genus *Lactococcus* is one of the genres that is part of normal microbiota snails [9], claim that matches our research, since both snails living in captivity and free life strains were isolated from *Lactococcus lactis.*

The other authors do not mention the presence of species of the genus *Lactobacillus*, however in our study we have isolated three species belonging to this genre, matching the species *Lactobacillus plantarum* isolates from feces of snails farmed and free living.

In **Table 2** are provided the strains isolated on Agar Sabouraud chloramphemicol and incubated under aerobic conditions at 28˚C for 24 to 48 hours.

In this medium were isolated five strains presenting yeast morphology, being identified as: *Candida norvegensis*, *Candida guilliermondii* and *Cryptococcus humicola*. In thebibliography, no information is provided in the presence of yeast, as constituents of the normal microbiota snails.

Table 3 shows the results obtained from the Isolation on MacConkey Agar, incubated under aerobic conditions at 37˚C for 24 to 48 hours, snails stool had a deteriorated state of health enteric derivative processes.

Among the microorganisms which are part of the normal microbiota *Helix aspersa* species described in the literature, four groups of Gram-negative anaerobic bacteria, belonging to the families Enterobacteriaceae and *Pseudomonadaceae* [9]-[11]. In our study we isolated bacteria belonging to the family Enterobacteriaceae coinciding with the species described in literature.

Throughout our research, we isolated and identified two species belonging to the genus *Klebsiella*: *Klebsiella oxytoca* and *Klebsiella terrigena*, a strain of the genus *Pantoea Pantoea* sp., Two strains belonging to the genus *Citrobacter*, identified as *Citrobacter koseri/farmeri* and two strains belonging *Enterobacter*: *Enterobacter cloacae* and *Enterobacter amnigenus.*

Helix aspersa is a prolific and phytophagous species that is limited by the manifestation of bacterial gastrointestinal origin [9] and by certain bacteria that can behave as opportunistic pathogens [10]. Such is the case of bacteria isolated from apparently healthy animals as *Salmonella enterica* [12], *Aeromonas* sp. [11] and *Enterobacter* sp. [13].

Table 2. Strains isolated in Agar Sabouraud + chloramphenicol isolated from feces of snails.

STRAINS	IDENTIFICATION (API 20C AUX®)
Cal1	*Candida norvegensis*
Cal3	*Cryptococcus humícola*
Cal6	*Candida norvegensis*
Cal8	*Cryptococcus humícola*
Cal10	*Candida guilliermondii*

Table 3. Strains isolated from feces of snails with health troubles on Agar MacConkey.

STRAINS	IDENTIFICATION (API 20 E®)
Cap1	*Klebsiella oxytoca*
Cap2	*Pantoea* sp.
Cap3	*Enterobacter cloacae*
Cap4	*Citrobacter koseri/farmeri*
Cap5	*Citrobacter koseri/farmeri*
Cap6	*Raoultella terrígena*
Cap7	*Enterobacter amnigenus*

4. Conclusion

The results obtained in our study can contribute to the normal microbiota of the species *Helix aspersa* Müller, which is constituted mainly by species of the genera *Lactobacillus*, *Lactococcus* and *Pediococcus* and yeasts identified as *Candida norvegensis*, *Candida guilliermondii* and *Cryptococcus humicola*. In the case of snails with enteropathogenic symptoms, the presence of Lactobacillus and Lactococcus are reduced and in this case we found high prevalence of species of *Klebsiella*, *Pantoea*, *Citrobacter* and *Enterobacter*.

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