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# Dynamics of bacterial community in the gut of *Cornu aspersum*

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## ABSTRACT

The dynamics of the bacterial community in the intestinal tract of *Cornu aspersum* was investigated during different states of its life cycle. Two approaches were applied – culture and non-culture. The non-culture approach was performed by ARDRA of 16S rDNA using two of the six tested endonucleases. Data were analyzed by hierarchical cluster analysis. The restriction of 16S rDNA samples from the snail of different physiological states with endonucleases *Hinf*I and *Csp*6I resulted in generation of different profiles depending on the snail states. By the culture approach we found that the total number of cultivable bacteria, representatives of Enterobacteriaceae, lactic acid bacteria, amylolitic and cellulolytic bacteria were the most abundant in active state of the snails. Cellulolytic bacteria were not detected in juveniles of *C. aspersum*. *Escherichia coli*, *Clostridium perfringens* as well as bacteria from the genus *Salmonella*, *Shigella* and *Pseudomonas* were not detected. Bacteria of the genus *Aeromonas* were found in juveniles of *C. aspersum*, after that their number decrease and were not found in hibernating snails. On the base of the two applied approaches this study shows that the bacterial flora in the intestinal tract of *C. aspersum* is affected by the seasonal and environmental variations and undergoes quantitative and qualitative changes during the different states of the life cycle. The snails harbor in their gut intestinal bacteria, which possess biochemical potentiality to degrade the plant components.

**Key words:** ARDRA, bacterial community, cultural approach, *Cornu aspersum*, dynamics

## Introduction

*Cornu aspersum* (Gastropoda, Pulmonata) is an ectothermic hermaphrodite species with determinate growth pattern subjected to a wide range of different climatic and trophic conditions and exhibits cyclic behavioural rhythms of activity and dormancy (hibernation and aestivation). Dormancy, in general, is a behavior of land snails that ensures their survival under adverse environmental conditions through a set of modifications in their overall biology (Fields, 1992; Withers et al., 1997; Pakay et al., 2002; Storey, 2002; Reuner et al., 2008). Both dormancy states involve physiological adjustments to resist of cold or heat stress (Nicolai et al., 2010) because the land snails use environmental cues to predict the seasonal climate changes, and prepare the appropriate physiological and behavioural response (Kotsakiozi et al., 2012). Hibernation is triggered by shortening of photoperiod and low winter temperatures. Aestivation occurs during high summer temperatures and low humidity (Attia, 2004; Ansart et al., 2001b).

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*C. aspersum* is known to house a permanent bacterial population, essentially constituted of aerobes and facultative anaerobes, representatives of two taxa: γ-Proteobacteria (*Buttiauxella*, *Citrobacter*, *Enterobacter*, *Kluyvera*, *Obesumbacterium*, *Raoultella*) and the Firmicutes (*Enterococcus, Lactococcus* and *Clostridium)* (Charrier et al., 2006). The strains of Enterobacteriaceae, Aeromonacae and *Enterococcus* are generally assigned as predominating populations into enteric environments (Lesel et al., 1990; Watkins & Simkiss, 1990; Charrier et al., 1998; Charrier et al., 2006; Ansart et al., 2001a) but it lacks of information concerning the dynamics of these populations in different stages of the life cycle of the snail. Some authors suggest that the starvation and hibernation triggered a fall in bacterial counts, but a great diversity of bacteria survived in the snail gut during these periods (Watkins & Simkiss, 1990; Charrier et al., 2006). In particular, growth of the flora is observed in the mucous ribbon of hibernating *C. aspersum*, as it could be a nutritive medium for bacteria (Charrier, 1990).

The aim of this study was to investigate the dynamic of the intestinal microflora of the snail *C. aspersum* during the different life cycle states.

## Materials and Methods

### *Snail intestinal tract sampling*

Thirty samples of *C. aspersum* in state of activity and dormancy were taken from private snail farm in Bulgaria for investigation of culturable component of the gut bacterial community. The samples included juveniles of the snails (HB), snails in active state (H2Y), aestivation (H1Y) and hibernation (H). All investigated samples were kept under starvation conditions for approximately 2 weeks. The snails were treated according to Chung (1985), and then the shells were disinfected with ethanol (70$^{o}$C) and removed aseptically. Snails were washed with sterile physiological solution and whole intestinal tract (oesophagus to rectum) was aseptically handled to avoid contamination from the external surface of the snail body. Because of the small size after disinfection the entire snails of HB state were mechanically homogenized. The homogenates were used for total DNA extraction and to prepare dilutions (in 0.85% w/w NaCl) for bacterial isolation.

### *Culture media and culture conditions*

The following culture media were used: PCA (Difco), NB (Merck), MRS agar (Merck), M17 agar (Merck), Mac Conkey agar (Oxoid), Mac Conkey 2 agar (Oxoid), SS agar (Scharlau), SPS agar (Scharlau), Starch agar (Difco), GSP agar (SIGMA-ALDRICH), milk agar and cellulose liquid medium.

The milk agar for proteolytic bacteria contained the following constituents per litter: 10 g meat extract, 10 g peptone, 5 g NaCl and 20 g agar. The medium was sterilized at 121°C for 15 minutes, cooled to 45-50°C and 10% dry skimmed milk (sterilized separately) was added.

The cellulose medium used for cellulolytic bacteria contained the following constituents per litter: 1 g peptone, 1 g $KH_2PO_4$, 2 g $NaNH_4HPO_4$ x $4H_2O$, 0.3 g $CaCl_2$, 0.5 g $CaCO_3$, 0.5 g $MgSO_4$ x $7H_2O$ and filter paper strips as a source of cellulose. The medium was sterilized at 121°C for 15 minutes. The aliquots of 100 µl of the different dilutions of the homogenates were spread in triplicate onto agar media. The plates were incubated at 28$^{o}$C or 37$^{o}$C for 24-48 h in anaerobic conditions (Anaerocult, BioMerieux) in the anoxic jars. The number of colony forming units per mililiter (CFU) was counted after the incubation period.

The tubes containing cellulose medium were inoculated with 1 ml in triplicate for each dilution. The most probable number of the cellulolytic bacteria per milliliter was calculated.

### *Extraction of DNA*

For DNA extraction two approaches were applied. At the first approach the total DNAs (T-DNA) were isolated from 1 ml of homogenates, immediately of its preparation from snails in different states. The second approach included preliminary cultivation step - 1 ml of the total homogenates was cultivated into Nutrient broth and MRS at 28°C for 24 h after that the DNAs were isolated (C-DNA). Culturable DNA (C-DNA) was extracted from juveniles (HB) of *C. aspersum*, snails of active state (H2Y), and of aestivation state (H1Y). DNA was isolated by a DNA isolation kit [Scientific Technological Service (STS) Ltd. Sofia, Bulgaria] and Gene Matrix Tissue & Bacteria DNA Purification Kit (EURx) according to the manufacture's instruction.

### *ARDRA*

The amplification of 16S RNA gene was performed by the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') using thermocycler TC312 (Techne). The PCR reaction mixture (50 µl) contained (final concentration): 10X *Pfu* buffer with $MgSO_4$, 0.2 mM.

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of each dNTP's, 0.1-1.0 μM of each primers, 1.25-2.5 units *Pfu* DNA polymerase (Thermo Scientific) and 50 pg - 1 μg genomic DNA as a template. The reaction conditions were: a denaturation step at 95°C for 3 min followed by 35 cycles at 95°C for 30 s, 50°C for 3 min, 72°C for 3 min and a final elongation at 72°C for 10 min. The amplified products were restricted by six endonucleases - *Hinf*I, *Taq*I, *Csp*6I, *Tas*I, *Tru*1I and *Hin*6I (Fermentas), according to the instruction of the manufacturers. The products were analyzed electrophoretically (2 h at 100 V) in 2,5% agarose (Agarose, DNA grade, Electran). The gels were stained with ethidium bromide (1 μg/ml) and photographed under UV light. The lengths of the restriction fragment were calculated and compared using a VWR GenoSoft Image analyzer. To differentiate the samples according to their profiles the hierarchical cluster analysis was performed by the Ward's method through SPSS cluster analysis procedure. The matrix of similarity between the samples was calculated using the Binary Squared Euclidean measure.

## Results

### *Study of the bacterial diversity by ARDRA*

To trace the changes in the microbial diversity between the different states of *C. aspersum* the ARDRA was applied. Two types of DNA were extracted – from the whole content of the intestinal tract (T-DNA) and after cultivation of the homogenates into NB and MRS for a growth of saprophytic and lactic acid bacteria, respectively. The two culture media were mixed prior to isolation of DNA (C-DNA). DNA was isolated from juveniles (HB) of *C. aspersum*, and from snails in active (H2Y-C) and aestivation (H1Y) state. The PCR amplification of the two type of DNA resulted in a PCR product of approximately 1600 bp. The restriction was performed by endonucleases *Csp*6I, *Taq*I, *Tas*I, *Tru*1I, *Hinf*I and *Hin*6I. The restrictases *Taq*I, *Tas*I, *Tru*1I and *Hin*6I were not discriminative. Only endonucleases *Csp*6I and *Hinf*I revealed genetic differences within the cultivable and the total microbial communities of the snails gut (Figure 1).

The profiles formed after restriction with *Csp*6I were separated into five groups at 96% similarity (Figure 2) on the base of cluster analyses.

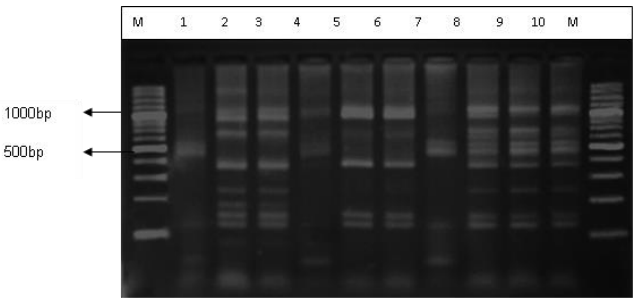


**Figure 1.** *ARDRA profiles obtained with endonuclease Csp6I. 1 - HB-T1; 2 - HB-C1; 3 - HB-C2; 4 - H2Y-T1; 5 - H2Y-C1; 6 - H2Y-C2; 7 - H1Y-T3; 8 - H1Y-C1; 9- H1Y-C2; 10- H1Y-C3; M – 100 bp DNA marker (Fermentas).*

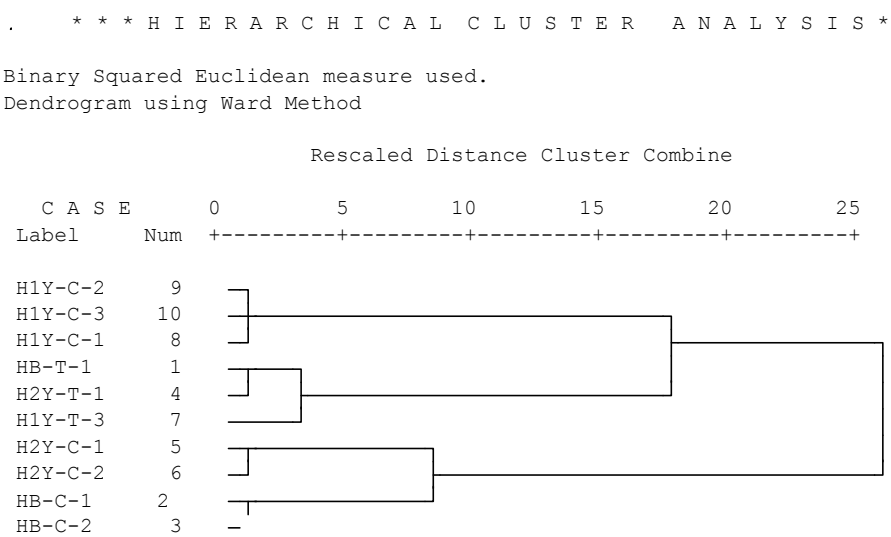


**Figure 2.** *Dendrogram based on the ARDRA profiles with endonuclease Csp6I obtained by hierarchical cluster analysis using Ward's method and the Binary Squared Euclidean measure.*

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The first group included three profiles from C-DNA isolated from snails in aestivation state (H1Y-C1, H1Y-C2 и H1Y-C3). The second group united two profiles from the restriction of T-ДНК samples isolated from juveniles of *C. aspersum* and snails in active state (HB-T1 и H2Y-T3). The profile H1Y-T3 resulted after restriction of the T-DNA from snails in aestivation state was separated independently and formed third group. The similarity between this profile and the two previous profiles was 88%. The fourth group was formed by two profiles identical to each other (H2Y-C1 и H2Y-C-2) received by the restriction of the C-DNA from snails in active state. The fifth group also included two identical profiles (HB-C1 and HB-C2) formed after the restriction of the C-DNA isolated from juveniles of *C. aspersum*.

Restriction analysis with endonuclease *Hinf*I (Figure 3) also formed profiles that show significant differences in the composition of the microbial community in the various states of the life cycle of *C. aspersum*.

The cluster analysis of ARDRA-profile obtained with endonucleases *Hinf*I (Figure 4) separated the restriction profiles into 6 groups at 96% similarity. The first cluster united profiles H1Y-C2 and H1Y-C3, which were formed after the restriction of the C-DNA from snails in aestivation. The profile H1Y-C1, which is also from snails in aestivation, was separated independently and showed 88% similarity to the profiles of the first cluster. We should note, however, that with the enzyme *Csp6*I H1Y-C1 sample formed common cluster with the first two profiles (Figure 4). The third group was comprised of two identical profiles - HB-C1 and HB-C-2 formed after the restriction of C-DNA of actively feeding snails. The similarity between it and the first two cluster was 68%. Profiles H2Y-C1 and H2Y-C2 formed the fourth group and were also obtained by restriction of C-DNA of actively feeding snails. The fifth cluster was formed by the profiles HB-T1 and H1Y-T3, which were identical to each other and were the result of restriction analysis of the T-DNA of juveniles of *C. aspersum* and snails in aestivation. Profile H2Y-T1 obtained by restriction of the T-DNA of actively feeding snails was separated independently and the similarity between it and the fifth cluster was 88%.

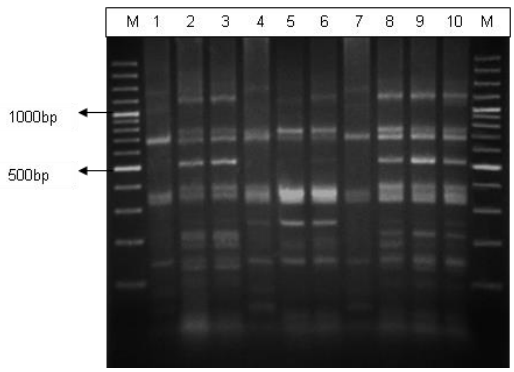


**Figure 3.** *ARDRA profiles obtained with endonuclease HinfI. 1 - HB-T1; 2 - HB-C1; 3 - HB-C2; 4 - H2Y-T1; 5 - H2Y-C1; 6 - H2Y-C2; 7 - H1Y-T3; 8 - H1Y-C1; 9 - H1Y-C2; 10 - H1Y-C3; M - 100 bp DNA marker (Fermentas).*

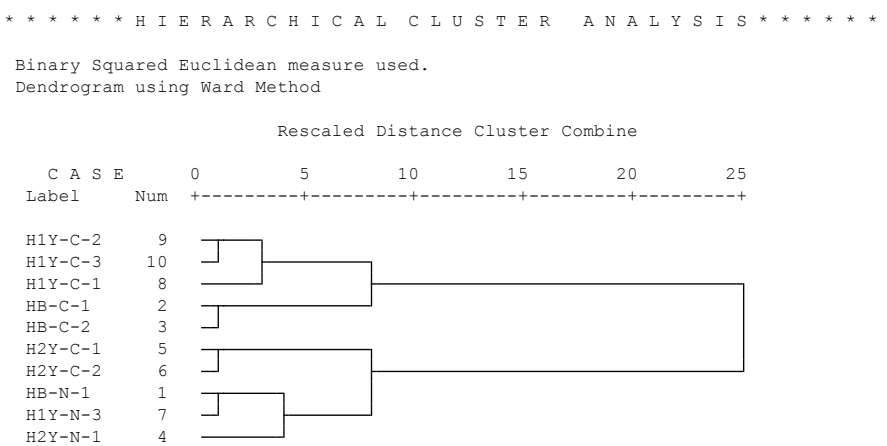


**Figure 4.** *Dendrogam of HinfI restriction obtained by hierarchical cluster analysis using Ward's method and the Binary Squared Euclidean measure.*

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***Dynamics of culturable components of the microbial community***

Dynamics of culturable component of the microbial community in the intestinal tract of *C. aspersum* in various stages of the life cycle was followed by the analysis of four samples designated as HB, H2Y, H1Y and H, which corresponded to the four stages of its life cycle – juvenile forms, active state, aestivation and hibernation, respectively.

By cultivation approach the total number of bacteria, as well as the number of certain physiological groups of bacteria was determined using different selective and non-selective media (Table 1). The total number of the culturable bacteria from the intestinal tract of the snails varied in the different stages of the life cycle. It was the highest during the active period ($1.6\times10^9$ CFU $ml^{-1}$) (Table 1), which coincided with the spring-summer season.

The analysis of the composition of culturable microflora (Table 1) showed that enterobacteria and lactic acid bacteria were presented in each stage of the life cycle of *C. aspersum*. The number increased during the active state and decrease in aestivation and hibernation. The number of the representatives of the family Enterobacteriaceae was determined using two media. On Mac Conkey 2 their number is an order higher (in active state) than that on Mac Conkey. It was due to the lower selectivity of the first medium, which allowed the development and homofermentative lactic acid enterococci.

The samples from different states of the snails were analyzed for the presence of representatives of the genera *Escherichia*, *Salmonella*, *Shigella*, *Aeromonas*, *Pseudomonas* and *Clostridium perfirngens*. On the used Mac Konkey agar medium we did not find colonies characteristic for *Escherichia coli*. It indicates that this bacteria were not been established as a stable component of the digestive tract of *C. aspersum*. The analysis of the colonies developed on the medium GSP showed that the number of the colonies with specific morphology for the genus *Aeromonas* (yellow colonies with yellow halos) was highest (Table 1) for juveniles of *C. aspersum*, then it decreased and during hibernation these bacteria were not established. On GSP medium the colonies specific for the genus *Pseudomonas* were not found. The characteristic colonies for the genera *Salmonella* and *Shigella* did not grow on SS agar, but there was a development of the colonies of different morphology with red halo, the number of which increased significantly in the active and aestivation state, and sharply reduced at hibernation (Table 1). We did not identify these bacteria, but they may be other members of the family Enterobacteriaceae. On SPS medium we found anaerobic bacteria which form colorless colonies, but did not possess the typical for *C. perfringens* morphology. The number of these bacteria was highest during the active states of *C. aspesum* after that it decreased, and during hibernation they are not established.

In connection to the feeding and the role of the intestinal microflora in the snail intestinal tract we analyzed quantitatively amylolytic, proteolytic and cellulolytic bacteria. The amylolytic bacteria are present throughout the life cycle, their number, and possibly their activity is highest in the active period.

**Table 1.** *Dynamics of the bacterial community of the intestinal tract of C. aspersum during the different life cycle states.*

| Medium | Group of bacteria | Physiological state of snails/number.$ml^{-1}$ | | | |
|---|---|---|---|---|---|
| | | HB | H2Y | H1Y | H |
| PCA | Total bacterial count | $5.3\times10^5$ | $1.6\times10^9$ | $5.0\times10^6$ | $6.5\times10^5$ |
| Mac Conkey | *Enterobacteriaceae* | $1.4\times10^5$ | $3.2\times10^6$ | $2.0\times10^5$ | $4.0\times10^5$ |
| Mac Conkey 2 | *Enterobacteriaceae* and enterococci | $2.1\times10^5$ | $1.1\times10^7$ | $1.2\times10^5$ | $1.0\times10^5$ |
| MRS | Lactic acid bacteria | $5.6\times10^5$ | $2.1\times10^7$ | $5.9\times10^6$ | $3.7\times10^5$ |
| M17 | Lactic acid bacteria | $2.4\times10^5$ | $9.0\times10^7$ | $1.8\times10^6$ | $3.2\times10^5$ |
| SS arap | *Salmonella*, *Shigella* and enterobacteria* | $6.5\times10^5$ | $3.0\times10^7$ | $1.3\times10^7$ | $1.7\times10^3$ |
| GSP | *Aeromonas* and *Pseudomonas* | $9.3\times10^7$ | $8.0\times10^6$ | $1.0\times10^4$ | - |
| SPS | *C. perfringens* | $3.6\times10^4$ | $3.4\times10^7$ | $3.9\times10^5$ | - |
| Starch agar | Amylolytic | $1.0\times10^2$ | $2.0\times10^7$ | $1.0\times10^4$ | $1.0\times10^5$ |
| Milk agar | Proteolytic | - | $1.9\times10^5$ | - | - |
| Media with cellulose | Cellulolytic | - | $1.3\times10^6$ | $2.5\times10^4$ | $4.5\times10^3$ |

Legend: (-) Growth is not detected.* *Salmonella* and *Shigella* were not detected

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In juveniles of *C. aspersum* both proteolytic and cellulolytic bacteria were absent. In the active state these two groups were the most numerous, the number of cellulolytic bacteria declined during the hibernation. Proteolytic bacteria were not detected both during aestivation and hibernation.

## Discussion

ARDRA analysis with endonucleases *Hinf*I and *Csp*6I and the hierarchical cluster analysis of the data showed that the composition of the bacterial community in the intestinal tract of *C. aspersum* was affected by the seasonal and environmental variation and responded with changes in its composition and structure.

During the active growth and nutrition, the intestinal tract of the snails was inhabited by both resident and transit microorganisms, which explained the largest amount of bacteria in this stage. The exogenous microflora which enters the intestinal tract of the snail by the food possesses enzymatic activities that assist in the digestion of plant cell walls (Lesel et al., 1990) and improve digestive processes. The dynamics of the total number of bacteria throughout the life cycle showed that it was lowest in juveniles of *C. aspersum* and in hibernation state. After the active state during which their number was highest in the aestivation state the total amount of bacteria decrease sharply (Table 1). During aestivation the snails lost a significant amount of water, which affects the viability of the bacteria and their number as well as rate of the metabolic processes (Nicolai, 2010). Probably this is the reason for the loss of transit bacterial populations. Low content of culturable microorganisms in young snails is maybe due to their diet, in which only the shells of eggs are used as a food resource. It is possible that the microbial community during this stage to characterize not only by low numbers, and the different component structure. Limiting factors in hibernation are significantly higher losses of water content of the body of the snail (more significant in comparison with the period of aestivation), reduction of food resources and low temperatures, which selected primarily psychrotrophic bacteria. Our results showed that the total amount of the intestinal microflora varied depending on the life cycle and the related physiological activity of the snails.

Some authors described as a component of the intestinal microflora of the different types of edible snail (*Achatina achatina*, *Achatina fulica*, *Helix pomatia*) bacteria such as *E. coli*, *Salmonella* sp., *Shigella* sp., *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Enterobacter* and others who may present a risk to the viability of the snails as well as to consumers (Adagbada et al., 2011). There are data which show that the high number of bacteria of the genus *Aeromonas* during hibernation is the cause of high mortality of snails in French farms (Kiebre-Toe et al., 2005). The colonies specific of the genus *Aeromonas* we established for juveniles of *C. aspersum*, but their number decrease in the next two stages. Hibernating snails did not contained bacteria of this genus. It is possible that the development of antagonists of these bacteria in the intestinal tract displaces them as a component of the intestinal microflora during hibernation. It is also possible certain changes in the environment to be important for their development.

Our study showed that bacteria of the genus *Salmonella*, *Shigella* and *C. perfringens* were not found. Parlapani et al. (2014) investigated the microbiological quality of raw and processed wild snails (*C. aspersum*, *Helix lucorum*) and cultured snails (*C. aspersum*) from indoor/outdoor type farms. The authors detected *Salmonella* spp. only in wild snail intestines and meat. Charrier et al. (2006) identified only one strictly anaerobe *Clostridium* species in *C. aspersum*.

The dynamics of the number of amilolytic, proteolytic and cellulolytic bacteria in *C. aspersum* during its life cycle could be explained by the diet of the snails and it can be assumed that the group of proteolytic bacteria was related to the nutrition and was a transient population. Oyeleke et al. (2012) found two bacterial isolates with ability to produce both protease and cellulase enzymes from the gut of *Archachatina marginata*. In the intestine of fed *C. aspersum*, Lesel et al. (1990) counted $10^7.g^{-1}$ CMC (carboxymethyl cellulose) degrading bacteria and $10^6 \cdot g^{-1}$ on native cellulose, while Watkins & Simkiss (1990) noted less than $10^6$ $g^{-1}$ bacteria growing on sterile paper. We confirmed the presence of bacteria able to degrade the cellulose in snails, although our results showed a lower number of these bacteria compared with the results of Lesel et al. (1990). Charrier et al. (2006) observed the absence of cellulose degrading strains, but they explained the result with the dissecting procedure, on one hand aerobically, on the other hand under anaerobiosis. Previous studies of Charrier & Rouland (1992) and Flarri & Charrier (1992) showed that the full enzyme set responsible for the degradation of native cellulose is active in the anterior tract of the snails. The study of Charrier & Rouland (2001) on mannan-degrading enzymes gave evidence that bacteria could not contribute significantly to the

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enzyme activities reported in the crop fluids. According to Charrier et al. (2006) it would appear that the breakdown of cellulose starts in the crop under the activity of endogenous cellulases and that the permanent intestinal flora could complement the cellulose hydrolysis. Transient populations of soil bacteria ingested with food might make plant cell walls more available to the host.

## Conclusion

In conclusion, this study shows that the bacterial flora in the intestinal tract of *C. aspersum* undergoes quantitative and qualitative changes during the different states in the life cycle of the snails. The snails harbor in their gut intestinal bacteria, which possess biochemical potentiality to degrade the plant components.

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