

# Potential interaction between the *Cepaea nemoralis* wild snail and *Citrobacter* spp. bacteria

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### Summary

The aim of this study was to determine the effect of various dietary supplements on the level of selected unsaturated fatty acids, with potential antibacterial properties, in the hepatopancreas of *Cepaea nemoralis* snails, and to perform a preliminary phenotypic characterization of *Citrobacter braakii* strains occurring in the intestinal tract of these snails, which are environmental reservoirs for these bacteria. Gas chromatography was used for fatty acid analysis of the snails. Biochemical characteristics of *Citrobacter braakii* isolates and their resistance profiles for six chemotherapeutics were determined. Giving *Cepaea nemoralis* food supplemented with *Allium sativum* (garlic), *Urtica dioica* (stinging nettle) and *Aloe vera* caused an increase in the total unsaturated fatty acids in the hepatopancreas. In the invertebrates studied, the *Aloe vera* and *Allium sativum* supplements caused an increase in arachidonic acid, while *Urtica dioica* increased oleic and linoleic acid content, which probably contributed to the 2-log CFU (colony forming unit) reduction of *Citrobacter* in the intestine. Wild snails of the genus *Cepaea* should be taken into consideration in epidemiological investigations as potential vectors for pathogenic strains of *Citrobacter* spp.

**Keywords:** *Cepaea nemoralis*, *Citrobacter*, fatty acids, epidemiological investigations, diet supplements

Mollusks as water and land organisms are constantly exposed to high concentrations of bacteria of which many may be pathogenic. These invertebrates continuously ingest bacteria from the soil and their environment and therefore have the potential to become contaminated with microorganisms, both internally and externally. Mollusks can play a role in the transfer of bacteria either by direct contact or by contamination with feces (17). In mollusks the response against microbial organisms is based on both cellular (phagocytosis, encapsulation) and humoral activities (lectins, agglutinins) (9).

The evolution of antibiotic-resistant bacteria has stimulated the search for novel antimicrobial agents from natural sources. A variety of antimicrobial factors have been isolated from mollusks (chlorinated acetylenes, terpenes, indole derivatives, glycerol derivatives, macrolides, lysozymes, proteins and peptides). Surprisingly, it has been found that compounds having antibiotic activity can be recovered from certain West-African snail species of the genera *Archachatina* and *Achatina*. A protein isolated and identified from the

yellowish mucus of the foot soles of these snails can be employed against a broad range of infectious pathogens. Terrestrial slugs and snails feed on a range of living and decaying plant and animal tissues. The intestinal tract of the animals is continuously exposed to bacteria present in the external environment. Once in the gut, these bacteria can either grow, be lysed by gut enzymes, or be excreted. Most of the proteolytic activity in the gut of snails results from enzymes produced by these invertebrates themselves, although a contribution from enzymes released from the gut microflora cannot be ruled out. Studies on soil invertebrates have resulted in the characterization of gut microbial populations from many species and have provided much information on host-microbe interactions (19). However, little attention has been paid to microbes found in mollusks. In the case of gastropod mollusks, most studies on gut bacteria have focused on their general ecology (3, 5, 20). Bacterial populations of the snail (*H. aspersa*) and the woodlouse (*O. asellus*) have already been studied and identified as belonging to *Pseudomonas*, *Xanthomonas*, *Acinobacter*,

*Vibrio* and *Enterobacteriaceae*. Some Gram-positive and endospore-forming bacteria of *Bacillus*, *Staphylococcus* and *Micrococcus* spp were also present (20).

Gram-negative bacteria of the genus *Citrobacter* are commensals that infrequently cause nosocomial infections of the urinary or respiratory tract in compromised hosts. Ingestion of food contaminated by strains from this genus may cause gastrointestinal infections in healthy persons (10).

The aim of this study was to determine the effect of various dietary supplements on the level of selected unsaturated fatty acids, with potential antibacterial properties, in hepatopancreas of *Cepaea nemoralis* snails, and to perform a preliminary phenotypic characterisation of *Citrobacter braakii* strains occurring in the intestinal tract of these snails, which are environmental reservoirs for these bacteria.

### Material and methods

Adult snails were taken from natural populations of *Cepaea (Cepaea) nemoralis* (Linnaeus, 1758). The snails were manually collected from one habitat characterized by low human impact and limited exposure to environmental pollutants, within the city of Lublin (N51°14'06,3"; E22°33'25,3"), from places covered with ruderal vegetation. Plants from the genera *Taraxacum*, *Galinsoga*, *Capsella*, *Plantago*, *Urtica*, *Achille* and *Sambucus* were predominant. Snails were collected once, in October of 2006. Specimens with shells of similar size were chosen for the study, which in environmental samples can offer a certain guarantee of similar age. The *Cepaea nemoralis* shell was on average about 19 mm high. The snails were carefully washed with distilled water and then placed in 15 × 15 × 5 cm perforated plastic containers, 10 in each container. The snails were kept for 90 days in a phytotron chamber (Biogenet) at a constant temperature of 15°C with 90% relative humidity under a photoperiod of 18 h L/6 h D.

Before feeding under laboratory conditions was begun, a control group of snails (control group I; C-I) taken directly from their environment was subjected to microbiological procedures and fatty acid analysis.

The experimental snails were given prepared food once a week. The food (food; F), containing ca. 5% dry mass, was prepared according to the following formula: 1 g agar powder (Difco), 3 g ground oven-dried carrots, 0.5 g dried skimmed milk (SM Siedlce PL), 0.5 g bran, 0.01 g CaCO<sub>3</sub> (BDH Ltd, UK) were mixed with 100 ml double-distilled water boiled with 1 g of agar (12, 13). This medium (20 ml) was poured into Petri dishes to form a thin layer. In addition to the standard medium, three groups of snails were given supplements once a week. The first group was given *ad libitum* crushed Aloe vera leaves – *Aloe vera* (L.) Burm.f. – in the form of a pulp, 30 µl per specimen (food + Aloe vera; FAV). The second group studied was given *ad libitum* crushed stinging nettle leaves – *Urtica dioica* L. – in the form of a pulp, 15 µl per specimen (food + *Urtica dioica*; FUD). The third group was given *ad libitum* garlic – *Allium sativum* L. in the form of a filtrate of homogenized garlic bulb diluted 1 : 5 with water, 10 µl per specimen

(food + *Allium sativum*; FAS). An experimental control group of snails was given the medium with no supplements once a week (control group II; C-II). Once a week the containers were washed carefully with hot water. After 12 weeks of feeding under laboratory conditions the snails were left for 48 h without food to cleanse their digestive system, and then they were frozen at –25°C for further analysis.

One snail from each group was excluded from further analysis and used for microbiological assays. After being carefully washed with sterile distilled water, the snails were placed in 7 × 7 × 14 cm plastic containers. After 1 hour the snails' feces were transferred to LB nutrient broth (Difco) and incubated at 37°C for 24 hours. Then the material was transferred to a *Salmonella Shigella* (SS) medium and a culture was grown at 37°C for 24 hours. After incubation on selective media, bacteria colonies from the snails were randomly chosen for further identification.

Cultures of *Citrobacter* were isolated and identified on the basis of key phenotypic characters using the Api 20 E system (bioMérieux). Sensitivity to chemotherapeutics was determined using Bauer and co-workers' disk-diffusion method on Mueller-Hinton agar (Difco) with bioMérieux disks. To determine the drug sensitivity of isolates, the following chemotherapeutics were used: tetracycline (TE30), trimethoprim sulfamethoxazole (SXT 1.25 + 23.75), streptomycin (S 10), ampicillin (AP 10), chloramphenicol (C 30) and nitrofurantoin (NI 300). After incubation for 24 hours at 37°C the results were interpreted according to NCCLS (National Committee for Clinical Laboratory Standards).

Total lipids were extracted from the snail tissues using a Soxhlet extractor. Methyl esters of fatty acids were prepared by saponification using KOH followed by transesterification. Any water present inhibits the reaction. Fatty acid methyl esters (FAME) were analyzed using a Varian 3800 (USA) gas chromatograph equipped with a flame ionization detector (FID) and a fused silica capillary column 60 m, 0.25 i.d. The injection port and detector were kept at 260 and 260°C, respectively. Column temperature was programmed at 2°C/min from 120 to 210°C. The carrier gas used was helium. Fatty acids were identified by comparing the relative retention times of FAME peaks of the samples with Supelco standards. Data were calculated as normalized area percentages of the total fatty acids. The results of the fatty acids profile were compared by variance analysis (Anova). Average values were compared using Tukey's test.

### Results and discussion

Traditional studies on food web dynamics have used gut content analyses and direct field observations to elucidate various aspects of population dynamics and community structure. The biological specificity of fatty acids, and the fact that they are transferred from primary producers to higher trophic levels, make these molecules suitable for tracing the origin and trajectory of organic matter in the ecosystem (1). Previous studies have found chemical defense associated with the eggs, embryos and larvae of many marine invertebrates (2). Sterols, such as cholesterol, are known to antagonize the antimicrobial activity of

fatty acids. The functional role of free lipids in mollusks varies among species according to both the composition of fatty acids and where they are located. Saturated acids with shorter chain lengths (e.g. C15) generally cause more cell death than those with longer chain lengths (e.g. C18). Oleic acid (C18:1) is at least as active as the polyunsaturated acids. Palmitoleic (C16:1), Oleic (C18:1), linoleic (C18:2) and arachidonic (C20:4) acids are known to possess antimicrobial activity against human pathogens. Structure-function relationship studies on the activity of free fatty acids against human pathogenic bacteria indicate that antimicrobial activity can depend on both chain length and degree of unsaturation (2).

The alimentary tract is a major site of interaction between a snail's ecosystem and its physiology. It may be permanently populated by microorganisms from its environment that could be beneficial or pathogenic in their influence (20). Because snails are capable of digesting food containing large quantities of cellulose fibers, many studies have been conducted recently concerning microorganisms that facilitate degradation of this type of compound and are associated with the digestive system of snails. Previous studies have shown a stable bacteria population in the snail gut, maintained from 1 to 7 days until it attained a concentration of  $10^9$  cells per gram of food content. Bacteria of the genus *Enterococcus* have also been of interest to microbiologists with regard to land snails. Nowadays, these bacteria play an important role in human clinical microbiology, mainly because of their frequent occurrence as nosocomial infectious agents as well as their increasing acquired antibiotic resistance (18). In recent years bacteria from the *Enterococci* family have been isolated from the gut of the brown garden snail *Helix aspersa*. The dominant isolate *Enterococcus casseliflavus* showed a very high fermentation ability (4). The presence of bacteria consumed with food in the snail gut depends on the physical and chemical conditions in the system. In many cases the food moves through the snail's digestive tract for as long as 30 hours, and little is known about the physicochemical properties of this microenvironment, especially its oxygen conditions (3, 5). In a study on aestivating snails in which two populations of *H. pomatia* were considered, cultivable bacteria dominated in the distal part of the intestine, with up to  $5 \cdot 10^9$  CFU  $g^{-1}$ . They were divided into two taxa: the Gamma Proteobacteria with *Buttiauxella*, *Citrobacter*, *Enterobacter*, *Kluyvera*, *Obesumbacterium*, *Raoultella* and the Firmicutes with *Enterococcus*, *Lactococcus*, and *Clostridium* (5). Ducklow et al. (7) found that relatively few aerobic heterotrophic genera were preponderant in wild and laboratory snails. Field snail populations have a greater diversity of microorganisms. *Enterobacteriaceae* are predominant in about one-third of all field snails, but are apparently out-competed when these snails are transported into the laboratory.

The species from the genus *Citrobacter* that have been most frequently connected with food contamination are *C. freundii* and *C. braakii*. Kaclikova et al. (10) have developed a method for detecting *Citrobacter freundii* and *C. braakii*, based on 5'-nuclease PCR oriented to the sequence of a *cfb* gene encoding a cyclopropane fatty acids synthase, which is specific for this species. Another reason for interest in *Citrobacter* is that some *Citrobacter braakii* strains (for example *C. braakii* YH-15) have produced phytase with specific activity of 3457 units/mg<sup>-1</sup>, which is 1.9 times higher than *E. coli* phytase, which had previously been recorded as having the highest specific activity. Its molecular weight was 47 kDa by SDS-PAGE gel (11). Phytase catalyzes the hydrolysis of phytate which is the main form of phosphate in foods or feeds of plant origin. Phytate also acts as an anti-nutrient because of its chelation of various metals and binding protein. Many attempts have been made to hydrolyze dietary phytate using phytase to decrease the amount of phosphate excreted by animals. Phytic acid (IP<sub>6</sub>), after hydrolysis of phytate, can act as a natural antioxidant. Exogenous phytic acid inhibited iron/ascorbate-induced formation of aldehydic lipid peroxidation products (22). A number of phytases have been detected in various bacteria, such as *Aerobacter aerogenes*, *Pseudomonas* sp., *Bacillus subtilis*, *Klebsiella* sp., *E. coli* and *Enterobacter* sp. (11).

Strains of *Citrobacter braakii* isolated from all the snails studied had similar biochemical characteristics. Based on the biochemical tests conducted, it can be stated that for the *Citrobacter braakii* isolates 4 of the 20 API tests had differentiation potential: production of  $\beta$ -galactosidase (ONPG), arginine dihydrolase (ADH) and decarboxylase ornithine (ODC), and citrate utilization (CIT).

The plate count method for determining the number of *Citrobacter* strain bacteria after the experimental feeding period indicated that CFU (colony forming unit) was reduced by two log units in comparison with the control. Analysis of the drug sensitivity of *Citrobacter braakii* isolates from the snail groups fed under laboratory conditions showed an increase in the number of strains resistant to ampicillin and nitrofurantoin compared with control group I (C-I) (tab. 1). It is interesting to note the resistance to streptomycin of all the isolates obtained from control group I and their semi-sensitivity to tetracycline. Strains from the *Mollusca* fed under laboratory conditions were either semi-sensitive or only 20% resistant to this antibiotic. Most effective against the *Citrobacter braakii* isolates from all the experimental groups were trimethoprim + sulfamethoxazole (100% – SXT) and tetracycline (TE). Most of the strains studied were semi-sensitive to chloramphenicol (C). Watkins and Simkiss (20) and Nicolai (15) have found that the most of the snail's microflora are picked up from the environment during feeding and modified by starvation and hibernation.

**Tab. 1. Effects of chemiotherapeutics (% resistant and sensitive) on growth of *Citrobacter braakii* isolates from *Cepaea nemoralis* faeces**

Chemiotherapeutic	C-I*		C-II		FAV		FUD		FAS	
	R	S	R	S	R	S	R	S	R	S
Ampicillin (10)**	20	20	60	0	100	0	100	0	100	0
Chloramphenicol (30)	0	0	0	20	0	0	0	20	0	0
Nitrofurantoin (300)	20	20	40	20	100	0	80	20	100	0
Streptomycin (10)	100	0	0	0	20	0	20	0	20	0
Tetracycline (30)	0	0	0	80	0	80	0	60	0	100
Trimethopr. + Sulfmethox. (1.25 + 23.75)	0	100	0	100	0	100	0	100	0	100

Explanations: \* – explanation of abbreviations in Material and methods; \*\* – figures in brackets denote amount of chemiotherapeutic in disk; R – resistant, S – sensitive

When starved or kept under clean conditions the bacterial population of the snail gut falls and the thickness of the alimentary tract decreases. All indications are that the bacteria in the alimentary tract of the snail are indiscriminately accumulated from the natural environment and are transient populations. The numbers of bacteria increase towards the posterior of the alimentary tract, although the numbers in the hepatopancreas are more constant (20). The bacteria in the snail appear to be similar to those found in soil and show wide resistance to antibiotics. Animal guts show morpho-anatomical and physiological adaptations to the diet, and this is especially obvious in primary consumers. Adaptation of snails feeding on a diet high in lignocellulose is manifested by a lengthening of the digestive tract and the presence in it of microorganisms producing enzymes which decompose food of plant origin (3).

The *Aloe vera* pulp given *ad libitum* caused an increase in arachidonic acid (20:4), docosatetraenoic acid (22:4) and docosapentaenoic acid (22:5) compared to both control groups, but a decrease in linoleic acid (18:1). In laboratory studies on rats, the administration of *Aloe vera* afforded a significant restoration of polyunsaturated fatty acid composition, which is presumably mediated by the scavenging of free radicals and the control of lipid metabolism (16).

The main constituents identified in the nettle herb are as follows: flavonoids – glucosides and rutinosides of quercetin, kampferol and isorhamnetin; caffeoyl-esters – caffeoylmalic acid, chlorogenic acid, neochlorogenic acid; caffeic acid, scopoletin, sitosterol, polysaccharides, minerals and fatty acids (6). Guil-Guerrero (8) found that the dominant polyunsaturated fatty acids in *Urtica dioica* leaves were  $\alpha$ -linolenic acid (40.7%) and linoleic acid (11.6%). In the group of snails given nettle leaves, the hepatopancreas contained increased oleic acid (18:1) and linoleic acid (18:2) compared to both control groups, while the level of the remaining four acids studied decreased

**Tab. 2. Selected fatty acid profile of snail *Cepaea nemoralis* hepatopancreas\***

Fatty acids	F**	C-I	C-II	FAV	FUD	FAS
18:1 n-9	17.35	24.82 <sup>b</sup>	25.20 <sup>ab</sup>	19.33 <sup>c</sup>	31.40 <sup>a</sup>	23.46 <sup>b</sup>
18:2 n-6	14.57	13.88 <sup>c</sup>	14.35 <sup>b</sup>	13.91 <sup>c</sup>	15.48 <sup>a</sup>	15.89 <sup>a</sup>
20:4 n-6	0.03	7.54 <sup>b</sup>	7.66 <sup>b</sup>	10.43 <sup>a</sup>	5.04 <sup>c</sup>	8.56 <sup>b</sup>
20:5 n-3	–	0.69 <sup>b</sup>	0.72 <sup>b</sup>	0.91 <sup>a</sup>	0.36 <sup>c</sup>	0.73 <sup>b</sup>
22:4 n-6	–	1.98 <sup>a</sup>	1.82 <sup>a</sup>	2.17 <sup>a</sup>	1.06 <sup>b</sup>	1.66 <sup>ab</sup>
22:5	–	1.01 <sup>b</sup>	1.03 <sup>b</sup>	1.48 <sup>a</sup>	0.61 <sup>c</sup>	1.18 <sup>b</sup>
ΣSFA	61.43	31.85	30.26	29.30	26.63	28.03
ΣUFA	38.58	68.15	69.80	70.69	73.36	71.95
UFA/SFA	0.64	2.14	2.44	2.59	2.89	2.71

Explanations: \* – results expressed as percentage of total fatty acid methyl esters; \*\* – explanation of abbreviations in Material and methods; values followed by different letters in the same line are significantly different ( $p < 0.05$ )

(tab. 2). Liu's (14) *in vitro* experiments determined that dietary supplementation of garlic decreased incorporation of acetate into fatty acids in various tissues. It is therefore reasonable to speculate that the inhibition of TG synthesis by garlic may in part be attributed to an impairment of fatty acid synthesis. A total of 70 fatty acids have been determined in *Alium sativum* tissues (21). Linoleic, palmitic and  $\gamma$ -linolenic acid are the three major fatty acids. Garlic filtrate given to one experimental group caused an increase in linoleic acid and arachidonic acid in the snails' hepatopancreata compared to the control groups. There was a slight decrease in oleic acid and docosatetraenoic acid.

In the three experimental groups, the dietary supplements caused an increase in all the unsaturated fatty acids in the complete fatty acids profile compared with the control groups. The UFA/SFA ratio was highest in the hepatopancreas of *Cepaea nemoralis* that were given *Urtica dioica* leaves (2.89 – FUD). Compared with control group I (C-I), the UFA/SFA ratio was higher not only in the groups with the supplemented diet but also in the laboratory control group (C-II). Future studies should endeavor to address whether or not unsaturated fatty acids are bioavailable in the *Gastropoda* material, thus providing a mechanism for defending against pathogens.

## Conclusions

The dietary supplements given to *Cepaea nemoralis* snails did not eliminate *Citrobacter* spp. strains from their digestive tracts. Combinations of data at various levels of biological and taxonomic levels of organization can be helpful in understanding the role of many factors that influence the defense mechanisms of invertebrates, including pulmonate snails, against potential pathogens. The results of this study provide additional insights into the nature of the microfloras of snails.

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