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Isolation and characterization of cultivable fermentative bacteria from the intestine of two edible snails, *Helix pomatia* and *Cornu aspersum* (Gastropoda: Pulmonata)

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ABSTRACT

The intestinal microbiota of the edible snails *Cornu aspersum (*Syn: *H. aspersa)*, and *Helix pomatia* were investigated by culture-based methods, 16S rRNA sequence analyses and phenotypic characterisations. The study was carried out on aestivating snails and two populations of *H. pomatia* were considered. The cultivable bacteria dominated in the distal part of the intestine, with up to 5.10^9 CFU g^{-1}, but the Swedish *H. pomatia* appeared significantly less colonised, suggesting a higher sensitivity of its microbiota to climatic change. All the strains, but one, shared ≥ 97% sequence identity with reference strains. They were arranged into two taxa: the Gamma Proteobacteria with *Buttiauxella*, *Citrobacter*, *Enterobacter*, *Kluyvera*, *Obesumbacterium, Raoultella* and the Firmicutes with *Enterococcus*, *Lactococcus,* and *Clostridium*. According to the literature, these genera are mostly assigned to enteric environments or to phyllosphere, data in favour of culturing snails in contact with soil and plants. None of the strains were able to digest filter paper, Avicel cellulose or carboxymethyl cellulose (CMC). Acetogens and methanogenic archaea were not cultivated, so the fate of hydrogen remains questionable. This microbiota could play important roles in the digestive process (fermentation) and the energy supply of the snail (L-lactate, acetate). The choice of cereals and plants by snail farmers should take into account the fermentative abilities of the intestinal microbiota.

Key terms: *Clostridium*, edible snails, Enterobacteriaceae, Enterococci, gut microbiota, Lactococci

INTRODUCTION

Terrestrial gastropods feed upon fresh plants with high protein and calcium contents (Iglesias and Castillejo, 1999; Chevalier et al.,2003) and may also participate, with other soil invertebrates, in the decomposition of leaf litter (Hatziioannou et al., 1994). Consequently, they need a large set of polysaccharide depolymerases and glycoside hydrolases for the digestion of plant materials. Previous works (Myers and Northcote, 1958; Charrier and Rouland, 1992; Flari and Charrier, 1992) demonstrated that native cellulose, laminaran and mannan are highly degraded by Helicid snails while starch, pullulan, α-glucosides, xylan and β-xylosides are weakly hydrolysed.

The dependence of pulmonates on microbial activity within their gut would explain their extraordinary efficiency in plant fibre digestion (60-80%) (Davidson, 1976; Charrier and Daguzan, 1980). A survey of the dominant cultivable bacteria in the digestive tract of snails revealed mainly Enterobacteria (Lesel et al., 1990; Watkins and Simkiss, 1990) and Enterococci (Charrier et al., 1998). Some authors suggested the role of bacteria in fermentative

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processes of the hosts (Charrier et al., 1998) in particular the breakdown of chitin and soluble cellulose (Lesel et al., 1990; Brendelberger, 1997). Others argued that bacteria are transient populations able to interact with snail intake of soil pollutants (Simkiss, 1985; Simkiss and Watkins, 1990). Only recently were described the environmental characteristics (pH, O_2) in Helicid guts (Charrier and Brune, 2003). The authors revealed a pH gradient along the gut, from acidic in the crop to neutral or alkaline in the intestine. The anoxic status of the whole gut was also demonstrated. Unfortunately, most of the microbiological data concerned aerobically cultivable bacteria and focused on crop fluids and digestive gland-intestine mixed extracts. Moreover, the breakdown of polysaccharides and oligosaccharides predominates in the anterior tract (oesophagus, crop and stomach) where bacteria appeared mainly aerobic, at the level of $1.3\ 10^6$ CFU g^{-1} tissue (Charrier et al., 1998). In contrast, the bacterial densities stay at a high level ($2\ 10^8$ CFU g^{-1} tissue) in the intestine of fed and starved animals.

A better knowledge of the roles of the intestinal microbiota in snail nutrition is needed and data should improve intensive rearing of these animals. Consequently, the aims of the present paper were: (i) to isolate the predominant cultivable bacteria of the two edible snail species *Helix pomatia* and *Cornu aspersum* (Syn: *Helix aspersa*), (ii) to identify the strains at the species or genus level using phylogenetic analysis of 16S rRNA sequences, and (iii) to determine the biochemical profiles of the strains. In order to assess the influence of the environment on the bacterial densities and communities, the species *Helix pomatia* was investigated from two populations of different geographical origins.

MATERIAL AND METHODS

Snails

Two species of snails were used: the brown garden snail *Cornu aspersum* and the vineyard snail *Helix pomatia*. *C. aspersum*, originated from Luberon (southern France) was collected in a garden covered with Poaceae, *Trifolium repens* and *Taraxacum* sp. *Helix pomatia* was taken from two distinct populations, one living in Götaland (southern Sweden) and the other in Auvergne (central France). They lived on mixtures of meadows and woods that consist of deciduous trees (oaks, beechs) and herbaceous plants (Poaceae, *Urtica dioïca*, *Taraxacum sp*., *Ranunculus sp*. in Auvergne, *Aegopodium podagraria*, *Mercuralis perennis* and *Poa nemoralis* in Götaland). The snails were collected in aestivation during summer.

Preparation of the intestinal extracts

The snails were anaesthetised with 0.1 mM succinyl chloride in 2% $MgCl_2$ (Chung, 1985). They were dissected in five sets of three (*H. pomatia*) or six (*C. aspersum*) individuals inside an anaerobic glove box, as previously described (Charrier et al., 1998). The intestine, embedded with the flat lobe of the digestive gland, comprised two parts defined on histological features. The proximal intestine (PI) ended along the kidney base and the distal intestine (DI) was pressed between the glandular acini and emerged by the rectum. They were illustrated elsewhere (Charrier and Brune, 2003). For the cultivation experiments (three sets of snails), PI and DI were separately placed into screw-capped tubes containing the anaerobic mineral solution of Bryant and Burkey (1953). For the study of plant fermentation (two sets of snails), PI and DI were put together into flasks containing the mineral solution with sterilized plant fragments.

Cultivation experiments

(i) Anaerobic counts. All the cultures were performed in triplicate 500 µl aliquots, according to the anaerobic technique of Bryant (1972). Serial decimal dilutions of the gut extracts were prepared under O_2 - free CO_2 in the mineral solution.

A complete solid medium (Leedle and Hespell, 1980) was used at pH 7 to quantify the total viable counts of bacteria as the

number of colony forming units (CFU). The medium, outgassed with a nitrogen-carbon dioxide mixture (80: 20%) was dispensed into 16 x 150 mm Hungate tubes sealed with butyl rubber stoppers (Bellco Glass In Vineland NJ). They were incubated for 7 days at 25°C before enumeration. The values were expressed in log CFU g^{-1} fresh weight ± SD and compared by Kruskal-Wallis (KW) and Tukey non parametric tests.

Cellulolytic bacteria were counted in the RGCA medium (Fonty et al., 1989) without agar, in which glucose and cellobiose were replaced by cellulose as a strip of filter paper (Whatman N° 1). Methanogenic archaea were counted in Balch tubes in the liquid medium of Balch et al., (1979) under H_2 / CO_2 (80: 20, v/v, 202 kPa). The presence of methanogenic archaea in each tube was determined by GC analysis of CH_4 production and the cultures were observed with epifluorescence microscopy. Acetogenic bacteria were enumerated on the AC-11 liquid medium (Breznak and Switzer, 1986) according to the method described by Morvan et al., (1996). The media for cellulolytic bacteria, methanogens and acetogens were incubated for three weeks at 25°C. Their numbers were estimated as the most probable number (MPN).

(ii) Aerobic counts. Concurrently, duplicate Petri dishes were inoculated with 1 ml aliquots since high oxygen-uptake rates were reported near the apical intestinal epithelium (Charrier and Brune, 2003). Total viable counts were performed on medium C (Raibaud et al., 1966) and compared by a Mann-Whitney test with enumerations obtained under anaerobiosis. We focused also on three specific media (Difco): the DesoxyCholate Agar medium for Enterobacteria, the Streptococcus medium for Streptococci and the Rogosa SL Agar medium for Lactobacilli. The media were incubated for three days at 25°C. The counts were expressed in log CFU g^{-1} fresh weight ± SD and the values were compared by Kruskal Wallis (KW) and Tukey non-parametric tests.

Eighteen strains were purified from the culture media inoculated with the highest dilutions. They were identified according to the following criteria: morphology, gram staining, motility, 16S rRNA sequencing, biochemical and nutritional characteristics.

Phylogenetic analysis

(i) 16S rRNA sequencing. The purified strains were sent, in 50% glycerol, to Macrogen (Seoul, Korea) for sequencing. The 16S r RNA genes were amplified by PCR using the bacterial primers 27f (5' - AGAGTTTGATCCTGGCTCAG- 3') complementary to position 8-27 (*E. coli* numbering) and 1492r (5' - GGTTACCTTGTTACGACTT- 3') complementary to position 1510-1492 (*E. coli* numbering). These primers were described by Lane (1991). Sequence assemblies were obtained with the CAP program (http: //www.infobiogen.fr) and pairwise sequence comparisons to 16S rRNA available in DNA sequence databases (Genbank, EMBL, DDBJ and PDB) were performed using BLASTN 2.2.10 program (http: //www.ncbi.nlm.nih.gov/). The sequences were edited and aligned manually using the Bioedit program (Hall, 1999). For distance and phylogenetic comparisons, 16S rRNA sequences of 17 type strains from DNA databases, that showed the highest similarity level with the snail gut strains, were added to the data matrix. The snail strain sequences were deposited in the public GenBank database (http: //www.ncbi.nlm.nih.gov/; see acc no in Table II). Due to incomplete sequencing and ambiguous alignments, several nucleotide sites were removed at both 5' and 3' sequence extremities to adjust the data matrix. 1422 aligned nucleotide sites were then used for phylogenetic analysis using PAUP program version 4.0b10 (Swofford, 2002). The parsimony method was used to construct rooted phylogenetic trees, the outgroup being represented by the 16S rRNA sequence of *Borrelia anserina* (acc no U42284), a spirochaete. For graphic representation, the strict consensus tree was drawn (Margush and McMorris, 1981). Robustness of the clades in the phylogenetic trees was estimated by bootstrap analysis with 1000 replicates.

(ii) Criteria for identification. When the 16S rRNA sequence similarity was over 99% with that of the reference strain from GenBank, the snail strain was given the species name of the prototype. A 16S rRNA sequence similarity ≤ 99% and ≥ 97% allowed identification of the strain at the genus level (Drancourt et al., 2000).

Biochemical and nutritional characteristics

Routine biochemical tests were performed anaerobically in Hungate tubes on the RF 20 medium (pH 7.0; 8 ml / tube) (Fonty et al., 1987). The ability of the strains to grow and produce acid from carbon sources were determined in triplicate by using the following substrates: L-arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, melibiose, raffinose, sucrose, xylose, adonitol, glycerol, mannitol, sorbitol, arabinogalactan, Avicel cellulose, CMC, chitin, pectin, pullulan, starch, xylan. Oligomers and polymers were purchased from Sigma Chemicals Co. (St Louis, MO), except the Avicel cellulose PH-101 from Fluka AG Interchim (Montluçon, France). The test of motility was performed at 25°C and 36°C, with API M Medium (bioMérieux, France).

Sterilized stock solutions of monomers, oligomers and polyols were added into the Hungate tubes (1969) containing the RF20 medium, at a final concentration of 0.5% (wt/vol). The polymers were added to the culture medium before sterilisation at a final concentration of 0.2% (wt/vol). The inoculated tubes were incubated at 25°C and the results were recorded after 48h for the monomers and the oligomers and after 7 days for the polymers.

Bacterial growth was assessed visually by comparing the turbidity of the cultures to controls, one without the carbon source and another without the strain. For polymers, this visual assessment was combined with phase-contrast microscope examinations and pH measurements. The use of the carbon source was considered positive if a change of at least 0.2 pH units occurred in the culture medium compared to the uninoculated control (Fonty et al., 1987).

For some strains, the end-products of glucose fermentation were determined after five days of incubation. Volatile fatty acids (VFA) and gas were analysed by gas chromatography (Fonty et al., 1987) and lactate by the enzymatic method of Boehringer (Roche Diagnostics).

Plant fermentation by the intestinal flora

The *in situ* intestinal flora was incubated with crushed leaves of plants known as diets for the edible snails, either in nature (Iglesias and Castillejo, 1999; Chevalier et al., 2003) or in culture (Charrier and Daguzan, 1980). Fresh leaves of stinging nettle (*Urtica dioïca*) and of dandelion (*Taraxacum officinale*) were separately crushed in a mortar. 1 g (fresh weight) of dandelion or stinging nettle was put into each flask. The basal medium used for this study was the mineral solution that was boiled for 10 min, cooled under N_2, then distributed in 40 ml fractions per flask containing the crushed leaves. After sterilization (120°C, 20 min), 0.8 ml of Na thioglycholate stock solution (50 g l^{-1}) was added under N_2. This culture medium, adjusted at pH 7.0 with NaOH 3M, was incubated anaerobically with freshly dissected snail intestines (PI + DI), which were just opened along their longitudinal axis. Three assays per plant, each containing one intestine (*H. pomatia*) or two intestines (*C. aspersum*), were tested against two blanks, one for each plant. The flasks were incubated horizontally at room temperature (27 ± 3 °C) under a permanent stirring. Volatile fatty acids (assays with *T. officinale*), H_2, CH_4, CO_2 and N_2 were analysed after 15 days of incubation.

RESULTS

Bacterial counts

In *C. aspersum* from Luberon (L) and *H. pomatia* from Auvergne (A), the bacterial density reached up to 5.10^9 CFU (g fresh tissue) $^{-1}$ in the distal intestine (Table I). With the complete medium, the density of the cultivated bacteria in the proximal

intestine was 10 to 1000 fold lower than in the distal part, whatever the species ($p < 0.001$, MW test). No significantly more bacteria were cultivated under anaerobiosis (Roll tubes) compared to aerobiosis (plates) (MW test).

The *H. pomatia* from Götaland (G) was the less abundantly colonised. Its cultivable intestinal bacteria remained 100 to 1000 fold lower in size than those observed in *H. pomatia* (A) and *C. aspersum* (L) ($p < 0.001$, Tukey test, see lower-case letters in Table I).

No methanogenic archaea were detected in the intestine homogenates, by measurements of CH_4 (gas) and epifluorescence microscopy and no acetogenic bacteria were found by acetate production on culture supernatants. Cellulolysis was not detected in gut contents cultured with filter paper or cellulose.

The bacterial counts obtained on the three selective media incubated in aerobic conditions pointed out that cultivable Enterobacteriaceae and Lactobacilli were equally abundant while Streptococcal numerations remained lower, particularly in the Swedish *H. pomatia* ($<10^2$ CFU g fresh tissue $^{-1}$, $p = 0.002$ by a KW test between media).

16S rRNA sequence analysis and bacterial identification

The culture media supposed to be selective for Enterobacteria (DCA) and Lactobacilli (MRS) did not provide strains different from those enriched in the complete media. Only the KF medium for Streptococci facilitated isolation of three Enterococci. All the strains cultured on plates were noted P and those enriched from roll tubes were noted RT (Table II).

TABLE I

Bacterial counts in the two intestinal regions of the snails *Helix pomatia* and *Cornu aspersum* (Syn. *Helix aspersa*)

Medium	Snail species	Proximal intestine	Distal intestine
RT = anaerobic	*Helix pomatia* G $_{a}$	3.8 ± 1.1 $_{b, c}$	6.9 ± 0.8 $_{b, c}$
complete	*Helix pomatia* A $_{b}$	5.3 ± 0.5 $_{a}$	9.3 ± 0.2 $_{a}$
medium	*Cornu aspersum* L $_{c}$	6.4 ± 0.5 $_{a}$	9.2 ± 0.2 $_{a}$
P = aerobic	*Helix pomatia* G $_{a}$	4.8 ± 0.7 $_{b, c}$	6.9 ± 0.7 $_{b, c}$
complete	*Helix pomatia* A $_{b}$	7.3 ± 0.3 $_{a}$	9.7 ± 0.1 $_{a}$
medium	*Cornu aspersum* L $_{c}$	6.6 ± 0.1 $_{a}$	9.3 ± 0.1 $_{a}$
DCA	*Helix pomatia* G $_{a}$	6.7 ± 0.9	6.6 ± 1.2 $_{b, c}$
	Helix pomatia A $_{b}$	7.1 ± 0.5	9.6 ± 0.2 $_{a}$
	Cornu aspersum L $_{c}$	7.1 ± 0.7	9.3 ± 0.2 $_{a}$
MRS	*Helix pomatia* G $_{a}$	4.1 ± 1.4	5.9 ± 0.5 $_{b, c}$
	Helix pomatia A $_{b}$	6.2 ± 1.3	9.0 ± 0.5 $_{a}$
	Cornu aspersum L $_{c}$	7.0 ± 1.4	8.2 ± 0.6 $_{a}$
KF	*Helix pomatia* G $_{a}$	< 2.0 $_{c}$	< 2.0 $_{c}$
	Helix pomatia A $_{b}$	4.5 ± 1.0 $_{a, c}$	8.9 ± 0.3 $_{a, c}$
	Cornu aspersum L $_{c}$	< 3.0 $_{c}$	6.5 ± 0.5 $_{c}$

Media: A complete medium in Roll tubes (RT) and on plates (P); Three selective platable media, Desoxycholate agar (DCA) for Enterobacteria, Mann Rogosa Sharpe (MRS) for Lactobacilli and KF for Streptococci. All values are the mean log CFU g^{-1} fresh tissue ± SD for three snails. Abbreviations used *H. pomatia* G = Götaland (Sweden); *H. pomatia* A = Auvergne and *C. aspersum* L = Luberon (France). Lower-case letters ($_{a, b, c}$) indicate significant differences between the lines ($_{a, b, c}$) at $p < 0.001$ (Tukey non-parametric test).

TABLE II

Comparison of 16S rRNA gene sequences between the 18 snail strains and the reference strains giving the highest score of sequence similarity. RT = Roll Tube; P = Plate; PI = Proximal Intestine; DI = Distal Intestine

Snail	**Strain**	Gut region	Accession no.	16S rRNA based - identification of the snail strains	Similarity value (%)
Helix pomatia from					
Götaland (Sweden)	**RT1**	DI	DQ223869	*Buttiauxella agrestis*	99.6
	RT2	PI	DQ223882	*Citrobacter gillenii*	99.6
	P1	DI	DQ223871	*Buttiauxella agrestis*	99.8
	P2	PI	DQ223875	*Lactococcus lactis*	99.9
	P3	DI	DQ223868	*Kluyvera intermedia*	99.1
Helix pomatia from					
Auvergne (France)	**RT3**	PI	DQ223876	*Lactococcus lactis*	99.4
	RT4	DI	DQ223877	*Lactococcus* sp.	98.4
	RT5	PI	DQ223874	*Obesumbacterium proteus*	99.4
	P4	DI	DQ223879	*Enterobacter amnigenus*	99.7
	P5	PI	DQ223885	*Enterococcus raffinosus*	99.6
	P6	PI	DQ223886	*Enterococcus malodoratus*	99.9
Cornu aspersum from					
Luberon (France)	**RT6**	PI	DQ223870	*Buttiauxella noackiae*	99.7
	RT7	PI	DQ223883	*Clostridium* sp.	98.3
	RT8	DI	DQ223873	*Raoultella terrigena*	99.6
	P7	PI	DQ223878	Similarity with *E. amnigenus*	96.3
	P8	PI	DQ223881	*Citrobacter gillenii*	99.0
	P9	DI	DQ223887	*Enterococcus casseliflavus*	99.9
	P10	DI	DQ223880	*Citrobacter* sp.	97.1

The strict consensus phylogenetic tree revealed that all the cultivated bacteria sampled in this study belong to only two main lineages, the Gamma subclass of the Proteobacteria (clade A) and the subdivision of low GC Firmicutes (clade B) (Fig 1). The bootstrap values ≥ 90% indicated a high relatedness between the snail strains and their respective prototype strains.

Within the clade A, we observed six clusters, *Kluyvera, Raoultella*, *Citrobacter*, *Enterobacter, Buttiauxella* and *Obesumbacterium*. The phylogenetic position of the strain P10 was not clear between the *Citrobacter* cluster and a *Pantoea agglomerans* strain (JCM1236). Within the *Citrobacter* cluster, RT2 and P8 were well supported as close relatives to *C. gillenii*. The *Buttiauxella* cluster appeared monophyletic with on one side RT1 and P1 assigned to *B. agrestis* and, on the other side RT6 highly related to *Buttiauxella noackiae*.

Within the Firmicutes (clade B), RT7, cultured in *C. aspersum*, appeared as a sister group of the reference strains *Clostridium* sp. (strain DR6) and *D. guttoïdeum* (DSM4024), with similar sequence divergences (1.7%). The *Lactococci* occurred in *H. pomatia* guts, while the *Enterococci* were present in French populations of both species. The assignation of P9 to *E. casseliflavus* was highly sustained, whereas the intrageneric relatedness of P6 to the strain *E. malodoratus* or *E. raffinosus* remained uncertain. The position of P5 with *E. raffinosus* was more clearly established.

TABLE III

Main fermentative capacities of the snail strains in each cluster (Column A). A positive test (column B) was a substrate degraded within 48 hours (oligomers and polyols) or 7 days (polymers) at 25°C. Beyond these respective times, the test was noted negative (column C). The substrates degraded by all the cultured Proteobacteria and Firmicutes are indicated in the text

CLUSTER	Positive Tests*	Negative Tests *
Buttiauxella	Melibiose, chitin; arabinogalactan by RT1, P1	Lactose, adonitol, glycerol, sorbitol, pullulan, starch, xylan
Citrobacter	Cellobiose, maltose, glycerol	Melibiose, raffinose, adonitol; all the polymers by RT2
Enterobacter	Cellobiose, maltose, melibiose, raffinose	Lactose, adonitol, glycerol; all the polymers by P4
Kluyvera	All oligomers and polyols except one; pectin	Adonitol, all the polymers except one
Obesumbacterium	Substrates degraded by all the Proteobacteria tested	Cellobiose, lactose, maltose, melibiose, raffinose, sucrose, adonitol, glycerol, sorbitol. All the polymers tested
Raoultella	All the oligomers and polyols tested, pectin, xylan, pullulan within 24h	Arabinogalactan, chitin, starch
Enterococcus	All the oligomers tested, arabinogalactan; polyols by P5, P6; pullulan within 24h by P9	Xylan; adonitol, glycerol, sorbitol by P9
Lactococcus	Substrates degraded by all the Firmicutes tested	Melibiose, raffinose, polyols, all the polymers tested
Clostridium	Galactose, lactose, xylose (within 24h), pectin, arabinogalactan	Arabinose, melibiose, raffinose, polyols, pullulan, starch, xylan

* When several strains were present in a cluster, we reported the identical results, but also discriminant characters of some strains.

Phenotypic characterization of the snail strains

All the strains were facultative anaerobes, except RT7 that was strictly anaerobic. No strains able to degrade filter paper, Avicel cellulose and CMC were observed.

Within the Proteobacteria, all the strains were Gram negative rods, that were motile in the clusters *Buttiauxella*, *Citrobacter*, *Enterobacter* and *Kluyvera*. They utilized L-arabinose, fructose, galactose, glucose, mannose, sucrose (except RT5), xylose and mannitol . Within the Firmicutes, the strains were Gram positive cocci or rods, with only *E. casseliflavus* as motile strain. They fermented cellobiose and maltose. All but one (RT7) were also positive on fructose and mannose. None of the Firmicutes utilized xylan. The main fermentative capacities of the snail strains in each cluster are summarized in the table III. Fast growths (within 24h) on xylose (RT7), on pullulan for RT8 (*R. terrigena*) and P9 (*E. casseliflavus*), three strains found in *C. aspersum*, were noticeable. Polyols and polymers appeared useful substrates to discriminate between the strains. The snail strains belonging to the clusters *Buttiauxella, Citrobacter*, *Clostridium* and *R. terrigena* produced H_2 in pure cultures.

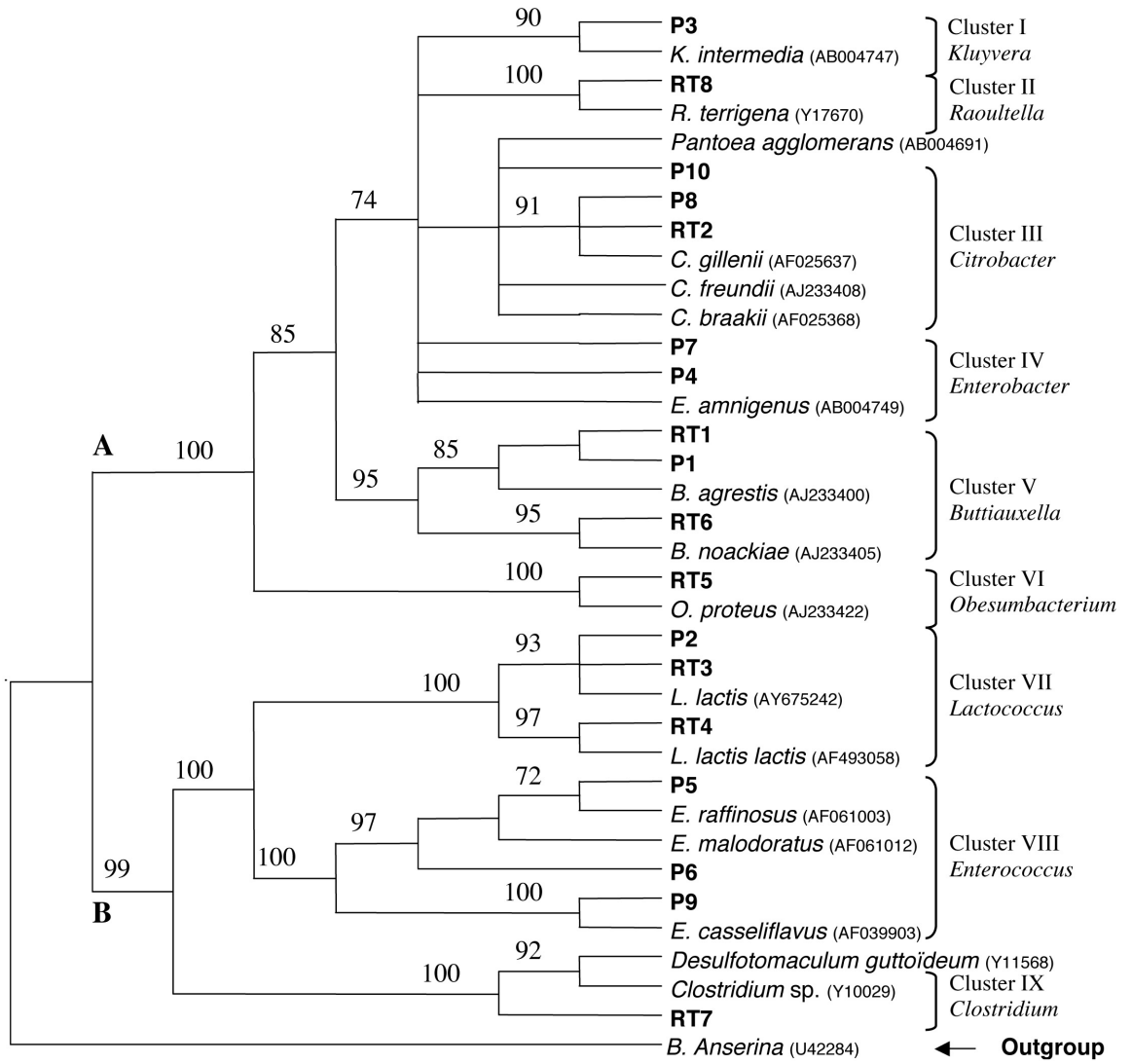

Figure 1: Strict consensus cladogram derived from the analysis of the 16S rRNA gene sequences of 18 cultivable strains isolated from the gut of two terrestrial snail species, *Helix pomatia* and *Cornu aspersum*. The GENBANK accession numbers of the prototype strains used for sequence alignments are noted in brackets and *Borrelia anserina* figures the outgroup. Bootstrap values are from 1000 replicates and the values lower than 70% are not figured. Clade A= Gamma Proteobacteria; Clade B= Firmicutes, Low G+C content of DNA.

Products of fermentation by the intestinal flora

After 15 days of incubation, no significant differences were observed between the assays. The pH in the culture media, compared to the respective blanks, decreased from 1.5 to 2.1 units during the process of fermentation whatever the plant source and reached acidic values between 4.5 ± 0.1 and 5.3 ± 0.5. Fermentations produced mainly CO_2 in the range of 70-74%, but also liberated some H_2 up to 7.5%. All the assays were negative for methane production. The main volatile fatty acid produced by the intestinal fermentation of dandelion was acetate (about 20 ± 8.8 mM) in both species, while ten times less propionate and butyrate were obtained.

DISCUSSION

Bacterial densities and communities

This study was carried out with snails collected during summer, in aestivation. In this environmental condition, the gut of *H. pomatia* from Götaland appeared significantly less abundantly colonised. Aestivation might cause a great stress by selecting for bacterial eurytherms over stenotherms. In that sense, microbiota of the Swedish *H. pomatia* would be more sensitive to climatic change than those of the French population. On the contrary, the natural population of *C. aspersum* studied here appeared more densely (x 100) colonised than the population reared in laboratory by Charrier et al., (1998).

Whatever the snail species and the population, facultative anaerobes emerged dominant from cultivation. This is in accordance with the possibility of a microoxic region in the vicinity of the apical epithelium (Charrier and Brune, 2003). We failed to cultivate acetogens, methanogens and cellulolytic anaerobes. Moreover, none of the purified strains was able to digest filter paper, Avicel cellulose or CMC. It is possible that cellulolytic bacteria were present but unable to grow on the culture media used. Robert and Bernalier-Donadille (2003) observed that some true cellulolytic bacterial species present in the colon of non-methanogenic human subjects did not grow on these substrates. In contrast, absence of detection of methanogenic archaea and reductive acetogens reflects more accurately the reality of the situation. No H_2 and CO_2 utilisation (acetogenesis) occurred in the culture tubes. No methane was detected by gas chromatography in the gut extracts and the *in vitro* incubations of intestinal contents. This result, already reported in *C. aspersum* (Charrier et al., 1998) was established in this study also for *H. pomatia*. In agreement, intact fed snails *H. pomatia* (n = 5) maintained for 24 hours inside a closed glass bell did not produce methane (data not shown). In this aspect, the structure of the microbiota and in consequence the functioning of the digestive microbial ecosystem of the snails differs markedly from those of vertebrates, especially herbivores.

Phylogeny of the cultivated bacteria

In this study, a value > 97% 16S rRNA sequence identity was used as the lowest level for genus identification (Drancourt et al., 2000). All the strains cultivated, except one, were differentiated by their 16S rRNA sequence, at the species or at the genus level. The cultivation methods allowed identification of two taxa, the Gamma Proteobacteria and the Firmicutes of low G+C DNA content. All the Proteobacteria cultivated were *Enterobacteriaceae*, a large family where recent phylogenetic works pointed out the existence of strong DNA relatedness between strains previously named differently after biochemical tests (*Hafnia alvei* and *O. preoteus* (Spröer et al., 1999; Olsson et al., 2004), *Enterobacter intermedia* and *K. cochleae* (Pavan et al., 2005). Drancourt et al., (2001) indicated the polyphyletic positions of *Enterobacter* and *Klebsiella* species, dividing the last one into two genera, *Klebsiella* and *Raoultella*. *R. terrigena* was present in *C. aspersum*. Within the Firmicutes, Stackebrandt et al., (1997) revealed the misclassification of *D. guttoïdeum*. In agreement with the authors, we showed that the strain RT7 was genotypically close to *D. guttoïdeum* as well as to the *Clostridium* strain DR6B. The other snail strains were more clearly established in three monophyletic clusters, *Buttiauxella* (Spröer et al., 1999) *Lactococcus* and *Enterococcus*.

Do the snails harbour autochthonous microbes in their gut?

An extensive work on snail microbiota does not exist. To our knowledge, this is the first analysis of bacterial strains found in the gut of *H. pomatia*. *C. aspersum* was already investigated by Simkiss and Watkins (1990), by Lesel et al., (1990). *Citrobacter*, *Klebsiella, Enterobacter, Staphylococcus, Streptococcus* and *Vibrio* were facultative anaerobes previously noticed in the snail gut, but briefly described.

Among the snail strains cultivated, *Enterobacteriaceae* and Enterococci are generally assigned to "enteric" environments, but only a few actually reside in animal guts: (i) *E. casseliflavus* was already described as a predominant species in the gut of *C. aspersum* (Charrier et al., 1998; (ii) Müller et al., (1996) underlined the profuse occurence of the genera *Butiauxella* and *Kluyvera* in snails and slugs and proposed the pulmonate gut as their natural niche; (iii) *C. gillenii* was detected in human stools (Brenner et al., 1993). Other *Enterobacteriaceae (E. amnigenus, E. malodoratus, R. terrigena)* have been isolated from plants, water, sewage and unpolluted soils. The strains *L. lactis* SL3 and *L. lactis* subsp. *lactis* were found in plants. *H. alvei* exists in different kinds of food products (Olsson et al., 2004). These data are in favour of a development of snail farming on soil and plants. Regarding *D. guttoïdeum,* it was first described in the upper layer of mud (Gogotova, 1983), but the closely related *Clostridium* strain (DR6B) was detected in the rumen of the red deer (Genbank information). Furthermore, *B. agrestis, E. amnigenus, K. intermedia, O. proteus* and *R. terrigena* are psychrotrophs (Gavini et al., 1976; 1983; Drancourt et al. 2001; Olsson et al., 2004). Strains phenotypically close to *Buttiauxella* sp., *Kluyvera* sp. and *Tatumella* sp. and isolated from fed *H. pomatia* (from Auvergne) showed a high resistance to freezing, with temperatures of crystallization lower than -9°C (Nicolai et al., 2005). We observed also the *Clostridium* strain entering a metabolically inactive state by forming spores during cold, like other members of the genus do (Balows et al., 2005). *L. lactis* possesses a surface protein (HtrA), which is a key factor in the response to specific stress conditions (Foucaud-Scheunemann and Poquet, 2003). These different potentialities are consistent with the survival of a great diversity of bacteria in the snail gut during hibernation. Since this study considered snails during aestivation, we may suppose that we have selected eurythermic strains which can reasonably be considered autochthonous species of the snail intestines. However, cultivation might cause a significant artefact on bacterial selection (generalists *vs* specialists), suggesting that a molecular approach needs to be applied in future on snail samples directly.

The roles of these bacteria in feed digestion and host nutrition

In the intestine of fed *C. aspersum*, Lesel et al., (1990) counted 10^7 g^{-1} CMC degrading bacteria and 10^6 g^{-1} on a native cellulose, while Simkiss and Watkins (1990) noted less than 10^6 g^{-1} bacteria growing on sterile paper. Apparently at odds with these results are the work of Charrier et al., (1998) and the present study where none of the snail strains were able to degrade the three celluloses tested (CMC, filter paper, and Avicel, a microcrystalline cellulose). These contrasting results may be explained by the dissecting procedure, on one hand aerobically, on the other hand under anaerobiosis. Previous studies showed that the full enzyme set responsible for the degradation of native cellulose is active in the anterior tract (Charrier and Rouland, 1992; Flari and Charrier, 1992). Another study on mannan-degrading enzymes (Charrier and Rouland, 2001) gave evidence that bacteria could not contribute significantly to the enzyme activities reported in the crop fluids. Therefore, 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. In addition, transient populations of soil bacteria ingested with food might make plant cell walls more available to the host. The presence of a microoxic compartment close to the intestinal epithelium (Charrier and Brune, 2003) argues in favour of bacterial potentialities under aerobic conditions.

Among the other carbohydrates tested, pullulan, starch and xylan were selective since only few snail strains grew on them. *C. aspersum* harboured at least one strain able to ferment these substrates. The occurrence of strong pullulanases in *R.*

terrigena and *E. casseliflavus* suggests to culture *C. aspersum* with amylopectin-rich cereals (maize, rice, sorghum) and to favour plant decaying for infestation by pullulan-rich fungi, like basidiomycetes, items of gastropod food (Speiser, 2001). In the gut of *H. pomatia*, strains were found able to ferment arabinogalactan and, either starch (French snail) or pectin (Swedish snail). Since the gut fluids of Helicid snails exhibit weak enzymatic activities on arabinogalactan, starch and xylan (Myers and Northcote, 1958; Charrier and Rouland, 1992; Flari and Charrier, 1992) and no activity on pectin (personal observation), the present data suggest the importance of some intestinal bacteria in the degradation of plant cell walls (arabinogalactan, pectin, xylan) and starch reserves. Several strains were able to grow on chitin, in particular *C. gillenii*, *B. agrestis*, *B. noackiae* and *E. malodoratus*. Jeuniaux (1955) had already given evidence for the presence of chitinolytic bacteria in the intestine of *H. pomatia* and estimated their density around 10^6 CFU (g fresh tissue)$^{-1}$. Animals might be a type of food eaten, since remains often are detected in gastropod faeces (Speiser, 2001). Chitin and its derivates (chitosans) could serve as a readily available nitrogen source for the bacteria and their host. The body of phytophagous gastropods consists of 10% nitrogen, while food plants only contain 4% (Speiser, 2001).

The production of hydrogen observed *in vitro* (incubation of intestines with plants) and in pure cultures by some strains (*Buttiauxella, Citrobacter*, *Clostridium* and *R. terrigena*) is in accordance with the accumulation of this gas *in situ* (Charrier and Brune, 2003). This bacterial production of H_2 raises the question of its fate. The absence of methanogenic archaea, already pointed out (Charrier et al., 1998; Charrier and Brune, 2003) and of reductive acetogenic bacteria supports the idea that sulphate-reducing bacteria (not enumerated in this work) might be the most important H_2-utilizing community in the snail gut. Simkiss (1985) isolated a sulfate-reducer from the crop of *C. aspersum* and Dallinger (1993) noticed that sulphides accumulated in the excretory cells of the digestive gland. Mucins present all along the gut lumen might be an important source of sulphate, but only few bacteria are known to digest sulphated mucin (Rho et al., 2005).

In conclusion, this study clearly demonstrates that phytophagous helicid snails harbour in their gut intestinal bacteria composed of Gamma Proteobacteria and Firmicutes. This microbiota possesses the biochemical potentiality to degrade and ferment the main plant components: soluble sugars, polyols as well as polymers except cellulose and to digest fungal material. We gave arguments that the most abundant bacteria we have cultivated are likely to play important roles in the digestive process and energy supply of the host. However, a question that arises from this study concerns the plasticity of the bacterial community according to the trophic resources available. Furthermore, there is currently no hypothesis to account for the real diversity of the gut microbiota in snails. Culture-independent approaches appear necessary to determine the fraction of the bacterial community which escape cultivation. This is the subject of the future study.

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