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TITOLO TESI

Survey on the spirilar flora of Lagomorphs

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Esame finale anno 2010

To **Mirko**:

“As armas e os barões assinalados,
Que da ocidental praia Lusitana,
Por mares nunca de antes navegados,
Passaram ainda além da Taprobana,
Em perigos e guerras esforçados,
Mais do que prometia a força humana,
E entre gente remota edificaram
Novo Reino, que tanto sublimaram;”

Luís Vaz de Camões (“Os Lusíadas” – Canto I)

"Não me queiram converter a convicção: sou lúcido!"
Álvaro de Campos (Fernando Pessoa, "Sou lúcido")

"A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales."
Marie Currie

Ad astra per aspera

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Background and objectives

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Members of the genera *Campylobacter* and *Helicobacter* have been in the spotlight in recent decades because of their status as animals and/or humans pathogens, both confirmed and emerging, and because of their association with food-borne and zoonotic diseases.

In 1991, the taxonomy of *Campylobacter* and related organisms was thoroughly revised, since this revision several new *Campylobacter* and *Helicobacter* species have been described and well-founded crediting a polyphasic taxonomic approach.

There is a paucity of data regarding the occurrence of spiral shaped enteric flora in leporids.

The main research aims of the present study were (i) to address to the characterization of *Campylobacter*-like organisms isolated from rabbits epidemiologically not correlated, by using a polyphasic approach; (ii) the definition of the prevalence of *Campylobacter* and *Helicobacter* in leporids; (iii) the study of some virulence determinants and finally (iv) to monitor of antibiotic susceptibility both in *Campylobacter* isolates and indicator bacteria.

Overview of the thesis

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The objectives laying this thesis focused on the survey of spirilar enteric flora of Lagomorphs, in particular *Campylobacter* and *Helicobacter* species in rabbits and hares. The following chapters can be read individually or together. In order to study and understand the significance of the survey, the animals sampled were not correlated epidemiologically.

The first part of the thesis presents an overview of the literature relating to the content of this work. It includes a general introduction to bacterial taxonomy (**I**) and the bacterial species concept and the taxonomy of members of the Epsilon Proteobacteria class (**II**), giving an overview of the known species and respective methods currently used for classification and identification. Furthermore, a general introduction to the lagomorphs (**III**) biology and diseases as well as known zoonotic agents is presented.

The second part presents an overview of the experimental work performed in the framework of this thesis. **Chapter 1** describes a new species, belonging to the group of thermotolerant *Campylobacter*, i.e. *Campylobacter cuniculorum* isolated from rabbits. **Chapter 2** resolves the prevalence of this new species as well as other genus members and *Helicobacter* species in leporids, i.e. rabbits and hares, epidemiologically not correlated. **Chapter 3** focuses on the study of some virulence determinants of *C. cuniculorum*, including the effects of Cytolethal Distending Toxin (CDT) on mammalian cells, the presence of the gene ecoding CDT (*cdtB*), and adhesion and invasion properties. In **Chapter 4**, results on monitoring antibiotic susceptibilities both in *Campylobacter* species and indicator bacteria (*Escherichia coli*) are presented.

The third part of the thesis comprises a general discussion and conclusion of the presented work.

The fourth part presents the summary of this work in English, Italian and Portuguese.

Part 1: General Introduction – literature review

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This part will whine (**I**) bacterial taxonomy followed by a short description of the (**II**) Taxonomy of the ε-*Proteobacteria*. Within this taxonomic overview, a short history introduction will be presented and members of the *Campylobaceriacea* and *Helicobactereaceae* will be described in detail. Subjects regarding the lagomorphs (**III**), in particular rabbits and hares, will be presented including a brief introduction, production, normal gut microbiota and respective disorders. Moreover, a short synopsis will illustrate the current knowledge on ε-Proteobacteria in leporids and some known zoonoses associated with lagomorphs.

I. Bacterial taxonomy

Attempts to bring order through categorization to the bewildering variety of organisms have been an ongoing human endeavor (Gevers et al., 2005). Microorganisms are fantastically diverse, and the amount and depth of genetic diversity represented among the named microbial species is greater than that represented among the animals and plants combined (Buckley and Roberts, 2007). In microbiology, bacterial taxonomy (or biosystematics) is an essential discipline that may be defined as the scientific study of the diversity of organisms with the objective of characterizing and arranging them in an orderly scheme. Within biosystematics, three sub disciplines arise: classification, nomenclature and identification (Owen, 2004; Vandamme et al., 1996). Regarding classification, it relates to the orderly arrangements of taxonomic units or taxa into groups on the basis of similarities or defined relationships. Nomenclature instead is referred to the assignment of taxonomic names to the taxa. Finally, identification is naming a distinct taxonomic unit based on the common characteristics or properties that distinguish them from other organisms. Indeed, classification precedes identification, given that it is necessary to describe and characterize the basic taxonomic unit before an isolate can be identified as a member of that unit (Buckley and Roberts, 2007; Gevers et al., 2005; Owen, 2004; Vandamme et al., 1996). In order to completely define modern biosystematics, two additional disciplines are needed: phylogeny (history, origin and evolution of a set of organisms) and population genetics (the variability of populations of bacteria and the formulation of theories that account for its variability) (Buckley and Roberts, 2007; Gevers et al., 2005; Vandamme et al., 1996). Moreover, it is currently a common practice to apply a polyphasic approach in taxonomic studies, incorporating genetic (e.g., DNA-DNA hybridization), phenotypic (e.g., the results of biochemical tests) and phylogenetic data (e.g. rRNA gene sequences), with the aim to create a consensus classification by integrating different kinds of data and information into a classification of the biological entities that contains a minimum of contradictions (Buckley and Roberts, 2007; Gevers et al., 2005; Vandamme et al., 1996).

In the twentieth century technical breakthrough changed the field of bacterial taxonomy in a groundbreaking way by defining a species as the basic taxonomic unit (Staley, 2006). For decades, classification and identification of microorganisms was based on morphological and biochemical characteristics, and a species was defined as "the type culture together with such other cultures or strains of bacteria that are accepted by bacteriologists as sufficiently closely related" (Buchanan, 1955). Nevertheless, there was no effective way to

determine "sufficiently closely related" and bacteriologists are differently competent to pass judgments on all kinds of bacteria (Buckley and Roberts, 2007; Cowan, 1965). However, on the late twentieth century with the introduction of DNA-DNA hybridization method, the species concept was defined as a group of organisms exhibiting 70% or greater DNA relatedness and less than 5% difference in their melting temperature (ΔTm) (Wayne et al., 1987). The DNA-DNA hybridization was believed to allow investigating evolution relationships through a pair wise comparison of the whole-genome sequence content between microorganisms. There are conversely some major drawbacks associated with using of DNA-DNA hybridization for species demarcation (Vandamme et al., 1996). Major disadvantages are: the laborious nature and time-consuming of the technique; different methods are used to determine the level of DNA-DNA hybridization and these methods do not always give the same (quantitative) results; finally, there is the impossibility of establishing a central database (Cho and Tiedje, 2001; Vandamme et al., 1996). Also, the 70% cut-off limit is an empirical value, created to correspond largely with the species designations in use at that time, and is not supported by a theoretical framework of what a prokaryotic species should entail (Gevers et al., 2005).

With the establishment of rapid sequence analysis of either 16S ribosomal RNA or 23S ribosomal RNA (rRNA), gene sequence analysis has become the most common molecular marker used in phylogenetic studies. The reasons for that are their universal presence, constant functionality and the fact that have mosaic structures of highly conserved and more variable domains (Gevers and Coenye, 2007; Vandamme et al., 1996). The analysis of the 16S rRNA gene sequence provides a phylogenetic framework that is particularly useful above species level. However, it has been stipulated that strains exhibiting 97% sequence similarity or less represent separate species. In addition, strains exhibiting >97% may or may not represent a single species, which should then be evaluated by determining DNA-DNA hybridization values (Stackebrandt and Goebel, 1994). Recently, it was proposed that the limit of the 16S rRNA sequence similarity should be increased to 98.7%-99%, since DNA-DNA hybridization is considered as mandatory for testing the genomic uniqueness of a novel species and lower 16S rRNA similarity values frequently do not corroborate a new species delineation (Stackebrandt and Ebers, 2006). However, overreliance on a single character, like 16S rRNA gene sequences, may lead to erroneous conclusions as verified in some members of the ε-Proteobacteria; more than 3% sequence divergence for strains belonging to the same species (as confirmed by DNA-DNA hybridization) has been demonstrated in *Campylobacter hyointestinalis* (Harrington and On, 1999), and several

Helicobacter species (Hänninen et al., 2005; Hänninen et al., 2003; Vandamme et al., 2000).

The introduction of several innovative methods, including MultiLocus gene Sequence Analysis (MLSA), whole genome sequencing and rapid DNA typing methods, prompted a re-evaluation of the bacteriological species definition (Stackebrandt et al., 2002). Figure 1 presents the taxonomic resolution of some techniques used for the classification of bacteria.

At present, the innovations mentioned above, MLSA and whole genome sequencing in particular, have been employed on a wider scale. A plethora of new papers has been evaluating diversity within and among species by exploiting whole genome information content and the bacterial species concept emerged from obscurity into the spotlight of high impact factors journals (Doolittle and Papke, 2006; Gevers et al., 2005; Konstantinidis and Tiedje, 2007).

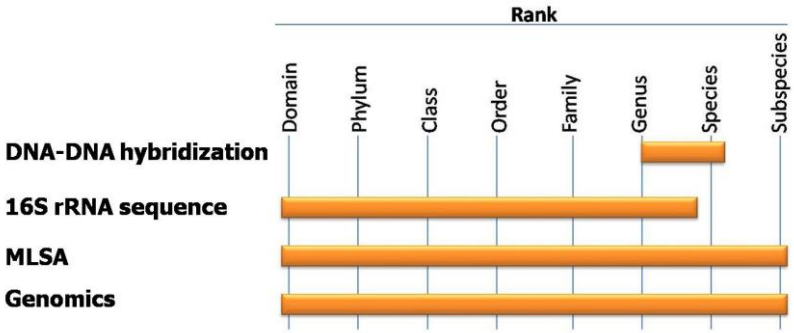

Figure 1: Taxonomic resolution of some of the techniques used for the classification of bacteria. Adapted from Vandamme et al. (1996) and Staley (2006).

Although 16S rRNA gene sequencing is important in identifying a strain to the family or genus level, it is of very little utility for differentiation of species (Staley, 2006), MLSA can be used for analysis below the genus level and for species identification, and such may be a suitable candidate to replace DNA-DNA hybridization in the future (Gevers et al., 2005). In order to fulfill the requirements of suitability of phylogenetic marker, alternative loci have been proposed, such as housekeeping genes. In MLSA, several housekeeping genes are combined to provide a buffer against the distorting effects of recombination at a single locus (Gevers et al., 2005). Although there is no MLSA scheme, various MLSA protocols have been published for numerous genera, with varying combinations of these alternative markers. Some particular examples of the use of housekeeping genes reported for their usefulness as phylogenetic markers in different genera are: *rpoB* (Korczak et al., 2006) and *groEL* (Kärenlampi et al., 2004) for *Campylobacter* genus, *gyrB* (Hännula and Hänninen,

2007) and *groEL* (Mikkonen et al., 2004) for the *Helicobacter* spp., *ureAB* for the gastric *Helicobacter* species (Hänninen et al., 2005; O'Rourke et al., 2004). However, these genes are informative within a given genus or family and may not be useful or even present in other taxa. As an example, *gyrB* is a good phylogenetic marker for *Helicobacter* species (Hännula and Hänninen, 2007) but not for *Vibrio* species (Thompson et al., 2007). Nevertheless some genes may be informative in more than one group, and these more widely distributed genes could provide tools for broader comparisons (Gevers and Coenye, 2007). Even though there is quite some sepsis about the existence of a universal alternative marker in addition to the 16S and 23S rRNA genes, the *rpoB* gene, coding for the RNA polymerase β subunit, has been suggested as a possible candidate (Adekambi et al., 2008; Case et al., 2007). It was demonstrated that it has similar or superior resolution compared to the 16S rRNA gene and showed the highest correlation, next to 16S and 23S, to the whole-genoma based parameter average amino-acid identity (AAI), for inferring phylogenetic relationships above the species rank (Adekambi et al., 2008; Case et al., 2007; Konstantinidis and Tiedje, 2005b).

Of all the technical advances thus far, the emergence of whole–genome sequences may prove to have the biggest impact on bacterial systematics. New derived parameters have been introduced, which compare conserved genes among two organisms as measure to establish evolutionary relatedness (Konstantinidis and Tiedje, 2005a, b). Nevertheless, to evaluate genetic relatedness among ranks above species the use of core genomes is near to impossible, since finding conserved genes among more distantly related genomes is problematic, and even if orthologs are found, they are difficult to compare. Despite the fact that some methods have been developed in order to avoid this problem, and the great utility of those in aiding to create a classification system that is more predictive of genetic and biochemical relatedness (Konstantinidis and Tiedje, 2005b), it would be unrealistic to expect that in the near future bacterial species will be described based on the whole-genome sequences only. Reasons to support the non viability of the whole-genome sequence are mainly the costs as well as the real significance of the method. It has been demonstrated (Konstantinidis et al., 2006) that phylogenetic trees based on MLSA data can bear the same phylogenetic signal as trees based on sequence analysis of the entire common gene pool. This implies that it is not needed the entire genome sequences for all strains under comparison of designed taxonomic frameworks. Nevertheless, in order to understand how to use MLSA data to define species and respective subdivisions from poorly or newly discovered taxa, it is necessary to provide pragmatic criteria.

The current knowledge is very limited in terms of understanding the genetic mechanisms that drive the diversification of prokaryotes, and efforts should be directed at creating a better insight in to these phenomena. However, at the same time it could be concluded that the existing classification system is congruent with the current genomic information and that the existing, primarily 16S rRNA gene sequence and DNA-DNA hybridization based system remains functional and pragmatic (Buckley and Roberts, 2007; Konstantinidis and Tiedje, 2007).

II. Taxonomy of the Epsilonproteobacteria

The prokaryotic domain "Bacteria" (or "Eubacteria") are subgrouped into 28 phyla. The epsilon (ε-) Proteobacteria comprise one of the five Classes within the phylum Proteobacteria (Gupta, 2006). This class is a complex phylogenetic lineage within the group of Gram-negative bacteria which includes either mucosal/animal associated or free-living species. Nowadays the order *Campylobacteriales* consists of two families *Campylobacteraceae* and *Helicobacteraceae* (Garrity et al., 2005b) (Figure 2). Moreover, several genera, composed by single free-living species, needs further studies in order to understand which allocation to bacterial family belong. Although the free-living *Sulfuricurvum*, *Sulfurimonas* and *Sulfurovum* have been proposed to be included in the family *Helicobacteraceae* (On and Owen, 2009), the members of *Helicobacteraceae* considered here are *Helicobacter*, *Wolinella* and *Thiovulum* as indicated in Bergey's Manual of Systematic Bacteriology (Garrity et al., 2005a).

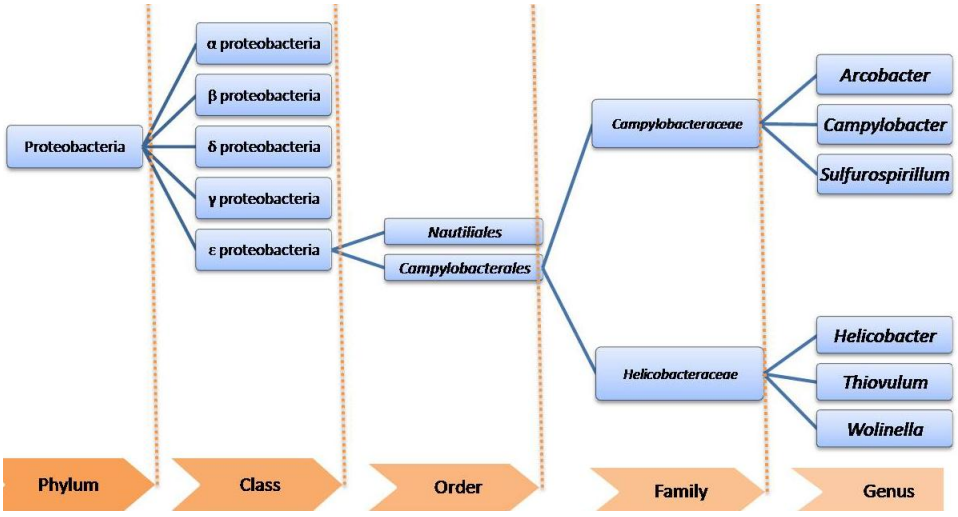

Figure 2: Taxonomic organization of Proteobacteria, with particular attention for Epsilon proteobacteria. Taxonomic ranks are presented on the bottom (orange).

In Figure 3, the phylogeny of several members of epsilonproteobacteria is presented.

A great deal of interest in some members of Epsilonproteobacteria stems from the fact that many of these species are host-associated (*Helicobacter* spp., *Campylobacter* spp., *Wolinella* sp.) and comprise important human and animal pathogens (Gupta, 2006).

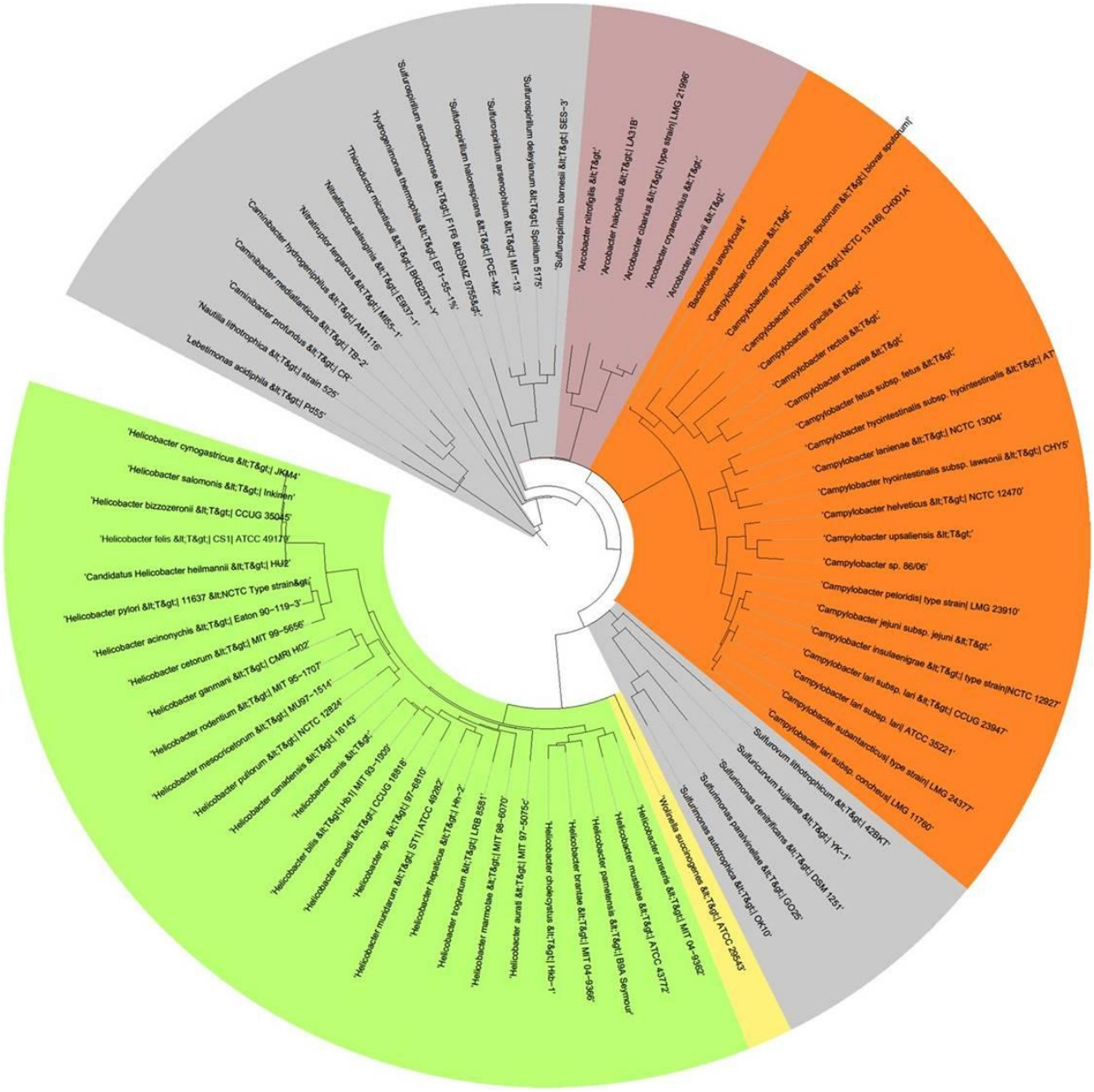

Figure 3: Phylogeny of Epsilon-proteobacteria based on 16S rRNA gene. The sequences were collected aligned from the Ribosomal Database Project (Cole et al., 2009). Tree was built using iTOL – Interactive Tree Of Life (Letunic and Bork, 2007). Branches for free-living environmental ε proteobacteria sequences are coloured in grey (■), *Arcobacter* in purple (■), *Campylobacter* in orange (■), *Wolinella* in yellow (■) and *Helicobacter* in green (■).

The family *Campylobacteraceae* currently comprises the mucosal-associated species belonging to *Campylobacter* genus, the environmental species from the *Arcobacter* genus and the free-living environmental bacteria that belong to the genera *Sulfurospirillum*.

The family *Helicobacteraceae* includes the type genus *Helicobacter*, and consists of animal-associated bacteria occurring as commensals or parasites. *Thiovulum majus* (the only member of the *Thiovulum* genus) are free-living environmental bacteria species. Finally, the *Wolinella* genus, have also only one species, *Wolinella succinogenes*, which are ruminal-associated bacteria.

Taxonomic history. The early history of this class of Gram-negative bacteria started with the first isolation of a *Vibrio*-like organism from aborted ovine fetuses by McFadyean and Stockman in 1913, later described as *Vibrio fetus* (Smith and Taylor, 1919). Subsequently, due to their low DNA base composition, their microaerophilic growth requirements and their nonfermentative metabolism, *Vibrio fetus* and *Vibrio bubulus* were transferred into the new genus *Campylobacter* as *Campylobacter fetus* and *Campylobacter bubulus* (Sebald and Véron, 1963). Ten years later, a more comprehensive study of the taxonomy of the microaerophilic *Vibrio*-like organisms was published, considering four distinct species in the genus *Campylobacter*: *C. fetus* (type species), *Campylobacter jejuni* (isolated from faeces of cattle with diarrhoea, blood of human with gastroenteritis and aborted sheep fetuses), *Campylobacter coli* (isolated from faeces of pigs with diarrhoea) and two subspecies of *Campylobacter sputorum* (subspecies *sputorum*, isolated from human sputum; subspecies *bubulus*, isolated from bovine vagina and semen) (Véron and Chatelain, 1973).

In the early 1980s, due to the availability of adequate isolation procedures [i.e. filtration technique (Butzler et al., 1973) and selective media (Skirrow, 1977)] manifold of *Campylobacter*-like organisms were isolated from a variety of human, animal and environmental sources and new species were described (Fox et al., 1989; Lawson et al., 1981; McClung et al., 1983; Neill et al., 1985). Nevertheless, the poor biochemical reactivity and the lack of clear-cut differential characters did not allow a clear classification of several CLOs and their taxonomic positions remained unsolved for many years (Vandamme et al., 2000; Vandamme and On, 2001).

Thanks to the introduction of bacterial phylogeny based on the degree of rRNA cistron similarity in the late 1980s, the taxonomy of *Campylobacter* began to be more comprehensible. The genus *Campylobacter* was found to be extremely heterogeneous comprising three distinct phylogenetic lineages which included members of the genera *Wolinella* and *Bacteroides* (Vandamme, 2000). The rRNA phylogeny together with phenotypic and other genotypic arguments led Goodwin and colleagues (1989) to move the gastric species *Campylobacter pylori* and *Campylobacter mustelae* from the genus

Campylobacter to a novel genus, *Helicobacter*. Two years later, a complete revision of the taxonomy and nomenclature of *Campylobacter* and related species (CLO, *Helicobacter*, "*Flexispira*", *Wolinella* and *Bacteroides*) based on 16S rRNA phylogeny and extensive DNA-rRNA hybridization study, allocated all these microorganisms in the rRNA superfamily VI (sensu De Ley), later renamed as ε-Proteobacteria (Vandamme et al., 1991). The genus *Campylobacter* was restricted to those species belonging to the rRNA homology cluster I containing *C. fetus*. The name *Arcobacter* was proposed for the aereotolerant *Campylobacter* species belonging to the rRNA homology cluster II containing *Campylobacter nitrofigilis* (reclassified as *Arcobacter nitrofigilis* – type species of the genus). Finally, the rRNA cluster III had included the novel *Helicobacter* genus, *Campylobacter cinaedi* and *Campylobacter fennelliae* (reclassified as *Helicobacter cinaedi* and *Helicobacter fennelliae* respectively) and *Wolinella succinogenes*. The genera *Campylobacter*, *Arcobacter* and the free-living CLOs, reclassified as *Sulfurospirillum*, were included in the same bacterial family *Campylobacteraceae* (Stolz et al., 2005; Vandamme and De Ley, 1991), sharing similar phenotypic and genotypic features. Only recently the family *Helicobacteraceae* was described and include *Helicobacter, Wolinella* and *Thiovulum* genera (Garrity et al., 2005a), as mentioned above.

The genus *Campylobacter* (Sebald and Véron, 1963). [adapted from Debruyne et al., 2008 and Euzéby, 2010a].

Members of the genus *Campylobacter* have following general characteristics. Cells are slender, spiral curved rods, 0.2-0.8 x 0.5-5 µm. They are Gram negative and do not form spores. Cells in old cultures may form spherical or coccoid bodies, considered to be degenerative forms difficult to detect using PCR methods.

They are typically motile, with a characteristic corkscrew-like motion performed by means of a single polar unsheathed flagellum at one or both ends of the cell. However, cells of some species are nonmotile (*Campylobacter gracilis*) or have multiple flagella (*Campylobacter showae*).

Campylobacter species grow under microaerobic atmosphere and have respiratory and chemoorganotrophic type of metabolism. Some species require anaerobiosis for optimal growth or grow only microaerobically in presence of fumarate or hydrogen as an electron donor (*Campylobacter concisus*, *Campylobacter curvus*, *Campylobacter rectus*,

Campylobacter mucosalis, *C. gracilis* and *C. showae*). Menaquinone-6 and methyl-substituted menaquinone-6 have been reported as major respiratory quinones in *Campylobacter* species.

Energy is obtained from amino acids or tricarboxylic acid cycle intermediates, not from carbohydrates which are neither fermented nor oxidized. *Campylobacter* spp. grow at 35-37ºC, not at 4ºC. Several species are able to grow at 42ºC (thermophilic or, more accurately, thermotolerant *Campylobacter* species). Gelatin, casein, starch and tyrosine are not hydrolyzed. Oxidase activity is present in all species except *C. gracilis*. Only *Campylobacter jejuni* and *Campylobacter avium*, as well as some strains of *Campylobacter curvus*, are able to hydrolize hippurate.

The G+C of the DNA ranges from 29 to 47 mol%. Plasmids have been described in a variety of species including *C. jejuni*, *Campylobacter coli*, *Campylobacter upsaliensis*, *C. mucosalis*, *Campylobacter hyointestinalis* and *C. fetus*. Tetracycline and kanamycin resistance were shown to be plasmid-mediated and transferable.

The type species of the *Campylobacter* genus is *Campylobacter fetus* (formerly *Vibrio fetus*, Smith and Taylor, 1919), and at present, there are 23 validly named *Campylobacter* species (Table 1). Within the genus *Campylobacter*, the group of thermophilic species, includes at present *C. jejuni*, *C. coli*, *Campylobacter helveticus*, *C. upsaliensis*, *Campylobacter lari*, *Campylobacter insulaenigreae*, *C. avium*, *Campylobacter peloridis, Campylobacter volucris* and *Campylobacter subantarcticus*, form a distinct 16S rRNA phylogenetic subcluster. *C. fetus* and *C. hyointestinalis* are also close relatives, while the remaining species form a loose assemblage of predominantly hydrogen-requiring organisms.

Table 1: List of the 23 *Campylobacter* species, isolation source(s) and human disease association.

***Campylobacter* species (Reference of the first description)**		**Source(s)**	**Pathogenicity (possible) for humans**
C. avium	(Rossi et al., 2009)	Poultry	?
C. canadensis	(Inglis et al., 2007)	Birds (*Grus americana*)	?
C. coli (formerly *Vibrio coli*)	(Doyle, 1948)	Pigs, poultry, cattle, sheep, birds, human	Gastroenteritis, septicaemia, abortions
C. concisus	(Tanner et al., 1981)	Human	Peridontal disease and gastroenteritis
C. curvus (formerly *Wolinella curva*)	(Tanner et al., 1984)	Human	Peridontal disease and gastroenteritis
C. fetus (formerly *Vibrio fetus*)			
subsp. *fetus*	(Smith and Taylor, 1919)	Cattle, sheep	Septicaemia, gastroenteritis, abortions, meningitis
subsp *venerealis*	(Florent, 1959)	Cattle	Septicaemia
C. gracilis (formerly *Bacteroides gracilis*)	(Tanner et al., 1981)	Human	Peridontal disease, abscesses, empyema
C. helveticus	(Stanley et al., 1992)	Cats, dogs	?
C. hominis	(Lawson et al., 2001)	Human	commensal species of the intestine
C. hyointestinalis			
subsp. *hyointestinalis*	(Gebhart et al., 1985)	Pigs, cattle, hamsters, deer, human	Gastroenteritis
subsp. *lawsonii*	(On et al., 1995)	Pigs	?
C. insulaenigrae	(Foster et al., 2004)	Marine mammals, human	Septicaemia and enteritis
C. jejuni (formerly *Vibrio jejuni*)			
subsp. *jejuni*	(Jones et al., 1931)	Poultry, pigs, cattle, sheep, dogs, cats, water, birds, rabbits, insects	gastroenteritis, septicaemia, meningitis, abortions, proctitis, GBS
subsp. *doylei*	(Steele and Owen, 1988)	Human	Gastroenteritis, septicaemia
C. lanienae	(Logan et al., 2000)	Cattle, pigs, humans	?
C. lari (formerly *C. laridis*)			
subsp. *lari* (formerly *C. lari*)	(Benjamin et al., 1983)	Birds, poultry, water, dogs, cats, mokeys, horses, seals, mussels	Gastroenteritis, septicaemia
subsp. *concheus*	(Debruyne et al., 2009c)	Molluscs, human	?
C. mucosalis (formerly *C. sputorum* ssp. *mucosalis*)	(Lawson and Rowland, 1974)	Pigs	?
C. peloridis	(Debruyne et al., 2009c)	Molluscs, human	?
C. rectus (formerly *Wolinella recta*)	(Tanner et al., 1981)	Human	Periodontal disease
C. showae	(Etoh et al., 1993)	Human	Periodontal disease
C. sputorum			
subsp. *sputorum* (formerly *Vibrio sputorum*)	(Prévot, 1940)	Cattle, sheep, pigs, human	Abscesses, gastroenteritis
subsp. *bubulus* (formerly *Vibrio bubulus*)	(Florent, 1953)	Cattle, sheep, pigs, human	?
C. subantarcticus	(Debruyne et al., 2009a)	Birds	?
C. upsaliensis	(Sandstedt and Ursing, 1991)	Dogs, cats, human	gastroenteritis, septicaemia, abscesses, abortions
C. ureolyticus (formerly *Bacteroides ureolyticus*)	(Jackson and Goodman, 1978)	Human, horses	Necrotic or gangrenous lesions
C. volucris	(Debruyne et al., 2009b)	Birds (*Larus ridibundus*)	?

There is no simple gold standard for the routine isolation of all *Campylobacter* species. Simultaneous application of a microareobic atmosphere containing hydrogen with a filtration method and a selective base medium is methodologically the optimal solution.

The first isolation method involves filtration of the cells through membrane filters with a pore size of 0.45, 0.65 or 0.8 µm using a nonselective agar medium or broth medium. For selective isolation, different selective media has been described, some using blood agar, others a blood-free agar base basal medium. However, none of these selective supplements supports growth of all of the *Campylobacter* species.

Incubation at 42ºC will increase selectivity by the elimination or inhibition of many, but not all, other intestinal organisms and is particularly useful for the isolation of the thermotolerant campylobacters. It will, however, inhibit growth of some other *Campylobacter* species.

Campylobacter species have been isolated from different sources (Table 1). They have been found in the reproductive organs, intestinal tract, and oral cavity of human and different animals.

Some species are pathogenic for humans and animals. In particular, *C. jejuni* is known worldwide as a major foodborne enteropathogen, with meat, milk and water as most important vehicles, causing as much enteric disease in man as *Salmonella* and *Shigella*. It infects people of all ages, more frequently diagnosed in children than adults and with higher incidence in summer season. The organism is found in the intestinal tract of a wide variety of animals and enteric *C. jejuni* associated disease has been reported also in animals. *C. jejuni* infection can trigger Guillain-Barré syndrome (an autoimmune disorder affecting the peripheral nervous system) due to similarity between its lipooligosaccharide and human gangliosides.

Other members of the thermophilic species group are *C. coli*, *C. lari, C. insulaenigrae* and *C. upsaliensis*, all of which are known to cause enteritis in human and to be carried in the intestinal tract of a variety of animals.

C. rectus and other oral species are associated with periodontal disease in humans and may cause infections in other part of the body.

C. fetus and *C. hyointestinalis* are primarily important in veterinary medicine, causing sporadic abortion and reproductive problems in cattle (*C. fetus* ssp. *fetus*, *C. fetus* ssp. *venerealis*) and sheep (*C. fetus* ssp. *fetus*) and enteric diseases in pigs (*C. hyointestinalis*). *C. fetus* ssp. *fetus* also can cause septicemia, meningitis, abortions and enteritis in humans, and *C. fetus* ssp. *venerealis* also can cause septicemia in humans.

The genus *Helicobacter* (Goodwin et al., 1989) [adapted from Euzéby, 2010b, On et al., 2005 and Garrity et al., 2005a)]

The *Helicobacter* genus consists of a group of microorganisms that colonize the mucus layer covering the epithelial surface of the gastrointestinal tract, i.e. oral cavity, stomach, cecum and colon, and internal organs, i.e. liver, of humans and a variety of animal species (Table 2).

Members of the genus *Helicobacter* are Gram-negative and cells may be curved, spiral, helical or fusiform shaped with 0.2-1.2 x 1.5-10 μm, although variant forms include short or tapered rods, and they are motile by means of flagella. The fact that flagella can be sheathed or unsheathed is one characteristic that helps to differentiate different species of *Helicobacter.* Spiral cells may be tightly or loosely wound depending on the species, and on the age and condition of the culture. Cells in old cultures or those exposed to air form coccoid bodies.

Helicobacter species grow under microaerobic atmosphere (with or without hydrogen) and have respiratory and chemoorganotrophic type of metabolism. Microaerobic conditions with hydrogen appear to enhance growth on culture media but it is not essential. In opposition to *Campylobacter* species, *Helicobacter* spp. do not have a complete Tricarboxylic Acid Cycle. Moreover, these species are assaccharolytic when the sugar catabolism is tested by standard methods, although it has been seen that *Helicobacter pylori* is able to perform glucose oxidation. They do not hydrolyze gelatin, starch, casein and tyrosine, and are negative for Methyl red and Voges-Proskauer. All species have oxidase activity, and most strains produce catalase.

The type species of the *Helicobacter* genus is *Helicobacter pylori* (formerly *Campylobacter pylori*, Marshall et al., 1984), and there are currently 10 validated *Helicobacter* species isolated from gastric tissue and 22 validated enterohepatic species (Tables 2). Moreover, some *Helicobacter* species may be commonly (*Helicobacter aurati*) or occasionally (*Helicobacter bilis* and *Helicobacter muridarum*) isolated from both gastric and enterohepatic sites. There is, in addition, a growing list of candidate and unvalidated species (data not shown).

Table 2: List of the *Helicobacter* species: 10 gastric and 22 enterohepatic species, isolation source(s) and human disease association.

	***Helicobacter* species (Reference of the first description)**		**Source(s)**	**Pathogenicity (possible) for humans**
Gastric	*Helicobacter acinonychis*	(Eaton et al., 1993)	Cheetahs	?
	Helicobacter baculiformis	(Baele et al., 2008b)	Cats	?
	Helicobacter bizzozeronii	(Hänninen et al., 1996)	dogs, cats, human	Gastritis
	Helicobacter cetorum	(Harper et al., 2002)	dolphins, whales, humans	?
	Helicobacter cynogastricus	(Van den Bulck et al., 2006b)	Dogs	?
	Helicobacter felis	(Paster et al., 1991)	cats, dogs, cheetahs, humans	Gastritis
	Helicobacter mustelae	(Fox et al., 1988)	Ferret, minks	?
	Helicobacter pylori	(Marshall et al., 1985)	human, cats, primates non-humans	Gastritis, MALT lymphoma, adenocarcinoma
	Helicobacter salomonis	(Jalava et al., 1997)	dogs, humans	Gastritis
	Helicobacter suis	(Baele et al., 2008a)	pigs, humans, primates non-humans	Gastritis
Enterohepatic	*Helicobacter anseris*	(Fox et al., 2006)	Goose (*Branta canadensis*)	?
	Helicobacter aurati	(Patterson et al., 2000)	Hamsters	?
	Helicobacter bilis	(Fox et al., 1995)	rodents, dogs, cats, sheep, pigs, humans	Hepatic diseases
	Helicobacter brantae	(Fox et al., 2006)	Goose	?
	Helicobacter callitrichis	(Won et al., 2007)	Marmoset	?
	Helicobacter canadensis	(Fox et al., 2000)	humans, gooses	Enteritis
	Helicobacter canis	(Stanley et al., 1993)	dogs, cats, humans	Enteritis, bacteraemia
	Helicobacter cholecystus	(Franklin et al., 1996)	Hamsters	?
	Helicobacter cinaedi	(Totten et al., 1985)	humans, hamsters, dogs, cats, monkeys, rats, foxes	Enteritis, bacteraemia, cellulitis
	Helicobacter equorum	(Moyaert et al., 2007)	Horses	?
	Helicobacter fennelliae	(Totten et al., 1985)	Humans	Proctitis, enteritis
	Helicobacter ganmani	(Robertson et al., 2001)	Mouses	?
	Helicobacter hepaticus	(Fox et al., 1994)	mouses, humans	Hepatic diseases
	Helicobacter marmotae	(Fox et al., 2002)	martmots, cats	?
	Helicobacter mastomyrinus	(Shen et al., 2005)	Rodents	?
	Helicobacter mesocricetorum	(Simmons et al., 2000)	Hamsters	?
	Helicobacter muridarum	(Lee et al., 1992)	rats, mouses	?
	Helicobacter pametensis	(Dewhirst et al., 1994)	birds, pigs, cats	?
	Helicobacter pullorum	(Stanley et al., 1994)	poultry, humans	Gastroenteritis
	Helicobacter rodentium	(Shen et al., 1997)	Mouses	?
	Helicobacter trogontum	(Mendes et al., 1996)	rats, pigs, sheep	?
	Helicobacter typhlonius	(Franklin et al., 2001)	Mouses	?

Within gastric helicobacters, *Helicobacter pylori* is the most well-known species. *H. pylori* is established as the primary cause of gastritis and peptic ulceration in humans and has been recognized as a major risk factor for mucosa-associated lymphoid tissue (MALT) lymphoma and adenocarcinoma. The prevalence of this species infection shows large geographical variations and in general it is around 80% in developing countries, whereas in industrialized countries, the prevalence is generally under 40% (Kusters et al., 2006). *H. pylori* is transmitted from human-to-human and it is mostly acquired in early childhood, most probably from another family member. Although this *Helicobacter* leads a quiet life in the gastric mucosa for the rest of the host's life, about 10–20% of people carrying *H. pylori* may develop gastrointestinal symptoms of infection (Kusters et al., 2006).

In gastric biopsies of a minority of patients with upper gastrointestinal symptoms (0.17-2.3%) long tightly coiled spiral rods, provisionally named as "*H. heilmannii",* can be observed (Baele et al., 2009). In contrast of the human-human transmission described for *H. pylori*, it has been suggested that "*H. heilmannii*" group [composed by "*H. heilmannii*' type one (*Helicobacter suis*) and *H. heilmannii*' type two (*Helicobacter felis*, *Helicobacter bizzozeronii,* and *Helicobacter salomonis*)] is transmitted by animals (Meining et al., 1998; Haesebrouck et al., 2009). Nevertheless, several groups have unsuccessfully attempted to recover "*H. heilmannii*' from human gastric biopsies in vitro. Up to now, *H. bizzozeronii* is the only "*H. heilmannii*' species successful cultivated from human gastric samples (Haesebrouck et al., 2009) on two different occasions: from a Danish patient in 1999 (Jalava et al., 2001) and from a Finnish patient with severe dyspeptic symptoms and chronic active gastritis in 2008 (Kivistö et al., 2010).

Transmission of gastric *Helicobacter* species between individuals is likely to occur via oral–oral contact, whereas enterohepatic *Helicobacter* species, that are shed in the feces of infected subjects, it is generally assumed that fecal–oral spread is the main route of natural acquisition of these *Helicobacter* infections (Moyaert et al., 2008).

Several enterohepatic *Helicobacter* species have been detected in humans, comprising *H. canadensis* (Fox et al., 2000; Laharie et al., 2009), *H. pullorum* (Laharie et al., 2009; Steinbrueckner et al., 1997), *H. fennelliae* (Hsueh et al., 1999), *H. cinaedi* (Matsumoto et al., 2007) and *H. canis* (Prag et al., 2007).

III. Lagomorphs

Lagomorpha. The order Lagomorpha is composed by two families: *Leporidae* (hares and rabbits) and *Ochotonidae* (pikas) (Matthee et al., 2004). These animals are distributed worldwide either as native or introduced species, showing their flexibility in adapting in different habitats (Chapman and Flux, 1990; Lombardi et al., 2007). Main external morphological differences within leporids, rabbits and hares, can be observed in Figure 4. Although both rabbits and hares share the general characteristic of caecal fermentation of digesta, hares have a smaller stomach and caecum as a proportion of body weight than the rabbit (Stott, 2008).

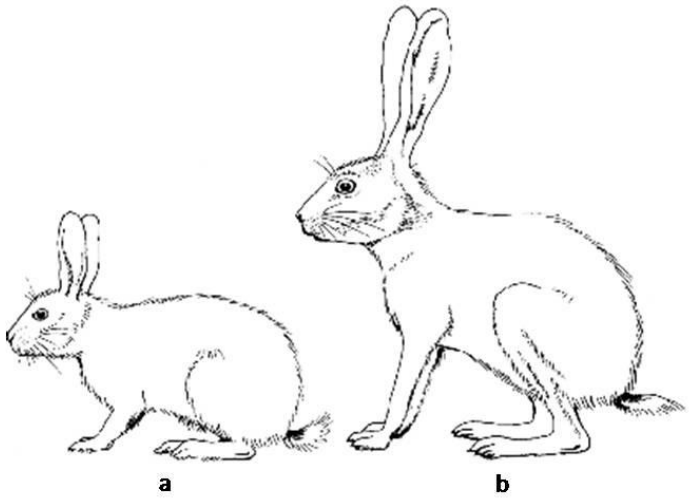

Figure 4: Comparative outlines of the rabbit (**a**) and hare (**b**). Main differences between genera are in leg and ear length. Adapted from Myers et al. (1989).

Leporids are herbivores and practive caecotrophy (Hirakawa, 2001). In fact, the gastrointestinal physiology is a very complex system that separates the digestible from the indigestible components of the diet in the proximal colon (Davies and Davies, 2003; Harcourt-Brown, 2002a). The caecum size of rabbit, is proportionally the largest of any mammal. It composes 40-60% of the total volume of the gastrointestinal tract and it is twice the length of the abdominal cavity (Davies and Davies, 2003; Harcourt-Brown, 2002a). A simplified diagram is presented in Figure 5, where it is possible to notice as well the respective topography of this tract.

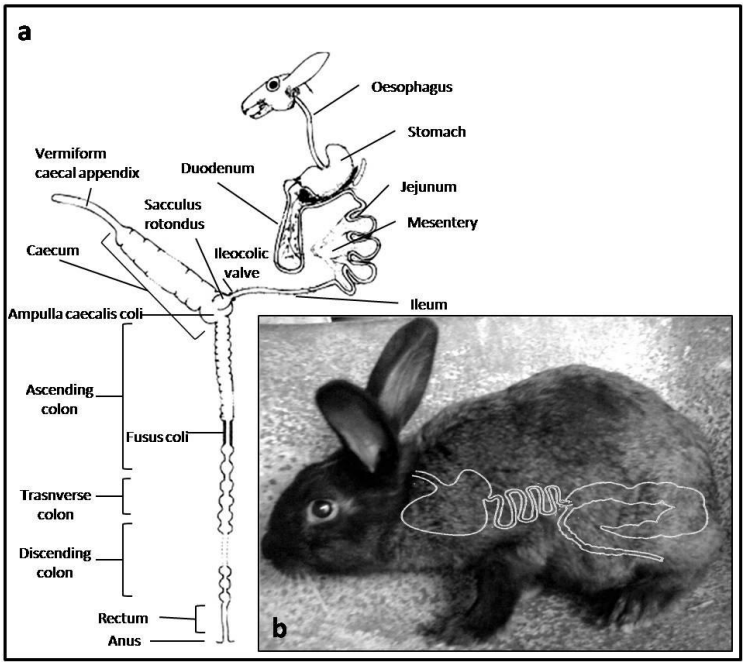

Figure 5: Simplified schematic diagram of the anatomy of gastrointestinal of the rabbit **a** (adapted from Harcourt-Brown, 2002a) and respective topography **b**.

Production. The leporids economic importance, especially in Europe, is the meat, fur and game. In addition, leporids play also a role in an ecological point of view, since they can serve either as prey for some predator species or by contributing to seed dispersal (Delibes-Mateos et al., 2007; Izhaki and Néeman, 1997). Nevertheless, rabbits are becoming important as pet animals (Wagner and Fehr, 2007) and are widely used in research as laboratory animals - in Europe over 260 000 rabbits each year are used in research (Seaman et al., 2008). In what regards meat consumption, rabbits have a bigger role than hares, for their worldwide production as food animal (Chapman and Flux, 1990; Myers et al., 1989). Nevertheless, leporid hunting date back thousands of years in Europe and even today it is important in several countries worldwide (Chapman and Flux, 1990; Cobos et al., 1995; Flux and Aneermann, 1990). Rabbit rearing in the world is mainly directed towards meat production. There are two types of production: industrial (intensive system) and rural (extensive system). Although intensive rearing systems are the most widespread, the rural production is still a common practice in different part of the world (Mendoza et al., 2004). According to FAO statistics, in 2008 China was the world leading production country of rabbit meat with 36% share, followed by Bolivarian Republic of Venezuela with 26% share, and Italy with 13% share of the world production. In Europe, Italy represents the 49% of the European production, followed by Spain with the 15% and France with the 11% (Food and Agriculture Organization of the United Nations, 2008). The Italian production can be seen in Figure 6. According to data from Italian National Union

"Associazioni di Produttori Avi-Cunicoli" (Coniglionline, 2010), in 2005 Italy produced 100.000.000 rabbits, with 3.700.000 total breeders, in which 68% breeders were from rural farm system production, whose production is addressed mainly to the domestic consumption.

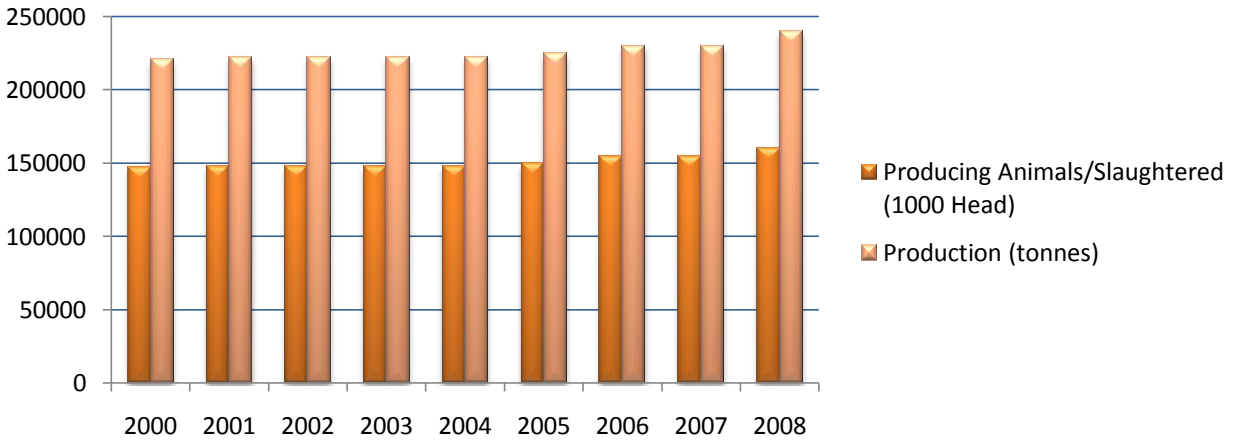

Figure 6: Rates of producing rabbits and slaughtered, and production in tones of Italy from 2000 until 2008. Data retrieved from the FAO Statistics database (Food and Agriculture Organization of the United Nations, 2008).

In what concerns consumption per person, Italy leads with around 4.6kg/year, followed by France with 3kg/person and Spain with more than 2Kg/person. In the other countries of the EU, the interest for the rabbit-rearing is rather limited and the consumption per person does not exceed 1 kg (Coniglionline, 2010).

In the intensive meat rabbit industry, control of bacterial disease is a major production challenge (Eady et al., 2007). In Europe, where rabbit farming is well established, approaches to disease control have relied on environmental hygiene, requiring large capital investment in housing and routine inclusion of antibiotics in rabbit feed (Eady et al., 2007; Rodriguez-Calleja et al., 2006; Rodriguez-Calleja et al., 2004). Although microbial contamination of raw meat has always been an important issue for food safety, available information for rabbit meat microbiology is very scarce (Kohler et al., 2008; Rodriguez-Calleja et al., 2006) thus rabbit meat is considered a safe product, since has not been incriminated in outbreaks of foodborne disease (Rodriguez-Calleja et al., 2004). Nevertheless, rabbit has the potential to carry food-poisoning organisms derived from different sources, like gut content or skin (Kohler et al., 2008; Rodriguez-Calleja et al., 2004). Management systems with a reliance on the use of antibiotics are under increasing

inspection due to the risk of microbial antibiotic resistance spreading to human pathogens (Eady et al., 2007).

Rabbit intestinal microbiota. The caecum is a finely balanced ecosystem composed of a variety of microorganisms (Harcourt-Brown, 2002b) and the digestive physiology of rabbits is influenced by the bacterial population in the caecum (Abecia et al., 2007). The type of bacteria which are necessary in this environment, with the specific physiological conditions such as those of the rabbit caecum (e.g., rate of passage, characteristics of the input substrate, content density) may differ significantly from those necessary in other herbivorous digestive tracts (Abecia et al., 2005). In a recent metagenomic study (Abecia et al., 2005), it was reported that the rabbit caecal microbiota is highly diverse compared to other herbivorous gut microbiota. Indeed, the majority of taxa detected in the Abecia and colleagues (2005) study represented not just novel species, but also new taxonomic lineages. Various bacterial species are normally found in rabbit cecum such as *Bacteroides* spp., streptococci, *Endophorus* spp., *Acuformis* spp., *Eubacterium* spp., *Fibrobacter* spp., *Ruminococcus* spp., *Clostridium*, *Peptococcus*, *Peptostreptococcus*, and *Fusobacterium* spp., and many unidentified anaerobic species (Crociani et al., 1984; Fann and O'Rourke, 2001; Harcourt-Brown, 2002b; Monteils et al., 2008). Moreover, coliform bacteria are rarely isolated from normal rabbit cecal contents (Blanco et al., 1994; Camguilhem and Milon, 1989).

The microbiota of the rabbit is affected by several factors such as age, diet and antibiotic usage, thus leading to gastrointestinal disorders (Fann and O'Rourke, 2001; Harcourt-Brown, 2002b). More frequently happens in intensive production systems where animals are under stress and eat artificial as well as medicated diets that may lead to the proliferation of pathogens (Abecia et al., 2005, 2007; Harcourt-Brown, 2002b; Lennox and Kelleher, 2009). The main bacterial pathogens responsible for these diseases are: *Salmonella enterica*, *Escherichia coli, Clostridum* spp. and *Lawsonia intracellularis* (Harcourt-Brown, 2002b; Lennox and Kelleher, 2009).

Epsilon-proteobacteria in Leporids. Although some members of ε-Proteobacteria, in particular *Campylobacter* and *Helicobacter* species, are frequently found in the gastrointestinal tracts of animals, so far there is a paucity of data concerning the presence of these bacteria in leporids. Nevertheless, *Campylobacter jejuni* have been isolated at low rates in hares (de Boer et al., 1983; Rosef et al., 1983) and in rabbits (Kohler et al., 2008;

Little et al., 2008; Meanger and Marshall, 1989; Prescott and Bruin-Mosch, 1981; Weber et al., 1982). In addition, only one study refers the presence of *C. coli* in 1% of the hares sampled (Wahlström et al., 2003). No other studies refer to the presence of other *Campylobacter* species in leporids, with the exception of one French study (Reynaud et al., 1993), that reports the occurence and isolation of a *Campylobacter*-like organism in high quantities from rabbits. In addition, few authors observed a high amount of spirilar forms in the caecum of rabbits probably referable to *Campylobacter*-like organisms, however without isolation (Hill, 1985; Ross et al., 1987, 1989).

Regarding *Helicobacter* species, so far there are no reports about the occurrence of these species in hares; however, there are two reports of occurrence in rabbits (Van den Bulck et al., 2005; Van den Bulck et al., 2006a), that presented results of the low presence of "*Helicobacter heilmannii*" and *H. canadensis* or *H. pullorum*.

In a recent study (Chamorro et al., 2010), that studied the effect of dietary supplementation of glutamine and glutamine and arginine in the intestinal microbiota profile of rabbits, authors report a frequency of detection of both *Campylobacter* and *Helicobacter* species by PCR-RFLP, although the authors were not performing isolation.

It is important to understand and study the occurrence of *Campylobacter* and *Helicobacter* in leporids, since some species of these genera have a zoonotic character. Leporids are important not only as food animals but also as pet animals, thus it is a priority to understand if they can be associated with zoonoses caused by these microorganisms.

Zoonoses. Leporids have been associated with a discrete number of zoonoses. Concerning wild leporids, the main bacterial diseases transmitted to humans are caused by *Yersinia pseudotuberculosis* (pseudotubercullosis), *Pasteurella multocida* (pasteurellosis), *Brucella* species (brucellosis), *Francisella tularensis* (tularemia), *Yersinia pestis* (plague) and *Borrelia burgdorferi* (Lyme disease) (Chomel, 1992; Harcourt-Brown, 2002c; Orloski and Lathrop, 2003; Telford and Spielman, 1989; Wibbelt and Frölich, 2005).

Regarding rabbits as pet animals, the diseases of major public health importance are rarely encountered and the risk is negligible. However, rabbits can bite, even though it is uncommon, and can inflict painful scratches that can become infected; moreover, owners can develop allergy to rabbit dander (Chomel, 1992; Harcourt-Brown, 2002c). Nevertheless, direct zoonotic bacterial transmission from domesticated rabbits to humans have been reported with the bacterial agents such as *Pasteurella multocida*, *Yersinia pseudotuberculosis*, *Bartonella alsatica*, *Bordetella bronchiseptica* and *Salmonella enterica*

(Chomel, 1992; Harcourt-Brown, 2002c; Raoult et al., 2006). Other diseases to which pet rabbits are susceptible, such as tularemia, are extremely rare and are more commonly transmitted to humans by wild animals (Chomel, 1992).

No viral zoonotic diseases are known that can be transmitted from leporids to humans; other zoonotic diseases caused by parasites and fungus have been reported, such as *Cheyletiella parasitovorax* (by handling infested animals), *Toxoplasma gondii* (by eating undercooked meat), and *Encephalitozoon cuniculi* (ingestion of spores that are shed in the urine of the infected animals) and dermatophytosis by *Trichophyton mentagrophytes* (Almeria et al., 2004; Chomel, 1992; Frolich et al., 2003; Harcourt-Brown, 2002c).

In what concerns food-borne diseases, the potential bacteria isolated from leporids meat and/ or carcasses are related to *Escherichia coli* EPEC, *Listeria* spp., *Campylobacter* spp., *Salmonella enterica* and *Sthaphylococcus aureus* (Kohler et al., 2008).

Nevertheless, it is also considered a zoonoses subject the worldwide emerging problem of antimicrobial resistance, since the spread of resistance microorganisms can occur, thus it is important to monitor the antibiotic resistance.

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Part 2: Experimental work

Chapter 1: *Campylobacter cuniculorum* sp. nov.

There is a paucity regarding the prevalence of *Campylobacter* species in leporids. Nevertheless, few studies (e.g. Ross *et al.,* 1987 and Reynault *et al.,* 1993) point to the presence of *Campylobacter*-like organisms (CLO) in high quantities in rabbit caeca.

In the present Chapter, the results of a polyphasic taxonomic investigation of eight strains of a CLO recovered from rabbits (*Oryctolagus cuniculus*) in Italy are shown. These isolates were obtained by using a broad spectrum of isolation methods, corroborating the importance of this approach in the understanding the prevalence of *Campylobacter* species in animal gut. The rabbit strains here indicated belong to a new *Campylobacter* species for which the name *Campylobacter cuniculorum* sp. nov., is proposed, with 150B^T (= LMG 24588^T = CCUG 56289^T) as the type strain.

Redrafted from:

Zanoni, R.G., Debruyne, L., Rossi, M., Revez, J., Vandamme, P. (2009). *Campylobacter cuniculorum* sp. nov. from rabbits. International Journal of Systematic and Evolutionary Microbiology. 59, 1666-1671.

Isolates. Eight *Campylobacter*-like unidentified isolates were recovered from the caecal contents of eight rabbits during routine bacteriological analysis. The isolates were obtained in 2005 (150B) and in 2007 (113/07, 114/07, 116/07, 117/07, 118/07, 119/07 and 120/07) from animals reared in intensive and rural farms of different Italian regions, thereby representing a temporally, geographically, and epidemiologically independent set of isolates. Isolations were made after 6-8 days of incubation at 37°C in a microaerobic atmosphere with hydrogen, on Nutrient Sheep Blood Agar [Nutrient Broth N°2 (Oxoid, LTD., Basingstoke, Hampshire, UK), with 1.5% Bacto Agar (Difco, BD-Becton, Dickinson and Company, Sparks, MD 21152, USA) and 5% sheep blood] plus Cefoperazone Amphotericin Teicoplanin Selective Supplement (CAT, Oxoid), on modified-Charcoal Cefoperazone Deoxycholate Agar (mCCDA, CM0739, Oxoid) and on Nutrient Sheep Blood Agar using a filter method (Zanoni et al., 2007). The microaerobic atmosphere with hydrogen was obtained by the gas replacement method using an anaerobic gas mixture (H_2 10%, CO_2 10%, N_2 80%) as described by Bolton et al. (1992).
After 6 days of incubation on Nutrient Sheep Blood Agar, colonies were 1-2 mm in diameter, greyish-green, flat with rough margins and slightly mucoid looking; sometimes resulted α-haemolytic and exhibited a tailing effect along the streak line. Cells were Gram negative, pleomorphic, typically sigmoid to allantoid in shape, 2.6±0.7 µm (mean±SD) in length and 0.3±0.1 µm in width when observed after Gram staining. Cells appeared coccoid after 10-12 days of incubation.

DNA extraction and PCR identification. Bacterial DNA was extracted by ChargeSwitch® gDNA Mini Bacteria Kit (Invitrogen Life Technologies, Carlsbad, CA). The strains were identified as *Campylobacter* using the genus-specific PCR described by Linton et al. (1996), but were not identified at the species level using species-specific PCR tests for *C. jejuni* and *C. coli* (Denis et al., 1999), *C. upsaliensis* and *C. helveticus* (Lawson et al., 1997), or *C. lari* (Linton et al., 1996).

16S ribosomal RNA. In order to establish the taxonomic position of the rabbit isolates, a phylogenetic analysis based on the sequences of 16S rRNA was carried out. The nearly complete 16S rRNA gene was amplified using universal primers p27f (5'-AGAGTTTGATCCTGGCTCAG-3') and p1492r (5'-TACGGCTACCTTGTTACGACT-5') and the PCR-amplified template was sequenced by primer walking (Primm s.r.l., Milan, Italy). Sequences were assembled with Vector NTI Software (Invitrogen) and then aligned in

BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) by ClustalW using publicly available *Campylobacter* reference sequences. The alignment was adjusted visually removing intervening sequence regions (IVS) and unknown bases and data were corrected for multiple base changes by the method of Jukes and Cantor (Jukes & Cantor, 1969). A phylogenetic tree was constructed in MEGA3 (http://www.megasoftware.net/) using the neighbour-joining method. Bootstrap analysis was performed with 1000 resembled data sets.

A fragment of 1283 bp of 16S rRNA gene was sequenced from each strain and a search of the NCBI database using MEGABLAST (http://www.ncbi.nlm.nih.gov/BLAST/) determined that the strains were most closely related to taxa within the genus *Campylobacter*, confirming their generic identification. The pairwise comparisons of 16S rRNA gene sequences showed that all rabbit isolates were genetically highly related, exhibiting 99.1% to 100% sequence similarity. Furthermore, the neighbour-joining dendrogram (Figure 7) indicated that all strains formed a robust clade (100% bootstrap support) which was clearly distinct from all other *Campylobacter* species. Pairwise sequence comparisons of strain 150B^T with the type strains of the most closely related species revealed similarities of 96.6%, 96.5% and 96.1% with *C. helveticus*, *C. jejuni* and *C. upsaliensis*, respectively.

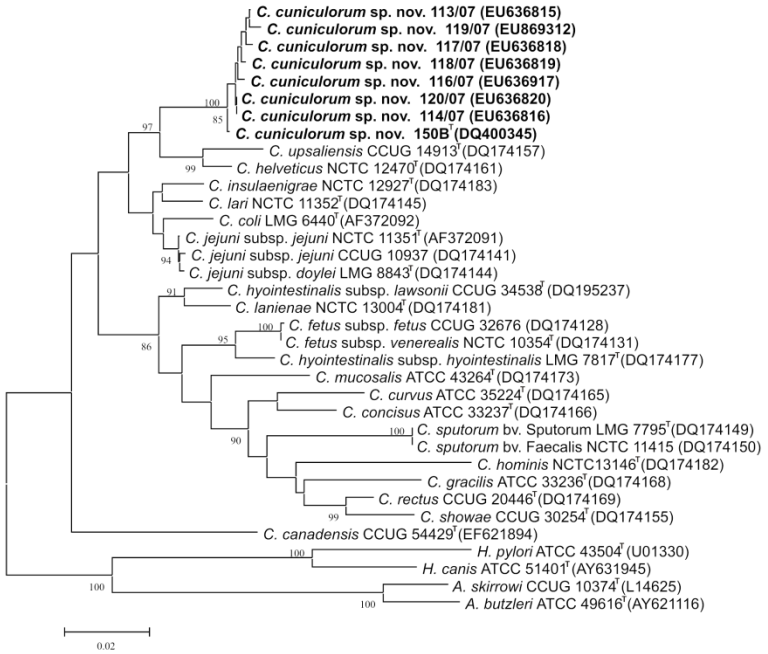

Figure 7. Unrooted tree, based on 16S rRNA gene sequences, showing the phylogenetic relationships of eight strains of *C. cuniculorum* sp. nov. Bar, 0.02 nucleotide substitutions per base. Numbers at nodes (≥ 85 %) indicating support for the internal branches within the tree obtained by bootstrap analysis (percentages of 1000 bootstraps).

Housekeeping genes *rpoB* and *groEL.* In view of the low 16S rRNA sequence divergence between the unidentified strains and other *Campylobacter* species, the phylogenetic relationships were further examined by *rpoB* (Korczak *et al.* 2006) and *groEL* (Kärenlampi *et al.* 2004) sequence analysis. Sequences were processed as described above. The phylogenetic trees based on the partial nucleotide *rpoB* and *groEL* of eight strains and the reference *Campylobacter* strains are shown in Figures 8 and 9, respectively.

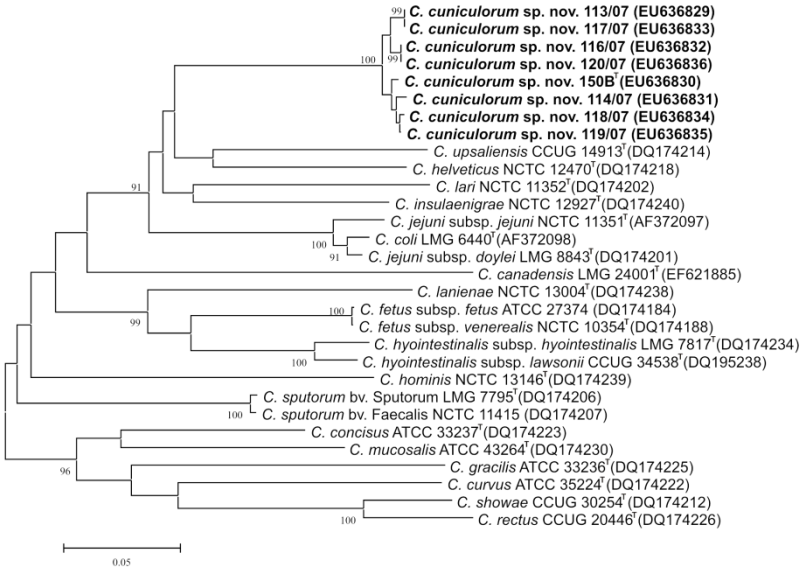

Figure 8. Unrooted tree, based on *rpoB* gene sequences, showing the phylogenetic relationships of eight strains of *C. cuniculorum* sp. nov. Bar, 0.05 nucleotide substitutions per base. Numbers at nodes (≥ 91 %) indicating support for the internal branches within the tree obtained by bootstrap analysis (percentages of 1000 bootstraps).

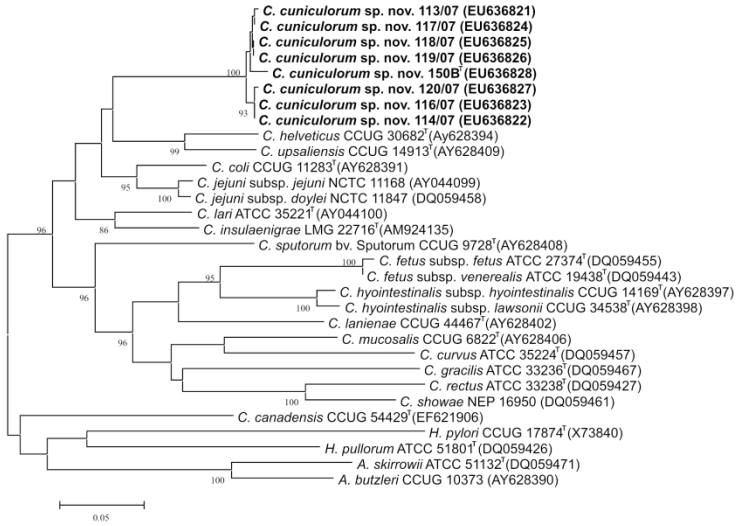

Figure 9. Unrooted tree, based on *groEL* gene sequences, showing the phylogenetic relationships of eight strains of *C. cuniculorum* sp. nov. Bar, 0.05 nucleotide substitutions per base. Numbers at nodes (≥ 86 %) indicating support for the internal branches within the tree obtained by bootstrap analysis (percentages of 1000 bootstraps).

In both trees, all unidentified strains clustered together in a tight clade clearly separated from all other *Campylobacter* species (100% bootstrap support). The *rpoB* sequence similarity within the clade of the rabbit strains ranged from 97.9 to 100%, while similarity values from 60.5 to 80.5% were obtained towards the other *Campylobacter* species. Likewise, pairwise comparisons of the *groEL* sequence similarities among the rabbit strains ranged from 97.7% to 100%, and values between strain 150B^T and other *Campylobacter* species were below 86%. Similarly to Korczak *et al.* (2006) and Kärenlampi *et al.* (2004), we observed a good congruence between *rpoB*, *groEL* and 16S rRNA gene sequence results since each of the phylogenetic trees showed a similar topology. However, compared to 16S rRNA, the *rpoB* and *groEL* sequence analysis showed lower interspecies similarity.

Although all sequence data demonstrated that the eight isolates represent a coherent taxon, whole-cell protein electrophoresis and DNA-DNA hybridisations were performed to further examine the relationships between the isolates.

Whole-cell protein electrophoresis. Whole-cell protein electrophoresis was performed after culturing strains on Mueller-Hinton agar supplemented with 5% horse blood at 37°C for 48h in microaerobic conditions with hydrogen. Protein extraction and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Pot *et al.* (1994). The similarity between the normalized whole-cell protein patterns was determined by the Pearson product moment correlation coefficient, after which clustering was performed by the Unweighted Pair Group Method with Arithmetic Averages (UPGMA), using GelCompar version 4.2 (Applied Maths, Belgium). For numerical analysis a variable dense band region (36.1 – 43.2 kDa) was excluded to increase species discrimination (Vandamme *et al.* 1991). As in many other *Campylobacter* species, a prominent protein band with variable position was present in the profiles of the rabbit isolates (Figure 10). Excluding this variable dense band region from the numerical analysis to enhance species level discrimination resulted in a clear grouping of the rabbit isolates.

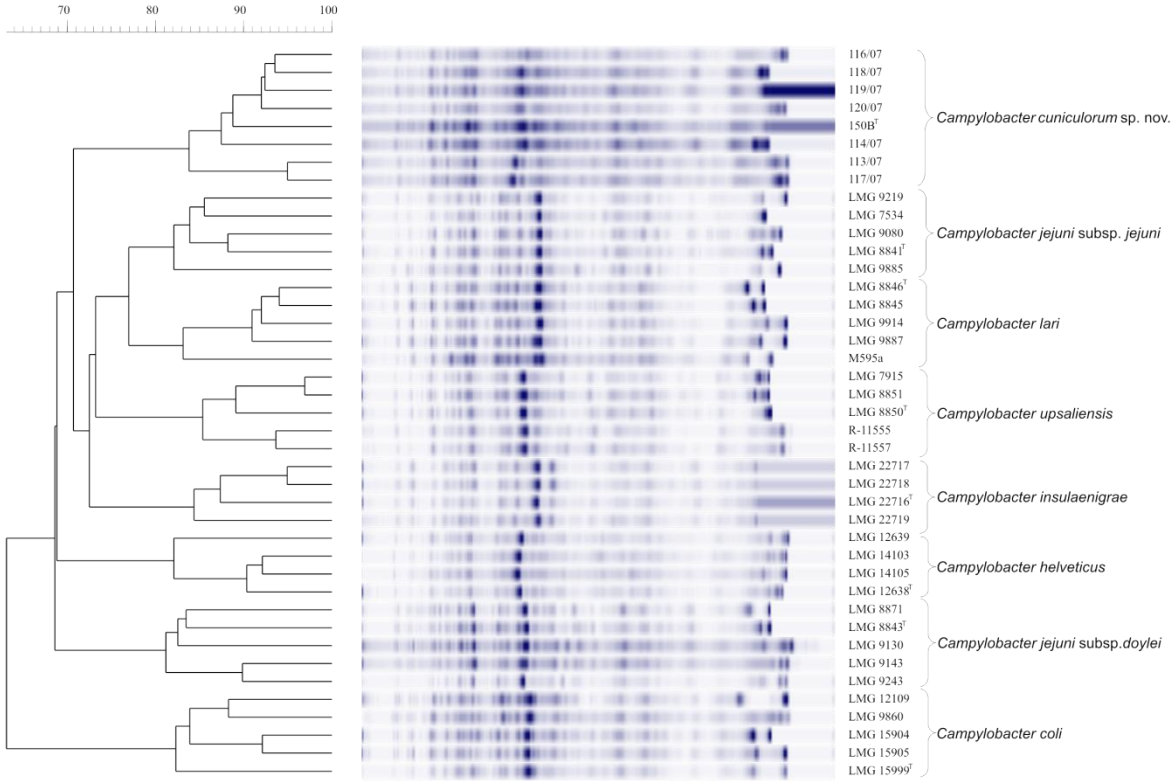

Figure 10. Dendrogram of the eight strains of *C. cuniculorum* sp. nov. based on UPGMA cluster analysis of 1D SDS PAGE cell protein profiles.

DNA-DNA hybridization. DNA–DNA hybridizations were subsequently performed between strains 150B^T and 116/07. For this purpose, DNA was extracted from 0.25–0.5 g (wet weight) cells as described by Pitcher *et al.* (1989). DNA–DNA hybridizations were performed with photobiotin-labelled probes in microplate wells (Ezaki *et al.*, 1989), using an HTS7000 Bio Assay Reader (PerkinElmer Inc, Waltham, Massachusetts 02451, USA) for the fluorescence measurements. The hybridization temperature was 30°C. A DNA–DNA hybridization value of 92% was calculated.

Phenotypic characteristics. The physiological characters of the novel species, determined using standard methods (On & Holmes, 1991a; 1991b; 1992; Ursing *et al.*, 1994; On *et al.*, 1996) along with all *Campylobacter* reference strains are represented in Table 3 and in the description of the novel species below. These characteristics allowed differentiation of the rabbit isolates from other established *Campylobacter* species.

Table 3. Phenotypic characteristics of *Campylobacter* species.

Taxa: 1, *Campylobacter cuniculorum* sp. nov.; 2, *Campylobacter canadensis;* 3, *Campylobacter coli*; 4, *Campylobacter concisus*; 5, *Campylobacter curvus*; 6, *Campylobacter fetus* ssp. *fetus;* 7. *Campylobacter fetus* ssp. *venerealis;* 8, *Campylobacter gracilis;* 9. *Campylobacter helveticus*; 10, *Campylobacter hominis*; 11, *Campylobacter hyointestinalis* ssp. *hyointestinalis*; 12, *Campylobacter hyointestinalis* ssp. *lawsonii;* 13, *Campylobacter insulanigrae*; 14, *Campylobacter jejuni* ssp. *doylei*; 15, *Campylobacter jejuni* ssp. *jejuni*; 16, *Campylobacter lanienae;* 17, *Campylobacter lari*; 18, *Campylobacter mucosalis*; 19, *Campylobacter rectus;* 20, *Campylobacter showae;* 21, *Campylobacter sputorum*; 22, *Campylobacter upsaliensis.* Data for reference species were taken from On *et al.* (1996), Foster *et al.* (2004), Vandamme *et al.* (2005) and Inglis *et al.* (2007): +, 90-100% of strains positive; -: 0-10% of strains negative; (+): 75-89% strains positive; (-): 11-25% of strains positive; V: 26-74% of strains positive. NA, No data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
α-Haemolysis	+	-	(-)	(-)	(-)	-	V	-	+	NA	V	V	NA	+	+	+	V	-	+	+	+	+
Oxidase	+	+	+	V	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	V	+	+
Catalase	+	V	+	-	-	+	(+)	V	-	-	+	+	+	V	+	+	+	-	(-)	+	V	-
Alkaline Phosphatase	-	-	-	V	V	-	-	-	-	-	-	(-)	NA	-	-	+	-	(+)	-	-	-	-
γ-Glutamyltranspeptidase	-	(+)	-	-	NA	-	NA	NA	-	NA	-	-	NA	-	-	NA	-	NA	NA	NA	-	-
Urease production	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	V	-	-	-	V*	-
Hippurate hydrolysis	-	-	-	-	(-)	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-
Indoxyl acetate hydrolysis	+	-	+	-	V	-	-	V	+	-	-	-	-	+	+	-	-	-	+	-	-	+
Nitrate reduction	+	V	+	(-)	+	+	+	(+)	+	-	+	+	+	-	+	+	+	-	+	+	+	+
Selenite reduction	-	NA	V	(-)	-	(+)	-	-	-	-	+	+	NA	-	+	+	+	-	+	+	+	+
TTC reduction	V	NA	+	-	V	-	-	-	-	NA	-	-	NA	V	+	NA	+	-	-	-	-	V
Trace H_2S/TSI	-	V	-	-	(-)	-	-	-	-	-	+	+	-	-	-	-	-	+	-	V	+	-
Growth at/in/on:																						
25ºC (microaerobic)	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37ºC (microaerobic)	+	+	+	+	V	+	+	-	+	+	+	+	+	+	+	+	+	+	-	V	+	+
42ºC (microaerobic)	(+)	+	+	(+)	V	(+)	-	V	+	(-)	+	+	-	-	+	+	+	+	(-)	V	+	+
37ºC (anaerobic)	-	+	-	+	+	(-)	V	+	-	+	-	+	-	-	-	+	-	+	+	+	+	-
37ºC (aerobic)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nutrient Agar	+	-	+	(-)	+	+	+	+	(+)	NA	+	+	NA	+	+	NA	+	+	(-)	V	+	+
CCD	(+)	+	+	(-)	(+)	+	+	V	+	NA	+	+	NA	+	+	NA	+	+	-	+	(+)	+
MacConkey agar	-	+	V	-	(+)	(+)	V	(+)	-	-	V	V	NA	-	-	+	-	(+)	-	+	V	-
1% glycine	-	V	+	(-)	+	+	-	+	V	+	+	V	+	(-)	+	-	+	V	+	V	+	+
2% NaCl	-	NA	-	(-)	V	-	-	V	-	+	-	-	-	-	-	-	(+)	+	V	+	+	-
1% bile	V	NA	(+)	-	-	+	+	-	+	NA	+	(+)	NA	+	+	NA	+	+	-	-	V	+
Requirement for H_2	-	-	-	+	+	-	-	+	-	+	V	V	NA	-	-	-	-	+	+	+	-	-
Resistance to:																						
Nalidixic Acid	V	V	-	(+)	+	+	V	V	-	V	+	+	+	-	-	+	V	(+)	(+)	-	(+)	-
Cephalotin	(+)	-	+	-	-	-	-	-	-	-	(-)	-	+	-	+	+	+	-	-	-	-	(-)

Transmission Electron Microscopy. Morphological characteristics were determined using transmission electron microscopy (TEM). For TEM, 48h old cells were negatively stained with 1% (w/v) phosphotungstic acid (Sigma Chemical Co., St. Luis, MO63178, USA) and examined using a ZEISS E 900 TEM microscope. Cells were spiral shaped, with bipolar unsheathed flagella; periplasmic fibers on the surface were not observed (Figure 11).

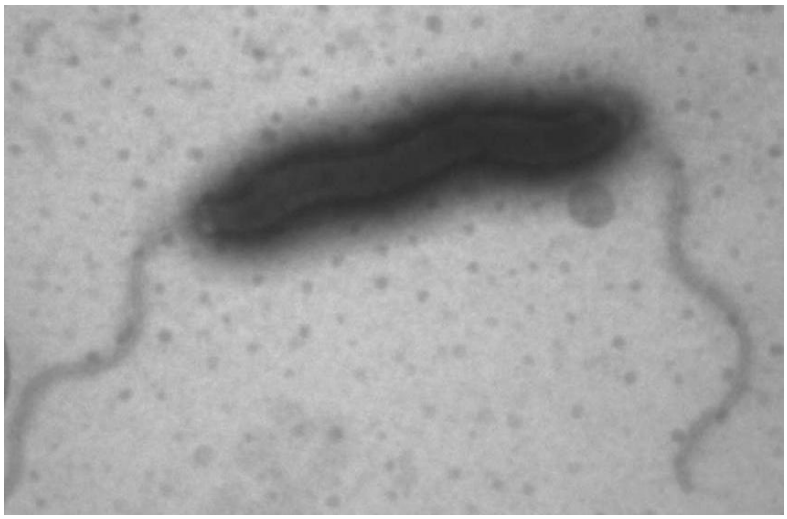

Figure 11. TEM image of *C. cuniculorum* sp. nov. strain 150B^T (Magnification: X10 000).

G+C content. For the determination of G+C content, DNA was enzymatically degraded into nucleosides as described by Mesbah & Whitman (1989). The nucleoside mixture was separated by HPLC using a Waters SymmetryShield C8 column maintained at 37°C. The solvent was 0.02M $(NH_4)H_2PO_4$ (pH 4.0) with 1.5 % acetonitrile. Non-methylated λ-phage DNA (Sigma) was used as the calibration reference. The DNA G+C content of strain 150B^T was 32.4 mol%. This value is within the range reported for genus *Campylobacter*, i.e. 29-47% (Vandamme *et al.,* 2005).

Conclusion. The results of the polyphasic taxonomic study conducted and described here indicate that the isolates recovered from caecal contents of rabbits represent a homogenous novel species within the genus *Campylobacter*, for which we propose the name *Campylobacter cuniculorum* sp. nov.

Description of *Campylobacter cuniculorum* sp. nov.

(cu.ni.cu.lo'rum. L. gen. pl. n. cuniculorum, of rabbits)

Cells are spiral, Gram negative rods, motile, 0.2-0.4 µm wide and 1.9-3.3 µm long, possessing a single flagellum in both poles. After subculturing on Nutrient Sheep Blood agar colonies are greyish-green in colour, flat with rough margins and slightly mucoid-looking; smooth, α-haemolytic, 1-2 mm in diameter at 37°C after 72-96h under microaerobic conditions. Colony appearance on modified-Charcoal Cefoperazone Deoxycholate Agar (mCCDA) and Cefoperazone Amphotericin Teicoplanin Selective Medium (CAT) is similar to that on Nutrient Agar, but growth on the first media is slightly restricted. The bacterium is strictly microaerobic, able to grow at 37°C and most strains at 42°C, but not at 25°C, or under anaerobic or aerobic conditions. Hydrogen is not required for growth. Oxidase and catalase are produced, but not urease, γ-glutamyltranspeptidase and alkaline phosphatase. Strains hydrolyse indoxyl acetate but not hippurate, reduce nitrate but not selenite; some strains reduce triphenyl-tetrazolium chloride (TTC), growth on Nutrient Agar without blood but not on MacConkey. No growth occurs in the presence of 1% (w/v) glycine and 2% (w/v) NaCl and only few strains grow in the presence of 1% (w/v) bile. Most strains are resistant to nalidixic acid (30 µg) and cephalothin (30 µg) by disk diffusion test.

Pathogenicity unknown. Strains have been recovered from rabbit caecal contents. The type strain is 150B^T (= LMG 24588^T = CCUG 56289^T) which was isolated from a rabbit in 2005.

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Chapter 2: Occurence of *Campylobacter* spp. and *Helicobacter* spp. in leporids

Campylobacter and *Helicobacter* species are frequently found in the gastrointestinal tracts of animals, however there is a paucity regarding the prevalence of these genera in leporids. Since some bacterial species of these genera are considered as zoonotic agents, it is important to monitor the prevalence of these microorganisms.

The objectives laying in this Chapter is to define the occurence of *Campylobater* spp. and *Helicobacter* spp. in leporids epidemiologically not correlated from the cecal contents of farmed and/or wild animals from Italy, Portugal and Denmark.

Partial data has been presented at the 9th World Rabbit Congress:

Revez, J.; Rossi, M.; Renzi, M.; Zanoni, R. G. Occurrence of *Campylobacter* spp. in Italian rabbit farms. *in* Proceedings of the 9th World Rabbit Congress - Pathology and Hygiene section. Verona, Italy, 10-13 June 2008.

Introduction

The consumption of rabbit and hare (as small game animal) meat is considerably high not only in south Europe (Malta, Italy, Portugal, France, Spain and Greece), but also in China and in several countries of America and Middle East. According to FAO statistics, in 2008, China was the world leading production country of rabbit meat with 36% share, followed by Bolivarian Republic of Venezuela with 26% share, and Italy with 13% share of the world production (Food and Agriculture Organization of the United Nations, 2008). Rabbit meat is considered a safe product, since has not been incriminated in outbreaks of foodborne disease. Nevertheless, rabbit has the potential to carry food-poisoning organisms derived from different sources (Rodríguez-Calleja et al., 2004). Likewise hare, as small game animal, may harbour many zoonotic agents, and as an example tularaemia are one of the most serious and dangerous under conditions of careless, handling of affected animals as well as foodborne diseases (Deutz and Kofer, 2000; Müller et al., 2007). Furthermore, rabbits are markedly gaining importance as pet animals (specially in North Europe and USA) and since they can often live in intimate contact with the adoptive family, precautionary measures are necessary to prevent zoonotic transmission of pathogens (Mani and Maguire, 2009; Van den Bulck et al., 2005).

So far there is a paucity of data concerning *Campylobacter* and *Helicobacter* isolation/detection from leporids. *Campylobacter* and *Helicobacter* species are frequently found in the gastrointestinal tracts of animals and some species of these genera are ascribed to have a zoonotic character.

Regarding *Campylobacter* species, *Campylobacter jejuni* and *Campylobacter coli* are the best recognized species to cause gastrointestinal diseases to humans; however, several other species have been recognized as human pathogens, including *Campylobacter upsaliensis, Campylobacter fetus* and *Campylobacter lari*. There are few more reports of the isolation of *Campylobacter* species from rabbits. *C. jejuni* have been isolated occasionally from healthy and diarrheic animals (Kohler et al., 2008; Little et al., 2008; Prescott and Bruin-Mosch, 1981; Weber et al., 1982). However, there is a strong disagreement regarding the occurrence of spiral bacteria in the caecum of these

animals, given that some authors reported the presence of *Campylobacter*-like organisms in high quantities (Reynaud et al., 1993; Ross et al., 1987). Recently we described a new species, *Campylobacter cuniculorum* (Zanoni et al., 2009 and Chapter 1), which the prevalence in rabbits is unknown, showing however, similar to those reported by Ross and colleagues (1987) and Reynaud and colleagues (1993). *C. cuniculourm* requires different methodologies of isolation than those usually applied for *C. jejuni* or *C.coli* (Zanoni et al., 2009 and Chapter 1), and it could explain the controversial results obtained by some authors. Therefore, it is important to use a broad spectrum of isolation methods in order to understanding the prevalence of non-*jejuni/coli Campylobacter* species in animal guts, otherwise there may be an undervaluation of the potential hazard of these species.

There are few reports regarding the occurrence of *Campylobacter* species in hares as well: low rates *C. jejuni* have been found (de Boer et al., 1983; Rosef et al., 1983) as well as *C. coli* (Wahlstrom et al., 2003).

In what concerns *Helicobacter,* several species, either gastric (including the group "*Helicobacter heilmannii*") or enteric (*Helicobacter cinaedi*, *Helicobacter bilis*, *Helicobacter fennelliae*, *Helicobacter pullorum*), have been related with gastritis, gastric ulceration (gastric species) and enteric disease, bacteremia and pneumonic illness (enteric species) in humans (De Groote et al., 2000; Moyaert et al., 2008).

To our knowledge, there are only two reports on the detection of *Helicobacter* species in pet, industrial and laboratory rabbits made by molecular techniques (Van den Bulck et al., 2005; Van den Bulck et al., 2006), which found DNA referable to "*Helicobacter heilmannii"* and *H. canadensis* or *H. pullorum*, however with a low prevalence. Moreover, there are no reports on the occurrence of *Helicobacter* spp. in hares. As previously mentioned for *Campylobacter* spp., there is no simple gold standard for the routine isolation of *Helicobacter* species and the simultaneous application of different atmospheres and media is methodologically the optimal solution to isolate these microorganisms from the animal gut.

The objectives of this survey were to: (i) study the prevalence and (ii) co-infection of different species of *Campylobacter* and enteric *Helicobacter* in healthy and diarrheic leporids epidemiologically not correlated.

Materials and Methods

Rabbit Sampling. 32 different intensive and rural Italian and Portuguese farms were sampled. When possible, three animals per farm were sampled. However we were able to retrieve the complete intestinal tract of 85 rabbits, collected at the slaughterhouse or during necroscopies between 2007 and 2008. During necroscopies it was not always possible to recover three animals per farm. Samples were processed avoiding cross-contamination. The gut from each rabbit was obtained directly after evisceration and packed into a separate sterile plastic bag using fresh disposable gloves (Figure 12 a and b). Approximately 4g of the caecal contents were collected for cultural examination (performed within 4h after the death), and roughly 1g of feaces were conserved at -20ºC until processing for PCR analysis. Rabbit samples details are present in Table 4.

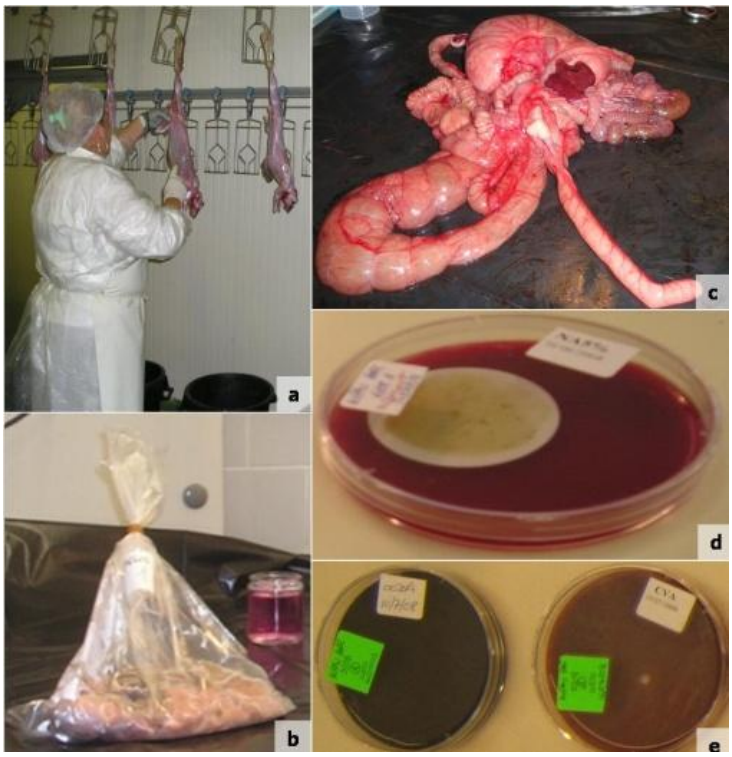

Figure 12: a. Slaughterhouse complete intestinal tract samppling; **b**. Intestinal tract packed into a separate plastic bag; **c**. opening the instestinal tract for obtaining caecal contents; **d**. Modified McDermott technique; **d.** inoculation onto two selective media mCCDA and CAT.

Table 4: Data regarding rabbit samples of the 32 farms examined with the respective information of provenience, category, farming system, health status (considered only if animals had or not enteritis) and number of anmals sampled. Samples with PT mean that were from Portugal.

Farm nº	Origin	Category*	Farm System	Health Status	nº of animals	Year of sampling
1	Emilia-Romagna	Adults	Rural	Enteritis	1	2007
2	Piemonte	Young	Intensive	Enteritis	3	2007
3	Emilia-Romagna	Young	Intensive	Enteritis	3	2007
4	Emilia-Romagna	Young	Intensive	Enteritis	2	2007
5	Emilia-Romagna	Young	Intensive	Enteritis	2	2007
6	Veneto	Young	Intensive	Healthy	3	2007
7	Veneto	Adults	Intensive	Healthy	3	2007
8	Sicilia	Young	Intensive	Enteritis	3	2007
9	Emilia-Romagna	Adults	Rural	Healthy	3	2007
10	Emilia-Romagna	Young	Intensive	Healthy	2	2007
11	Emilia-Romagna	Young	Intensive	Healthy	3	2007
12	Emilia-Romagna	Adults	Intensive	Healthy	3	2007
13	Friuli Venezia Giulia	Adults	Intensive	Healthy	3	2007
14	Veneto	Young	Intensive	Healthy	3	2007
15	Veneto	Young	Intensive	Healthy	3	2007
16	Marche	Young	Intensive	Enteritis	2	2007
17	Marche	Young	Intensive	Enteritis	2	2007
18	Veneto	Young	Intensive	Healthy	3	2007
19	Marche	Young	Intensive	Healthy	3	2007
20	Marche	Adults	Intensive	Healthy	3	2007
21	Emilia-Romagna	Young	Intensive	Healthy	3	2007
22	Lazio	Young	Intensive	Healthy	3	2007
23	Emilia-Romagna	Young	Intensive	Healthy	3	2007
24	Beira Litoral (PT)	Adults	Rural	Healthy	3	2007
25	Algarve (PT)	Young	Rural	Healthy	2	2008
26	Emilia-Romagna	Adults	Rural	Enteritis	2	2008
27	Emilia-Romagna	Adults	Rural	Healthy	2	2008
28	Emilia-Romagna	Adults	Rural	Enteritis	2	2008
29	Emilia-Romagna	Adults	Rural	Healthy	3	2008
30	Lazio	Adults	Rural	Healthy	3	2008
31	Lazio	Adults	Rural	Healthy	3	2008
32	Lazio	Adults	Rural	Healthy	3	2008

* Adults: > 6 months; young: < 6 months.

Hare Sampling. The caecal contents of a total of 29 hares were sampled between 2007 and 2009 and conserved at -20ºC. Twenty six animals were captured from 2007 till 2009 in Denmark and brought to the Veterinary Institute of Danish Technical University (Aarhus, Denmark). Three animals were obtained during 2007 from State Veterinary Institute of Lombardia and Emilia-Romagna, in Italy. Because it was not

possible to obtain the cecal contents within 4-5h after the death of the animals, the samples were subjected only to PCR analysis for the detection of *Campylobacter* and *Helicobacter* spp. Hare samples details are shown in Table 5.

Table 5: Data regarding the individual 29 hare's samples examined with the region of capture origin and year of capture and health status (considered only if animals had or not enteritis). The 3 first samples are from Italy and the next 26 are Danish samples.

Animal n°.	Origin Capture	Year of Capture	Health Status
1	Emilia-Romagna	2007	Enteritis
2	Emilia-Romagna	2007	Enteritis
3	Emilia-Romagna	2007	Enteritis
4	Rønde	2007	Healthy
5	Rønde	2007	Healthy
6	Rønde	2007	Healthy
7	Rønde	2007	Healthy
8	Rønde	2007	Healthy
9	Rønde	2007	Healthy
10	Rønde	2007	Healthy
11	Rønde	2007	Healthy
12	Rønde	2007	Healthy
13	Rønde	2007	Healthy
14	Rønde	2007	Healthy
15	Rønde	2008	Healthy
16	Rønde	2008	Healthy
17	Rønde	2008	Healthy
18	Rønde	2008	Healthy
19	Rønde	2008	Healthy
20	Rønde	2008	Healthy
21	Rønde	2008	Healthy
22	Rønde	2008	Healthy
23	Rønde	2008	Healthy
24	Rønde	2008	Healthy
25	Kolind	2009	Healthy
26	Åbenra	2009	Healthy
27	Roslev	2009	Healthy
28	Holsterbn	2009	Healthy
29	Rønde	2009	Healthy

Cultural examination. Approximately 4g of caecal content from each rabbit were squeezed into 5 ml of sterile saline and shaken using a vortex mixer in order to obtain a homogenous suspension. Diluted samples were inoculated by streaking 10 µl of each

suspension directly onto on Nutrient Broth N°2 (Oxoid) with 1.5% of Bacto Agar (Difco) and supplemented with 5% sheep blood (NAB) plus CAT Selective Supplement (CAT, Oxoid), NAB plus Blaser-Wang Selective Supplement (Blaser, home made), NAB supplemented plus Skirrow Selective Suplement (Skirrow, home made) and modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA, Oxoid). In addition, an aliquot of 100 µl of each sample was diluted in 400 µl of a sterile mixture containing 100 µl of Bacto-Brain Heart Infusion (BD-Becton, Dickinson and Company), 300µl of inactivated horse serum and 30mg of glucose (Sigma). The diluted samples were then inoculated on NAB using the modified filter technique of Steele and McDermott [membrane technique, (Zanoni et al., 2007)] (Figure 12d). All plates were incubated at 37°C in a jar under a microaerobic atmosphere with hydrogen obtained by the gas replacement method with anaerobic gas mixture (H_2 10%, CO_2 10%, N_2 80%) (Bolton et al., 1992) and examined daily for up to 12 days. Colonies of Gram negative, curved or spiral rod organisms were subcultivated and cloned on NAB.

DNA extraction. The total DNA of the caecal contents of all rabbits was extracted using QIAmp DNA Stool Mini Kit (Qiagen) while the DNA of the caecal contents of hares was extracted using KingFish (Promega); both methods followed the manufacturer's instructions. The chromosomal DNA of bacterial isolates was extracted using REDExtract-N-Amp Tissue PCR Kit (Sigma).

Direct PCR analysis of the caecal contents. In order to define the occurrence of *Campylobacter* spp. and *Helicobacter* spp., the DNA samples obtained from the caecal contents of the rabbits and hares were subjected to genus specific PCRs as described by Linton et al (1996) and Al-Soud et al. (2003), respectively, using the REDExtract-N-Amp Tissue PCR Kit (Sigma). The annealing temperature of *Helicobacter* genus specific PCR (Al-Soud et al., 2003) was modified from 55ºC to 65ºC to increase its specificity. The DNA of the caecal content obtained from a broiler chicken positive for *Helicobacter* spp. and *Campylobacter* spp. (Zanoni et al., 2007) was used as a positive control. Moreover, spiked samples were done in order to confirm the sensibility of the method

with the DNA of *Campylobacter cuniculorum* LMG 24588^T and *Helicobacter pullorum* CIP 104787^T, respectively. The list of the primers used is presented in Table 6.

Table 6: List of primers used and respective references. The annealing temperature as well as the primers sequence and the amplicon size in bp are also shown.

PCR (reference)	Amplicon (size in bp)	Primers sequence (F, forward; R, reverse)	Annealing temperature
Campylobacter genus (Linton et al., 1996)	816	F, 5′ GGATGACACTTTTCGGAGC 3′ R, 5′ CATTGTAGCACGTGTGTC 3′	54°C
Helicobacter genus (Al-Soud et al., 2003)	780	F, 5′ CTATGACGGGTATCCGGC 3′ R, 5′ CTCACGACACGAGCTGAC 3′	65°C
C. jejuni/ C.coli (Denis et al., 1999)	857[a] 589[b] 462[c]	F, 5′ ATCTAATGGCTTAACCATTAAAC 3′ R, 5′ GGACGGTAACTAGTTTAGTATT 3′ F, 5′ CTATTTTATTTTTGAGTGCTTGTG 3′ R, 5′ GCTTTATTTGCCATTTGTTTTATTA 3′ F, 5′ AATTGAAAATTGCTCCAACTATG 3′ R, 5′ TGATTTTATTATTTGTAGCAGCG 3′	59°C
C. upsaliensis/ C. helveticus (Lawson et al., 1997)	878[d] 1225[e]	F, 5′ GGGACAACACTTAGAAATGAG 3′ R, 5′ CACTTCCGTATCTCTACAGA 3′ F, 5′ GGGACAACACTTAGAAATGAG 3′ R, 5′ CCGTGACATGGCTGATTCAC 3′	58°C
C. lari (Linton et al., 1996)	561	F, 5′ CAAGTCTCTTGTGAAATCCAAC 3′ R, 5′ ATTTAGAGTGCTCACCCGAAG 3′	64°C
C. cuniculorum (This study)	200	F, 5′ GGAAGAATTTGTCCGGTTGA 3′ R, 5′ TGCGTAGCGGTCAAATACAC 3′	54°C

[a]: *Campylobacter* genus; [b]: *C. jejuni*; [c]: *C. coli*; [d]: *C. upsaliensis*; [e]: *C. helveticus.*

Identification of bacterial isolates. The isolates underwent genus specific PCRs for *Campylobacter* (Linton et al., 1996) and *Helicobacter* (Al-Soud et al., 2003) as described above. Moreover, positive *Campylobacter* isolates were identified at the species level by *C. jejuni–C. coli* specific multiplex PCR (Denis et al., 1999), *C. upsaliensis–C. helveticus* specific duplex PCR (Lawson et al., 1997) and *C. lari* PCR (Linton et al., 1996), using REDExtract-N-Amp Tissue PCR Kit (Sigma). Isolates were also subjected to a multiplex PCR specific for *Campylobacter* genus and for *C. cuniculorum*, by using the primers described by Linton and colleagues (1996) for the genus and the primers specific for *C. cuniculorum* developed in the present study. The primers specific for *C. cuniculorum* were CUNrpoB1F 5′ GGAAGAATTTGTCCGGTTGA 3′ and CUNrpoB2R 5′ TGCGTAGCGGTCAAATACAC 3′. These primers were designed from *rpo*B gene of *C. cuniculorum* (accession numbers EU636829, EU636830, EU636831, EU636832, EU636833, EU636834, EU636835 and EU636836) and 40 pmol each were used to amplify 200 bp specific fragment using REDExtract-N-Amp™ PCR ReadyMix™. The thermal cycling conditions were: 94°C for 4 minutes, 30 cycles of 94°C for 60 seconds,

54°C for 60 seconds, and 72°C for 60 seconds, followed by 72°C for 10 minutes. The specificity of this PCR was tested against a range of thermophilic *Campylobacter* species (*C. jejuni, C. coli, C. helveticus, C. upsaliensis* and *C. lari*) and confirmed by comparing the sequences of the purified amplicons of 5 different positive isolates with deposited *rpo*B sequences using BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/). The list of the primers used is present in Table 6.

Results

Direct PCR analysis of the caecal contents. According to the PCR results, all the 85 rabbits examined, reared on 32 different farms epidemiologically not correlated, were positive for *Campylobacter* genus and 3 rabbits resulted positive for *Helicobacter* genus (from the rural farms number 9, 26 and 28). On the other hand, all hares tested resulted negative both for *Campylobacter* and *Helicobacter* genera. In order to identify the *Helicobacter* species detected on the three rabbit samples, the 780bp amplicons of these samples were extracted from the gel and sequenced using the PCR primers. All three samples showed clear unique sequences, indicating that the PCR amplified the 16S rRNA of a single *Helicobacter* taxon. The three sequences showed 99.7-99.9% of similarity among them, and 98% of similarity with Uncultured *Helicobacter* (accession number AM932602) and 97% of similarity both to *Helicobacter rodentium* (accession number AY631957) and *Helicobacter winghamensis* (accession number AF363063), using BLASTN against NCBI database. Although sequence analysis of the amplicons of these samples show that the animals had the same *Helicobacter* taxon, however it was not possible to identify which species was present, due to the low level of 16S rRNA similarity with known species.

Cultural examination and bacterial identification. In what concerns the isolation from the caecal contents of rabbits, all farms and 96.5% (82 out of 85) of the animals were positive to the isolation of *Campylobacter*-like organisms. From the positive animals, after 4-8 days of incubation, in CAT and mCCDA as well as NAB inoculated

using a filter method, large number of grey-green colonies with 1-2 mm in diameter, flat with rough margins and slightly mucoid-looking (>100 CFU) of Gram-negative slender curved rods referable to *Campylobacter* were present (Figure 13).

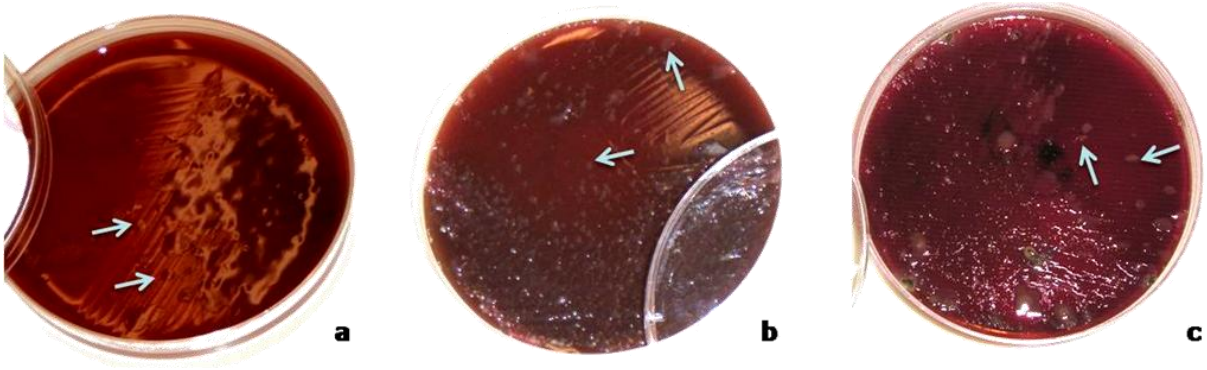

Figure 13. First isolation plates after 7 days of incubation under microaerophilic conditions. **a.** Membrane filter technique; **b.** and **c.** different samples inoculated on selective medium (NAB supplemented with CAT). Arrows indicate characteristic *Campylobacter*-like colonies.

No isolation of these colonies was possible either in Blaser or Skirrow plates. When possible, three colonies from each animal were selected for further identification. The suspected colonies were further identified as *C. cuniculorum* by multiplex PCR in all the animals. Regarding the PCR designed for *C. cuniculorum*, it was able to perceive only this species, showing high specificity (data not shown). Moreover, the 5 randomly chosen samples had their sequences of the purified amplicons 100% of identity with *C. cuniculorum* 150B^{T}. One gel image from this multiplex specific PCR is shown in Figure 14.

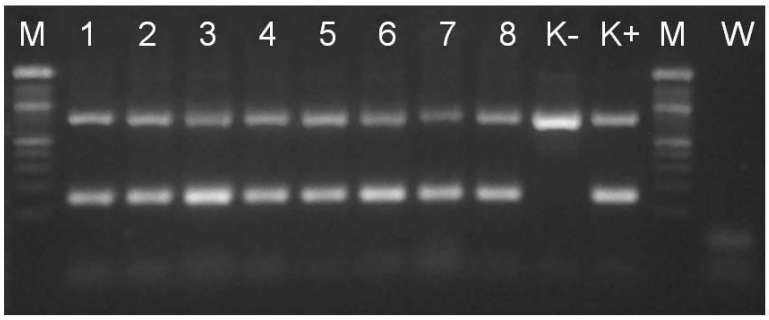

Figure 14. Gel electrophoresis of the multiplex PCR for *Campylobacter* genus (816 bp) and *C. cuniculorum* (200 bp). M: Marker; K-: *C.jejuni;* K+: *C. cuniculorum* 150^{T}; W: water; 1-8: *Campylobacter*-like clones here identified as *C. cuniculorum.*

Both rabbits from farm 28 (rural farm) resulted also co-infected with *C. coli.*
Although it was possible to detect the presence of *Helicobacter* species in 3 rabbits from the faeces by direct PCR, it was not possible to isolate these microorganisms.

Discussion

There is a paucity of data in the literature regarding the occurrence/ prevalence of *Campylobacter* and *Helicobacter* species in leporids.
Surveys on feces, carcasses and meat have been done. Kohler and colleagues (2008) performed a survey at the slaughterhouse and found out that all carcasses analyzed resulted negative for *Campylobacter* but two fecal samples out of 500 (0.4%) were positive for *C. jejuni*. However, the isolation methods used by these authors were those for the isolation of *C. jejuni* and *C. coli*, which included the incubation enrichment broth with Polimixin B (42°C for 48 hours), followed by plating at 42°C for a short incubation period (48h) in Campylobacter Selective Agar with Butzler Selective Supplement. Moreover, Little and colleagues (2008) examined the carriage of *Campylobacter* in rabbit meat and found that 1.96% of positivities (1 sample positive out of 51 meat samples). Again the isolation methods used were those applied for *C. jejuni* and *C. coli.*
In opposition to these authors, Ross and colleagues (1987) by searching the bacterial localization in the caecum of the rabbit, observed in very high numbers of helically shaped organisms (with lengths up to 4 µm and diameters of approximately 300 nm) that formed homogeneous assemblies. According to the *C. cuniculorum* description, these bacteria match the appearance as well as sizes. Moreover, Reynaud and colleagues (1993) reported the presence of a high number of an unidentified *Campylobacter*-like organism after an incubation period of 8 days at 37°C, and according to the authors description, it is likely that they were able to isolate *C. cuniculorum*.
In our study, 96,5% of the rabbits samples resulted positive for *C. cuniculorum* isolation and from only two animals (2.33%) *C. coli* was also isolated. Although from three animals were not possible to isolate *C. cuniculorum,* the caecal contents resulted positive to the *C. cuniculorum* specific multiplex PCR (data not shown).

The elevated number of colonies of *C. cuniculorum* in the first isolation media of all positive samples suggests that this microorganism, when present, colonizes the caecum at a high concentration. Indeed, the fact that other authors were obtaining low rates of detection of *Campylobacter* species it is probably linked to the isolation methodologies that may not support the growth of other potential pathogenic non-*jejuni* and non-*coli* *Campylobacter* species, leading to an undervaluation of human disease caused by other *Camyplobacter* species including *C. cuniculorum*. The isolation methods used in this study seems to be highly suitable for isolating *Campylobacter* from rabbit caecal contents. Nevertheless, no *C. cuniculorum* were recovered from either Blaser or Skirrow media; these media contains Polymixin B and the presence of this antibiotic appears to be not suitable for the isolation of this bacterium.

During the same period, faeces of 151 human patients of different age with gastroenteric disorder were collected from S. Orsola Hospital (Bologna, Italy) and subjected to the same isolation procedure described in this study (data not shown). Eight *Campylobacter* species, in which 4 were *C. jejuni* and other 4 were non thermophilic *Campylobacter* species were isolated. However, it was not possible to reveal the presence of *C. cuniculorum* in those samples. Although the number of human samples was not enough to exclude the role of *C. cuniculorum* as a disease-causing agent, the data did not suggest its responsibility in human enteric diseases. Nevertheless, if the role of *C. cuniculorum* as foodborne pathogens will be confirmed, the high frequency of isolation accompanied by the elevated intestinal concentration could represent a serious contamination risk of the rabbit carcasses.

In our study it was possible to isolate *C. coli* in 2 rabbits from the same farm (2.33% of the total animals examined). These isolations were from animals that came from rural farm system, where there is a strong promiscuity of rabbits with other animals, such as poultry. Similar hypothesis could explain the three rabbits resulted positive to the genus *Helicobacter*-specific PCR, although resulted negative in cultural examination. Since only three animals resulted positive for this bacterial genus, it is possible that they most likely merely reflect a passage of these bacteria through the caecum.

There are no reports on the occurrence of *Helicobacter* species and few reports regarding the occurrence of *Campylobacter* species in hares. Hares are leporids quite

similar to rabbits, and it would be expected that hares would carry *C. cuniculorum* as well, or even another *Campylobacter*-like organisms. Low carriages of *C. jejuni* (de Boer et al., 1983; Rosef et al., 1983) and *C. coli* (Wahlstrom et al., 2003) in hares have been previously reported. Although in this study a limited number of hares were sampled, no *Campylobacter* or *Helicobacter* species were detected; these animals appear to not have either *C. cuniculorum* or other *Campylobacter*-like organisms colonizing their gut.

Conclusion

This survey allowed reporting the high prevalence of *C. cuniculorum* in the caecal contents of rabbits epidemiologically and geographically not correlated. Since all rabbit farms analyzed resulted positive to *C. cuniculorum*, with a high intestinal concentration, this could represent a serious contamination risk of the rabbit carcasses. Moreover, *C. coli* was found in 2 rabbits from the same farm (2.33% of the total animals). In addition, 3 rabbits resulted positive for the detection of *Helicobacter* genus, although no identification was possible.

Regarding hares samples, they appear to not be carriers of either *Campylobacter* or *Helicobacter* species.

In this view, further studies are necessary to describe and evaluate the potential pathogenic role of *C. cuniculorum* on rabbits as well as for humans.

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Chapter 3: Virulence determinatns in *Campylobacter cuniculorum*

Campylobacter species frequently colonises the gut in both humans and animals gut. Several thermotolerant *Campylobacter* species are recognized as human pathogens, and their virulence determinants have been studied although not yet properly established.

This Chapter contains the study undertaken in 13 *C. cuniculorum* isolates from different sources to determine their potential cytotoxic, adhesion and invasion activities towards mammalian cells.

Partial data has been presented at the 15th International Workshop on *Campylobacter*, *Helicobacter*, and Related Organisms (CHRO):

Revez, J.; Zanoni, R. G., Bang, D. D. Cytolethal distending toxin like effects from *Campylobacter cuniculorum* strains. *in* Proceedings of the 15th International Workshop on *Campylobacter*, *Helicobacter*, and Related Organisms (CHRO2009), Niigata, Japan, 2-5 September 2009.

Introduction

Members of the genus *Campylobacter* colonise the gastrointestinal tract of both humans and animals, where they can be either commensal or act as pathogens. Although *Campylobacter jejuni* and *Campylobacter coli* constitute the majority cause of gastroenterities, other species (*Campylobacter upsaliensis*, *Campylobacter fetus*, *Campylobacter lari*) have also been responsible for human illness. The fact that isolation techniques currently used in many diagnostic laboratories may not support the growth of other potential pathogenic non-*jejuni* and non-*coli Campylobacter* species, can explain the underevaluation of human disease caused by other *Camyplobacter* species (Lastovica, 2006). Indeed, many campylobacters may play a greater role in causing human and animal disease; however it is still not understood. Several *Campylobacter* virulence determinants have been attributed to motility, adherence and invasion and to the ability to produce toxins, where the Cytolethal Distending Toxin (CDT) is the best characterized toxin (Asakura et al., 2007; Bang et al., 2003; Bang et al., 2001; Engberg et al., 2005). CDT toxin is encoded by three adjacent genes (*cdtA*, *cdtB*, and *cdtC*) which are genetically arranged as an operon (Asakura et al., 2007); although, the CDT production in several *Campylobacter* species (*C. jejuni, C. coli*, *C. fetus*, *C. lari* and *C. upsaliensis*) was first reported in 1988 (Johnson and Lior, 1988), only in 1996 the CDT genes of *C. jejuni* were cloned, identified and characterized and furthermore it was verified that *cdtB* was present in *Campylobacter* species mentioned above as well as in *Campylobacter hyointestinalis* (Pickett et al., 1996). The sensibility of different cell types to the CDT activity is variable, however in susceptible cell lines typically causes progressive distention by both elongation and swelling, enlargement of the nuclei, and ultimately cell death due to cycle blockage in G2 phase (Bang et al., 2003; Konkel et al., 2001; Pickett and Whitehouse, 1999).

The CDT production by *C. jejuni* as been reported to induce secretion of interleukin-8 release by intestinal cells and it can thus play an important role in the host mucosal inflammatory response (Van Deun et al., 2007). Although animal models have shown that CDT would play a role in disease development, its involvement in the human pathogenesis is still unknown. Other virulence determinants that have been extensively

studied, particularly in *C. jejuni*, include the ability to adhere and invade cells. Colonization or adherence of *Campylobacter* onto mucosal surfaces may facilitate invasion into host cells, althougt do not necessarily lead to invasion (Zheng et al., 2008). On the other hand, cell invasion can result in cellular injury, since invaded cells become swollen and rounded indicating changes in ion transport regulators, leading to reduced absorptive capacity of the intestine. This is probably due to the production of cytotoxin, enterotoxin or haemolysin (Konkel et al., 2001).

Despite the extent research on the virulence determinants of *Campylobacter* species, further elucidation of its pathogenic mechanisms as well as the roles of inflammation and toxin production are still needed.

The new thermotolerant species *Campylobacter cuniculorum* is a fastidous organism that requires prolonged incubation than *C. jejuni* or *C. coli,* and is unable to grow in some selective media (i.e. Skirrow and Blaser-Wang media). The *C. cuniculorum* appears to be highly adapted to the rabbit gastrointestinal tract, in the way that it has been isolated in high concentrations from rabbits with enteritis as well as from healthy individuals (Chapter 2). Moreover, the possible role of *C. cuniculorum* as a human gastrointestinal pathogen is not been accomplished since so far it has never been isolated from humans and their potential virulence determinants are not known.

The aims of this study were to investigate in a total of 13 *C. cuniculorum* strains the presence of *cdtB* gene and the effects on mammalian cells of cytotoxin, adhesion and invasion, which arguably might be involved in pathogenicity.

Material and Methods

Bacterial strains and grow conditions. The information regarding *C. cuniculorum* strains is included in Table 7. Strains were taken from -80°C stock and streaked on Calf Blood Agar (CBA, Oxoid) plates and incubated under microaerobic condition (5 % O_2, 15 % CO_2 and 80 % N_2; BOC) at 37°C for 4 days. The bacteria were sub-culture 2 more times in order to adapt to the media and finally then sub-cultured on a new CBA plate

for another 24h prior to experimentation. Care was taken to pass the strain no more than four times.

Detection of *cdtB* gene from *Campylobacter* isolates by PCR. Extraction of the DNA from *C. cuniculorum* strains was performed by using the QIAamp DNA mini kit according to the manufacturer's instructions. These exctracts were used as templates in PCR reactions with the primers VAT2 and WMI1 complementary to the *cdtB* gene as described by Pickett et al. (1996). The parameters of the PCR used for *cdtB* detection were described by Bang et al. (2001).

Preparation of mammalian cell cultures. The cells used were African Green monkey kidney (Vero, ATCC/CCL-81) cells, colorectal adenocarcinoma (Colo 205, ATCC/CCL-222) cells and cervix adenocarcinoma HeLa (HeLa, ATCC/CCL-2). Cells were propagated as monolayers within plastic tissue-culture flasks in Dulbecco's modified Eagle's growth medium (DMEM, Invitrogen) supplemented with 10% of Foetal Bovine Serum (FBS, Invitrogen) and 200 $U.mL^{-1}$ of penicillin and 0,2 $mg.mL^{-1}$ of streptomycin (Pen Strep, Gibco, Invitrogen). The maintenance of the cell cultures was performed as described elsewhere, with the incubation conditions at 37°C with 5% CO_2. Cell monolayers were detached by the addition of a trypsinizing solution [0.05% (w/v) trypsin, 0.02% (w/v) EDTA] and gentle tapping of the flask. For all assays, cell aggregates in the suspension were dispersed by sterile-pipetting before the cells were washed in fresh medium and suspended to a final viable cell density of $0,1x10^6$ $cells.mL^{-1}$. For the adhesion and invasion assay, the medium did not contain antibiotics.

Preparation of the bacteria-free supernatants for toxin assays. Bacterial-free supernates were prepared as described by Bang et al. (2001) with the following modifications. Briefly, the bacterial suspensions in Veal Infusion Broth (VIB, Difco) were adjusted to a defined optical density of 1, corresponding to 10^9 $CFU.mL^{-1}$ at OD_{620} on an ELISA reader (Multiskan MS Labsystem, Life Science Inter. Comp., Finland) to enable comparison of the CDT production of different isolates. The tubes were incubated at 37°C for 96h under microaerobic conditions. The cell-free bacterial culture supernatants

were collected by centrifugation at 13000 rpm for 5 minutes at 4°C and were subjected to filtration using cellulose filters (pore size 0.22 µm, Millipore Corp., USA). In order to test sterility, 10µL of each filtrate was plated on BA plates and incubated at 37°C under aerobic, microaerobic and anaerobic conditions for up to 12 days. Supernatants were stored at -20°C until required.

Detection of the CDT activity by cell culture assays. Cell culture suspension of Vero, Hela and Colo205 (120µL) were transferred, as described above, to each of 96 wells in a microtitration plate and incubated at 37°C, 5% CO_2 for 3 hours prior infection. Then 30µL of bacteria-free supernatant were added in duplicate to each well and twofold dilutions were performed (Figure 15). The plates were incubated at 37°C with 5% CO_2. The cytotoxic effects were monitored daily for 4 days using inverse optical microscopy and on the days 3 and 4, cells were stained with the double staining Hoechst 33258 and Propidium Iodine and analysed by fluorescence microscopy (Barriere et al., 2001). The CDT titre of a given sample was defined as the highest dilution that caused at least 30% of the cells in a well to be rounded or distended, as described previously (Bang et al., 2001). Duplicate assays were highly reproducible even after storage for 4-6 months at -20°C. Toxin production by each isolate was tested in at least three independent experiments. Repeat assays of a given isolate with bacterial supernatants prepared at different times did not vary by more than onefold dilution. Supernatant from a culture of *C. jejuni* DVI-SC181 was used as a positive control and Veal Infusion Broth (VIB) and Hank's Balanced Salt Solution (HBSS, Inivtrogen) were used as negative controls. The control samples were always included in duplicate wells on each plate.

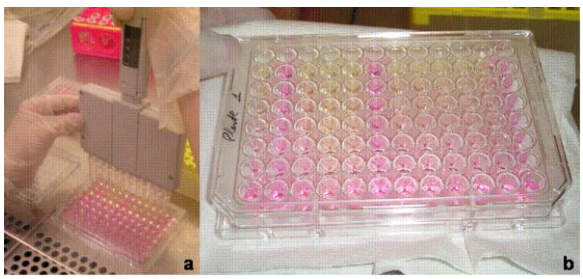

Figure 15. Preparation of the twofold dilutions of bacteria-free supernatant; **a)** shows the dilutions with a multichannel pipette and **b)** shows the ready plate after the dilutions.

Adhesion and Invasion. The number of intracellular bacteria per mammalian cell culture was assessed by the gentamicin protection assay (Everest et al., 1992), with slight modifications. Briefly, the bacteria were harvested and well suspended in room temperature Phosphate Buffer Saline (PBS; 10 mM, pH 7,3) to an OD_{620} equal to 1.0 (approximately 10^9 bacteria.mL^{-1}). Bacterial suspensions were then diluted 15 times in pre-warmed Dulbecco's modified Eagle's growth medium (DMEM, Invitrogen) containing 10% (v/v) Fetal Bovine Serum (FBS) without antibiotics to the desired density for inoculation of cell cultures at a Multiplicity of Infection (MOI) of 100 bacteria per cell.
Mammalian cell monolayers (Colo 205, HeLa or Vero) in 24-well tissue-culture plates were seeded at a concentration of $0,6x10^6$ cells per well. When a monolayer of cells had formed (approximately after 3 hours), the growth medium was removed, the cell monolayer was washed twice with sterile warmed (37ºC) DMEM and 300µL of the bacterial suspensions were added per well in duplicate tests. At 2h post inoculation (p.i.), 500µL of a solution of 200µL gentamicin mL^{-1} (Invitrogen) in DMEM was added to measure invasive organisms alone or DMEM alone for the estimation of adherent plus invasive organisms. After 1,5h of further incubation at 37ºC, all monolayers were washed twice with Hank's Buffered Salt Solution (HBSS, Invitrogen) and the bacteria were released by lysing the mammalian cells for 20 minutes with 500µL 0,2% (v/v) Triton X-100 in PBS per well. Tenfold dilutions of the released bacteria were made in PBS and viable counts were made on CBA. The bacteria counts were numerated by plate counting and are presented as a Colony Forming Unit (CFU) of invaded bacteria. The number of CFU of adherent cells alone was determined as CFU. of non-gentamicin culture (i.e. adherent+internalized CFU) – CFU of gentamicin-treated culture (i.e. internalized). *C. jejuni* DVI-SC181 was used as a positive control and VIB were used as negative controls.

Statistical analysis. All statistical calculations were performed with BioEstat v 5.0 software (GraphPad Software). Data were compared by one-way analysis of variance, followed by the Bonferroni comparison test (α =0.01). Statistical significance was established at a P value of <0.01.

Results

Detection of *cdtB* gene and CDT activity. Regarding the detection of the *cdt*B gene in *C. cuniculorum,* all strains tested resulted negative to the PCR analysis. However, cytotoxic-like effect was observed in all cell lines (HeLa, Vero and Colo 205) with all *C. cuniculorum* strains. Nevertheless, the strains showed different extends of toxicity to the cells. Some microscopic observations of the cell's morphology when inoculated with bacterial-free supernatants undiluted are presented in Figure 16.

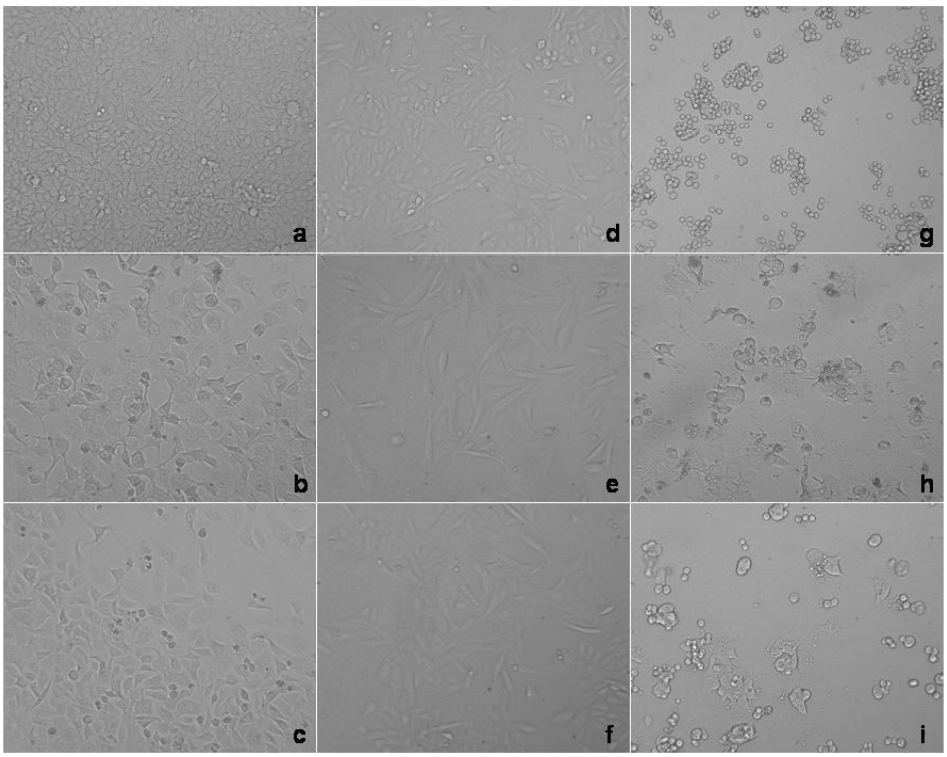

Figure 16. Morphological observations of the cytotoxicity assay after 4 days of incubation with titre 1 on HeLa (**a-c**), Vero (**d-f**) and Colo205 (**g-i**). VIB (negative control): **a**, **d** and **g**. *C. jejuni* DVI-SC181 (positive control): **b**, **e** and **h**. *C. cuniculorum* strain 6: **c**, **f** and **i**. Magnification 100x.

The effect caused to the cells by the crude bacterial free-supernanant from *C. jejuni* (positive control Figure 16 b,e and h) shown high rates of distended/ rounded cells as well as bigger cells were observed (around 5 times bigger). On the other hand, *C. cuniculorum* strains (strain 6; Figure 16 c, f and i) promoted a milder effect in respect of *C. jejuni.* However, when comparing *C. cuniculorum* and the negative controls (VIB; Figure 16 a, d and g), it is possible to observe that there is an increment of the size (around 3 to 4 times) in all the cell lines. Nevertheless, either with *C. jejuni* or *C.*

cuniculorum supernatants, there is a sharp decrease in the number of cells observed. Morphological changes of HeLa cells nuclei, when using different titres of *C. jejuni* DVI-SC181 bacterial-free supernatants, are presented in Figure 17. Similar data were obtained with Vero and Colo 205 cells (data not shown).

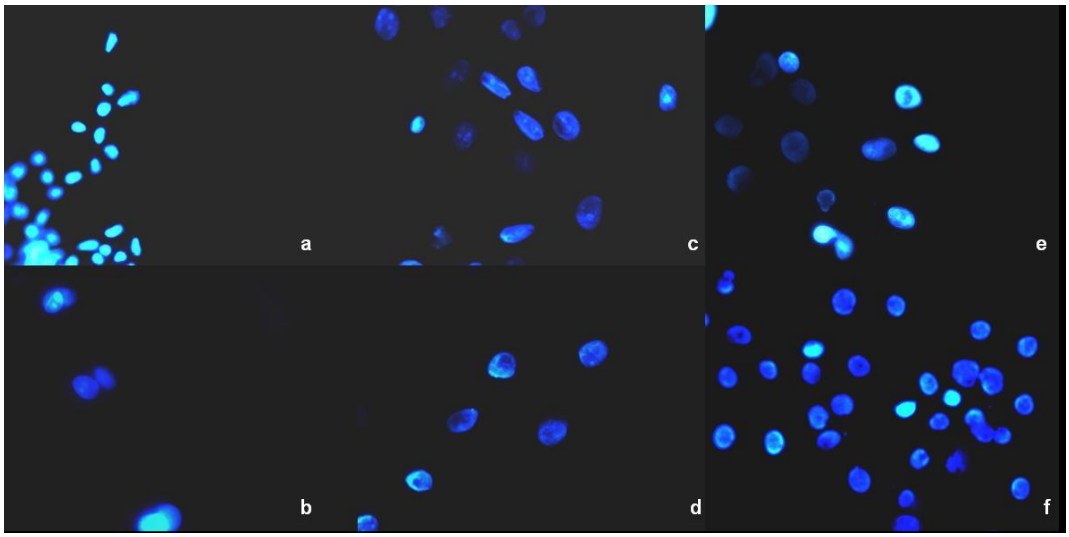

Figure 17. Morphological observation of HeLa cells after 72 hours by fluorescence microscopy infected with *C. jejuni* DVI-SC181 bacterial-free supernatants. a) cells inoculated with Titre 1 present nuclear condensation very strength when compared with control (f) and a sharp decrease in the number of cells observed; b-e) from titre 2 to titre 16 presenting nuclei condensation characteristic of apoptotic cells and further nuclei fragmentation (micronuclei); f) negative control. Magnification 200x

Morphological changes were determined by using the double staining with Propidium Iodine/Hoechst 33258, in which Propidium Iodide stains only necrotic cells and Hoechst stains both healthy and apoptotic cells. The control HeLa cells had regular and round-shaped nuclei, while the infected ones appeared more brightly indicating apoptosis. All *C. cuniculorum* supernatants showed a mild effect with the titre 1 of all *C. cuniculorum* strains corresponding to titre 4 or 8 of *C. jejuni* DVI-SC181. The nuclei of the cells when inoculated with the crude bacterial free-supernanant from *C. cuniculorum* (strain 6; Figure 18 c, f and i, for HeLa, Vero and Colo 205 respectively) showed evident chromatin condensation as well as an occasional fragmentation and formation of micronuclei (Figure 18 c).

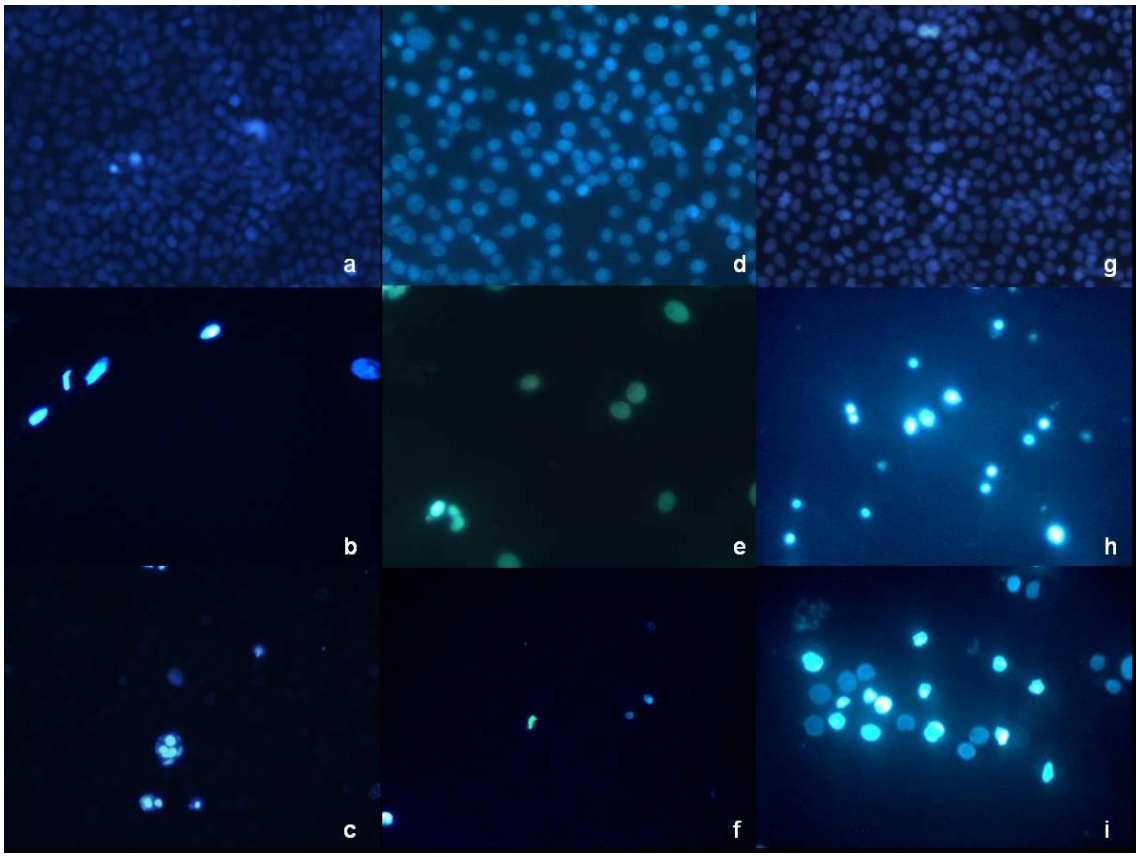

Figure 18. Morphological observations of the cytotoxicity assay after 4 days of incubation with titre 1 HeLa (**a-c**), Vero (**d-f**) and Colo205 (**g-i**). VIB (negative control): **a**, **d** and **g**. *C. jejuni* DVI-SC181 (positive control): **b**, **e** and **h**. *C. cuniculorum* strain 6: **c**, **f** and **i**. Magnification 200x.

However, the positive control (Figure 18 b, e and h) caused higher damage to the cells and greater nuclei change. It was remarkable that Vero cells were more sensitive to the *Campylobacter* bacterial-free undiluted supernatants compared to HeLa and Colo 205.

The Cytolethal distending toxin (CDT)-like titres produced by the different isolates were determined by performing two-fold serial dilutions of the bacterial-free supernatants and it was observed that cytotoxicity activities were quite similar within the isolates. The *Campylobacter cuniculorum* isolates titres on HeLa varied from 1 to 8; on Vero cells the titres ranged between 2 and 8 and on Colo 205 cells the titres were from 2 to 4. Moreover, the positive control, *C. jejuni* had highest titre values for all cell lines, and these values were 32 for HeLa and Vero and 16 for Colo 205 cells (Table 7). The cytoxicity effects on the Colo205 were given with lower titres rather than in Vero or HeLa cells.

Table 7. Source of the *C. cuniculorum* strains isolated from rabbits.Titres of cytoxicity effects of *C. cuniculorum* strains (codes 1-13) tested on the different cell lines. Positive control is *Campylobacter jejuni* DVI-SC181.

Strain Code	Farm nº.	Farm Type	Health Status	Provinience	Cytoxicity titres		
					HeLa	Vero	Colo205
1	*1*	Rural	Enteritis	Emilia-Romagna	4	8	2
2	*4*	Intensive	Enteritis	Emilia-Romagna	8	8	2
3	*6*	Intensive	Healthy	Veneto	4	4	4
4	*8*	Intensive	Enteritis	Sicilia	4	8	2
5	*9*	Rural	Healthy	Emilia-Romagna	4	4	2
6	*10*	Intensive	Healthy	Emilia-Romagna	4	8	2
7	*16*	Intensive	Enteritis	Marche	4	4	2
8	*17*	Intensive	Enteritis	Marche	4	4	4
9	*19*	Intensive	Healthy	Marche	1	4	2
10	*21*	Intensive	Healthy	Emilia-Romagna	2	4	2
11	*22*	Intensive	Healthy	Lazio	4	4	4
12	*26*	Rural	Healthy	Beira Litoral (PT)	2	8	2
13	*28*	Rural	Enteritis	Emilia-Romagna	2	8	2
		Positive control: *Campylobacter jejuni* DVI-SC181			32	32	16

Adhesion and Invasion. Detailed data of adhesion and invasion for each *C. cuniculorum* strains are shown in Figure 19. Adherence by *C. cuniculorum* isolates varied between 10^3-10^6 CFU.mL^{-1} for HeLa and Vero cells and between 10^3-10^5 CFU.mL^{-1} for Colo 205 cells. On the other hand, invasion counts varied between 10^1-10^5 CFU.mL^{-1} for HeLa cells and 0 to 10^5 CFU.mL^{-1} for Vero and Colo 205 cells. The adherence was generally higher with HeLa and Vero cells than with Colo 205 cells. Similarly, the capacity for cell invasion was most pronounced with HeLa and Vero cells and greater overall than the invasive capacity exhibited towards Colo 205 cells. For instance, some *C. cuniculorum* strains were not at all able to invade Colo 205 cells, but were able to invade HeLa and Vero (with the exception of *C. cuniculorum* strain 6 that was only able to invade HeLa cells).

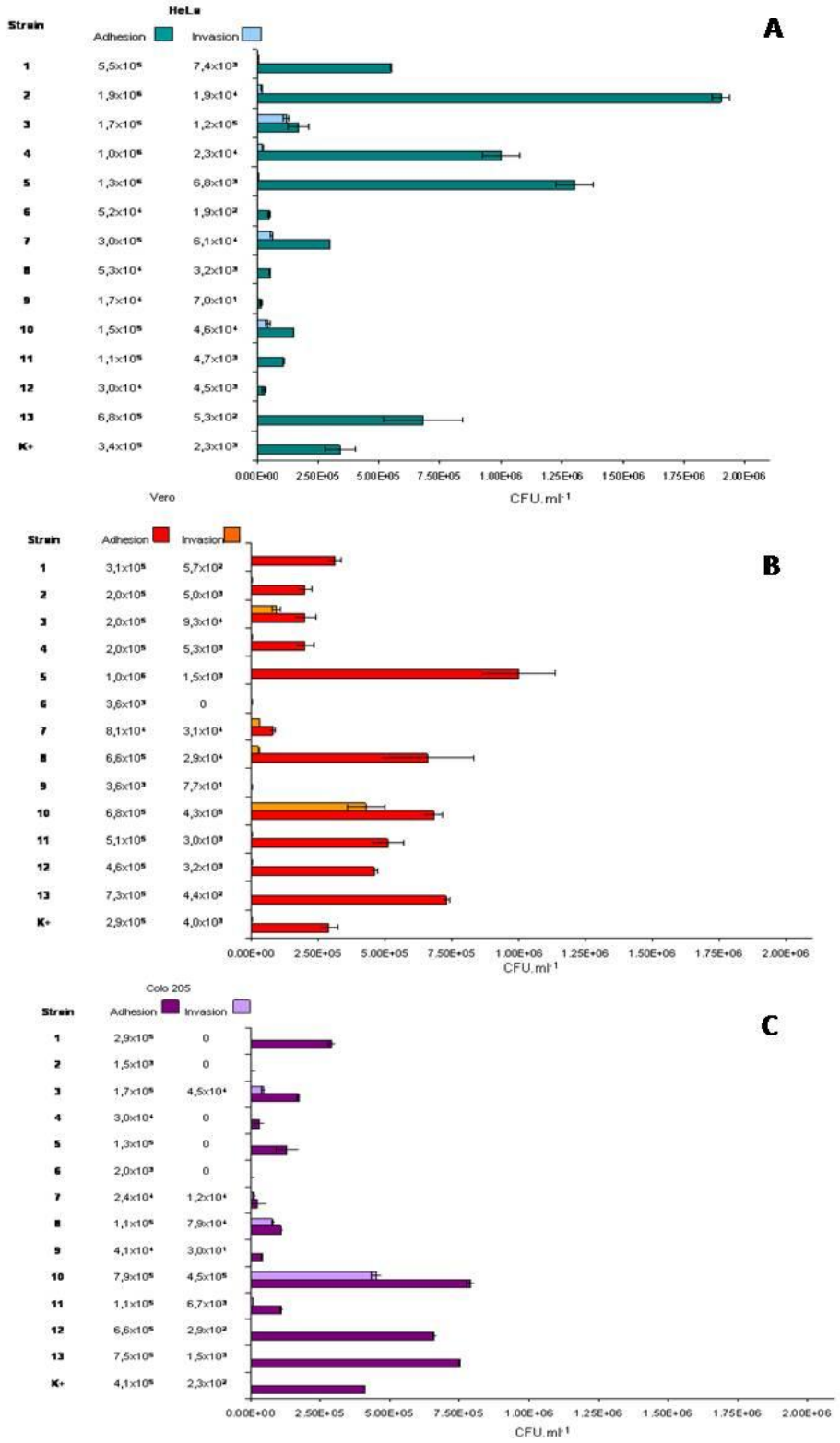

Figure 19. Data of the means of three duplicate tests of adhesion and invasion from *C. cuniculorum* isolates and *C. jejuni* DVI-SC181. **A.** results on HeLa cells. **B.** results on Vero cells. **C.** results on Colo 205 cells. K+ is the positive control *C. jejuni*.

Some *C. cuniculorum* isolates exibited higher adhesion and invasion rates than the positive control *C. jejuni* DVI-SC181. As an esample, in HeLa cells, *C. cuniculorum*

strains 1, 2, 4 and 5 had higher values of adhesion and invasion than the positive control. In Vero cells, adhesion and invasion rates of the strains 7 and 8 were also higher than in *C. jejuni.* In addition, *C. cuniculorum* strains 10 and 13 had higher values for adhesion and invasion in Colo 205 cells.

Discussion

C. jejuni is a major cause of human diarrhoeal disease and in spite of the lack of knowledge about specific virulence mechanisms, CDT is the most characterised of the putative *Campylobacter* toxins (Engberg et al., 2005). Although the *cdt* gene locus has been detected in several *Campylobacter* species, the frequency of the *cdtB* gene as well as CDT production appears not to be present in all *Campylobacter* strains among different species (Eyigor et al., 1999; (Engberg et al., 2005; Jain et al., 2008).

In this study it was not possible to reveal the presence of *cdtB* gene in any of the 13 isolates of *C. cuniculorum*, however a range of CDT-like effects on HeLa, Vero and Colo 205 cells were observed in all the strains. Indeed, the morphological changes observed in the cells treated with bacteria-free supernatants of *C. cuniculorum*: shown a marked enlargement (c. 3–4 fold) in comparison with control cells; were distended; presented nuclear fragmentation and apoptosis. There are common phenomena with susceptible cells when treated with the supernatant of CDT-producing bacteria, such as *C. jejuni* CDT producing strains (Engberg et al., 2005; Mooney et al., 2001). Moreover, another characteristic effect of CDT, confirmed by the double staining Hoectsh 33258/Propidium iodide, was that cytodistending cells underwent apoptosis rather than necrosis.

Although CDT-like effects were verified, very low titres were obtained to the three target cells (between 1 and 8) and it has been suggested that with low titres (usually between 2 and 8) the toxins should not be considered to have a role in the pathogenesis of *Campylobacter*-infections (Florin and Antillon, 1992). Indeed, no correlation was obtained between the cytotoxic effects and rabbits health status.

When studing the CDT production by campylobacters at least 2 different cell types should be used (Florin and Antillon, 1992) and cell lines which are considered to be sensitive include Vero and HeLa (Bang et al., 2001; Florin and Antillon, 1992). In this study, Vero cells showed greater titre values and were statisticaly different from HeLa

and Colo 205 cells. Our results are in accordance with other authors, which reported that Vero were more sensitive than HeLa (Florin and Antillon, 1992), and Colo 205 (Bang et al., 2004).

It is possible that the cytodistension, enlargement of the nuclei, nucleus fragmentation and apoptosis phenomena observed in this study are the result of bacterial factor(s) other than CDT. Given the importance of programmed cell death as a homeostatic mechanism for maintaining the balance of intestinal epithelial cells, the host cell mechanism(s) underlying *C. cuniculorum*-induced apoptosis and its relevance in vivo will be an important area for future investigation.

Other virulence determinants that have been extensively studied include adhesion and invasion. Adhesion of microbial pathogens onto mucosal surfaces may facilitate invasion (Zheng et al., 2008) which can result in cellular injury (Konkel et al., 2001). In this study, the isolates presented variations for both adherence and invasion in all three cell lines. Some strains were not able to invade at all the cells, however other *C. cuniculorum* isolates adhered and invaded cells more efficiently than the *C. jejuni* positive control, which Russell and colleagues (1998) also verified when studying *C. concisus* invasion. The only statistical significant differences obtained in our study were the rates of invasion between Vero and Colo205 cells. There was no statistically significant difference in the adhesion rate within the three cells tested and no correlation between CDT-like activity and cell adhesion/invasion was found. Nevertheless, a *C. cuniculorum* isolate (strain 2) with the higher cytoxicity in HeLa cells also showed the higher average rate of adhesion in those cells, suggesting a relation between the CDT-like effect and the ability of adhesion in these epithelial cells. Data from other studies show that the role of CDT in *C. jejuni* adhesion and invasion is rather controversial. In a recent study (Jain et al., 2008), adhesion and invasion attributes in HeLa cells of CDT-negative and CDT-positive *C. jejuni* were analysed, and the authors concluded that CDT-positive *C. jejuni* adhered (mean $2.7x10^4 \pm 3.5x10^4$) and invaded (mean $1.0x10^3 \pm 1.3x10^3$) more significantly than CDT-negative *C. jejuni* (which adhered with a mean of $2.7x10^2 \pm 1.9x10^2$ and invaded with a mean of $1.4x10^1 \pm 3.1x10^1$). In opposition, other authors (Biswas et al., 2006) reported that the production of cytotoxin does not play a role in the adherence, since no difference in adhesion to HeLa cells of

Δ*cdt* - mutants compared to wild-type *C. jejuni* NCTC11168 was found, however, pointing out that CDT may play a role in invasion.

The ability of *C. jejuni* to invade intestinal epithelial cells distinguishes this enteric pathogen from commensal bacteria in the gut (Zheng et al., 2008). Within *Campylobacter* genera, *Campylobacter concisus*, that appears highly adapted to the human gastrointestinal tract and human disease association is not clear, showed CDT-like effects on Vero cells (Engberg et al., 2005), however without evidence of *cdtB*. Moreover some strains of *C. concisus* are able to adhere and invade (Russell and Ward, 1998). Other members of the family *Campylobacteraceae* such as *Arcobacter* spp. that can be considered as water and food contaminants (Miller et al., 2009) have shown to be able to adhere to human epitelial cells (Ho et al., 2007). In partcular, *Arcobacter cryaerophilus* have adherence and invasion properties (Ho et al., 2007), and even if these species have been isolated from humans, some strains are considered to be non-pathogenic and are human commensals (Miller et al., 2009). In addition, regarding the presence and effect of CDT in *Arcobacter* spp., no CDT amplimers were detected but toxicity to HeLa and INT407 cells was observed (Johnson and Murano, 2002). Although virulence determinants have been studied both in *C. concisus* and in *Arcobacter* spp., the role of these species as human pathogens is still not clear. In our study, we found that *C. cuniculorum* strains promoted a CDT-like effects (similar to those founded *C. concisus*), and were able to adhere and invade but there is no linkage between the virulence properties of *C. cuniculorum* and the health status of the animals. Although, it is possible that *C. cuniculorum* play a role as commensals in rabbits, like some other *Campylobacteraceae* play in other animals and humans, the virulence determinants founded in this study could not exlude the potential pathogenicity towards humans as described in some genotypes of *C. concisus.*

Conclusion

This study focused on the description of some virulence determinants of *Campylobacter cuniculorum,* including the presence of *cdtB* and effects CDT-like of culture supernatants of this species on HeLa, Vero and Colo 205 cells. Moreover, adhesion and invasion properties were also studied in the same three cell lines. The *cdtB* was not

detected, however CDT-like effects were observed at a low titre, with no correlation between the cytotoxic effects and the health status of the rabbits.

There was no clear relationship between source of isolation or disease manifestation and possession of statistically significantly levels of particular virulence-associated factors although, cell adhesion and invasion occured.

Further studies should be carried on, in order to understand the pathological role of this new species.

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Chapter 4: Antimicrobial susceptibility

The awareness of the emerging worldwide problem of antimicrobial resistance is increasing. This alarming emergence is pointed out by European directive 2003/99/EC (monitoring of zoonoses and zoonotic agents) as a characteristic that should be monitored to cover not only zoonotic agents but also indicator organisms, since such organisms constitute a reservoir of resistance genes, which they can transfer to pathogenic bacteria.

The objectives in this Chapter are: (i) to present results of the antimicrobial susceptibility in *Campylobacter* species and *Escherichia coli*, isolated from rabbits epidemiologically not correlated and (ii) to correlate data regarding the antimicrobial resistance within *Campylobacter* species and the indicator bacteria.

Introduction

The use of antimicrobial agents constitutes an important factor that led to the global threat of antibiotic resistance. Antibiotics use promotes the selection and dissemination of resistance among commensal and pathogenic bacteria in the intestinal tracts of both humans and animals (Poeta et al., 2009; Stobberingh and van den Bogaard, 2000; Teuber, 2001; van den Bogaard and Stobberingh, 2000). The level of resistance in the endogenous flora is considered to be a good indicator for the selection pressure exerted by antimicrobial agents use as well as for the resistance problems to be expected in pathogens (Stobberingh and van den Bogaard, 2000). In fact commensal bacteria, that have been exposed to antibiotics and become resistant, can constitute an enormous reservoir of resistance genes and may transfer those genetic elements to pathogenic bacteria, such as *Salmonella* and *Shigella* (Angulo et al., 2004; Stobberingh and van den Bogaard, 2000). Additionally, resistance genes commonly encode resistance not just to a particular antibiotic, but to an entire class of antimicrobials and some genes might cause cross-resistance to compounds that are structurally different (Tollefson and Karp, 2004). Investigation of the prevalence of resistance of certain common intestinal bacteria, so called indicator bacteria, like *Escherichia coli* and *Enterococcus* spp. in the tract of animals and humans makes it feasible to compare the prevalence of resistance in different populations and to detect a possible transfer of resistance bacteria from animals to humans (van den Bogaard and Stobberingh, 2000). Moreover, molecular analysis of antibiotic resistance genes, plasmids and transposons, has demonstrated that identical elements are found in animals and humans microbiota (Teuber, 2001). Thus, the intestinal tract of animals can be considered as a reservoir of resistant bacteria and resistance genes (Moore et al., 2005; Poeta et al., 2009); the spread of resistance from animal microfloras to human microfloras can be done via the food chain or by direct contact with animals (Angulo et al., 2004; Moore et al., 2005; Teuber, 2001). Regarding food animals, resistant bacteria may spread among animals during transportation to the slaughter plant and during antemortem processing points. Furthermore, food products may become contaminated at the time of slaughter or post-

slaughter processing; on the other hand, raw animal products may cross-contaminate other foods during preparation (Tollefson and Karp, 2004).

Globally, an estimated 50% of all antimicrobials serve veterinary purposes. The veterinary use of antibiotics includes the use on pets and food animals (Teuber, 2001). In food animals, antibiotics are used in therapy and prophylaxis of bacterial associated infectious diseases, and were used in the past to increase growth and feed efficiencies (Teuber, 2001; van den Bogaard and Stobberingh, 2000). The main infectious diseases treated are enteric and pulmonary infections, skin and organ abscesses and mastitis (Teuber, 2001; Tollefson and Karp, 2004). Moreover, food animals and in particular what concerns the rabbit breeding, the intensive production system is mainly affected by an high incidence of enteric disorders and the problematic of the antibiotic resistance, along with legal limitations imposed by the European Commission as well as the susceptibility of rabbits to some commonly drugs originates significant economic losses (Poeta et al., 2009).

The antibiotics used in both veterinary and human medicine are: penicillins, cephalosporins, tetracyclines, chloramphenicols, aminoglycosides, spectinomycin, lincosamide, macrolides, nitrofuranes, nitroimidazoles, sulfonamides, trimethoprim, polymyxins and quinolones (Teuber, 2001; Tollefson and Karp, 2004). As a medical consequence, veterinary use of a wide range antimicrobials contribute to selective pressure, resistance reservoirs and routes of transmission (Teuber, 2001), thus threatening our ability to successfully treat certain infections (Tollefson and Karp, 2004).

The European directive 2003/99/EC (on monitoring of zoonoses and zoonotic agents), has as public health priority to monitor antibiotic resistance either in indicator organisms as zoonotic agents, such as *Salmonella* sp. and *Campylobacter* species. Indeed, *Campylobacter* species (mainly *C. jejuni* and *C. coli*) are recognized as the most common causative agents of bacterial gastroenteritis in the world (Alfredson and Korolik, 2007), and trends in antimicrobial resistance have shown a clear association between use of antibiotics in the veterinary industry and resistant isolates of *Campylobacter* spp. in humans (Alfredson and Korolik, 2007; Angulo et al., 2004). In addition, a continuos monitoring of susceptibility profiles of *Campylobacter* species to a

panel of antimicrobial agents (that should include ampicillin, amoxycillin, erythromycin, tetracycline, streptomycin, gentamicin, enrofloxacin and ciprofloxacin) is necessary to control either the rates of resistance to the agents of choice used in the treatment of clinical enteric infection either the emergence of multidrug-resistant organisms. Moreover, it is also important to study and search for the mechanisms for the transfer of resistance both within *Campylobacter* spp. and between different genera of enteric organisms (Caprioli et al., 2000; Moore et al., 2005). *Campylobacter* species possess genetic mechanisms for natural transformation and conjugation, so if antibiotic resistance genes associated with mobile elements are acquired, the trait is rapidly transferred between strains of the same species (Alfredson and Korolik, 2007). Moreover, although there is a paucity of data regarding the passage of resistance genes within different *Campylobacter* species, this phenomenon cannot be neglected.

The agar dilution method is considered to be the most valid method for testing the antibiotic susceptibility for *Campylobacter* species (Moore et al., 2005) and regarding indicator bacteria (e.g. *Escherichia coli*) either dilution or disk diffusion methods are accepted (Caprioli et al., 2000).

The aims of this study were to define the antimicrobial susceptibility in *Campylobacter cuniculorum* and *Escherichia coli*, isolated from rabbits, by using the agar dilution method and the disk diffusion, respectively. Moreover, one *Campylobacter coli* strain was included in this study.

Materials & Methods

***Campylobacter* species isolates.** Twenty nine *Campylobacter cuniculorum* strains from different farms were chosen randomly for the determination of antibiotic susceptibility (Chapter 2). These strains were chosen based on the diversity of the farming system, health status and provenience. A single *Campylobacter coli* isolate was included in this study. For the tests, isolates were plated on Nutrient Agar [Nutrient Broth Nº2 (Oxoid) and 1.5% of Bacto Agar (BD)] supplemented with 5% Sheep blood and incubated at 37ºC±1 under microaerobic conditions.

***Escherichia coli* isolates.** Ceacal contents of 85 animals, belonging to 32 farms, epidemiologically not correlated were collected between 2005 and 2008 (as indicated on Chapter 2). Cecal specimens were plated both directly and 1:2 diluted in sterile saline onto MacConkey agar and incubated for 24 hours at 37ºC±1. When possible, a mean of two *E. coli* colonies were selected from each rabbit and colonies with typical morphology and lactose positive were selected and presumptively identified with the Mackenzie test and confirmed by the IMViC assay (Indole, Methyl-Red, Voges-Proskauer and Citrate). After identification, strains were stored at -20ºC in Tryptic Soy Broth (BD) with 20% (v/v) glycerol. When needed, strains were streaked on Tryptic Soy Agar (BD) for the disk diffusion test analysis. For the antibiotic susceptibility testing, two colonies per farm were picked when possible.

Minimiun Inhibitory Concentration (MIC) determination of *Campylobacter* species. The MIC values of ampicillin, ciprofloxacin, chloramphenicol, erythromycin, gentamicin, nalidixic acid, enrofloxacin and tetracycline were determined according to the agar dilution method described by the Clinical and Laboratory Standard Institute (CLSI, formerly NCCLS) for *Campylobacter jejuni* and related species (NCCLS, 2002). The original method for *Campylobacter cuniculorum* was modified as described previously by Rossi and colleagues (2008). Briefly, (i) the medium used was represented by Nutrient Broth N.2 (Oxoid) supplemented with 1.5% Bacto Agar (BD) and 5% defibrinated sheep blood, (ii) the plates were incubated at 37 C±1 under microaerobic atmosphere with hydrogen and read after 48 and 72h of incubation. All antimicrobial agents were purchased from Sigma. The antibiotic concentrations ranged from 0.015 to 128 μg ml^{-1}. For all isolates, a final inoculum of 2 μl containing 5 log CFU was seeded as a spot on agar plates. *C. jejuni* ATCC 33560 was used as a quality control strain for ciprofloxacin, enrofloxacin, nalidixic acid, erythromycin, gentamicin and tetracycline (NCCLS, 2002) whereas *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as quality control strains for ampicillin and chloramphenicol (NCCLS, 2004). The Minimum Inhibitory Concentration (MIC) determination was performed in duplicate.

Disk diffusion. The antimicrobial susceptibility of *E. coli* isolates was tested by disc diffusion method (Kirby-Bauer) according to the Clinical Laboratory Standards Institute method (NCCLS, 2005) on Mueller-Hinton Agar II (BBL-BD), using 17 discs containing antibacterial substances: ampicillin 10 µg (AM10), amoxicillin–clavulanic acid 20 + 10 µg (AmC), amikacin 30 µg (AN30), chloramphenicol 30 µg (C30), colistin 10 µg (CL10), cefotaxime 30 µg (CTX 30), cephazolin 30 µg (CZ30), enrofloxacin 5µg (ENR5), florfenicol 30 µg (FFC30), sulphamethoxazole (G0,25), gentamicin 10 µg (GM10), kanamycin 30 µg (K30), marbofloxacin 5µg (MAR5), nalidixic acid 30 µg (NA30), streptomycin 10 µg (S10), trimethoprim–sulphamethoxazole 1.25/ 23.75 µg (SXT), and tetracycline 30 µg (Te30). All disks, except florfenicol (Mast diagnostic), were supplied by Oxoid Ltd. As recommended by CLSI, *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as quality control strains. Interpretative standards for the zone diameter were used, although strains with values within the intermediate category were considered in the resistant category.

Results

MIC determination of *Campylobacter* species. MIC values from each *C. cuniculorum* strains are shown in Table 8 while the distribution in Table 9.

C. cuniculorum strains isolated from rural/extensive farming system had lower MIC values rather than animinals from intensive/industrial system, thus being susceptible to all antimicrobial agents tested with the exception of nalidixic acid. The isolates from rural farm system had the following range of MIC values: ciprofloxacin 0.125-0.5 $\mu g.ml^{-1}$, nalidixic acid from 16-64 $\mu g.ml^{-1}$ (only one isolate with 64 $\mu g.ml^{-1}$ as MIC value), enrofloxacin 0.06-0.125 $\mu g.ml^{-1}$, ampicillin 1-16 $\mu g.ml^{-1}$, tetracycline 4-8 $\mu g.ml^{-1}$, gentamycin 0.06-0.25 $\mu g.ml^{-1}$, erytromycin 0.5-4 $\mu g.ml^{-1}$ (only one isolate had 4 $\mu g.ml^{-1}$ as MIC value) and chloramphenicol 8-16 $\mu g.ml^{-1}$.

A monomodal distribution for the MICs was found for all the antibiotics tested except for ciprofloxacin, enrofloxacin and erythromycin which showed a bimodal appearance

with a second peak between 4-128 μg.ml^{-1}, 1-16 μg.ml^{-1} and ≥ 128 μg.ml^{-1}, respectively (Table 9).

Table 8. MIC values from the 29 *C. cuniculorum* isolates and respective information (origin, category, farm system and health status).

Farm code	Origin/Region	Category*	Farm System	Health Status	MIC values (μg.ml^{-1}) for the Antimicrobials							
					CIP	NA	ENR	AMP	TE	GM	E	C
1	Emilia-Romagna	Alduts	Rural	Enteritis	0,125	16	0,06	2	4	0,25	1	16
2	Piemonte	Young	Intensive	Enteritis	0,125	128	0,06	8	32	2	>128	8
3	Emilia-Romagna	Young	Intensive	Enteritis	64	>128	16	2	32	4	>128	8
4	Emilia-Romagna	Young	Intensive	Enteritis	0,25	128	0,06	16	64	8	>128	8
5	Emilia-Romagna	Young	Intensive	Enteritis	32	>128	8	8	4	0,03	1	16
6	Veneto	Young	Intensive	Healthy	32	128	4	8	32	1	>128	16
7	Veneto	Alduts	Intensive	Healthy	32	>128	8	8	32	4	>128	8
8	Sicilia	Young	Intensive	Enteritis	0,5	64	0,125	8	32	1	>128	16
9	Emilia-Romagna	Alduts	Rural	Healthy	0,5	32	0,06	4	8	0,125	1	16
10	Emilia-Romagna	Young	Intensive	Healthy	8	>128	2	8	16	0,03	>128	4
11	Emilia-Romagna	Young	Intensive	Healthy	64	>128	8	4	32	0,5	0,5	16
13	Friuli Venezia Giulia	Alduts	Intensive	Healthy	64	>128	8	8	32	1	>128	16
14	Veneto	Young	Intensive	Healthy	128	>128	16	8	32	4	>128	16
15	Veneto	Young	Intensive	Healthy	16	64	1	2	8	0,125	1	16
16	Marche	Young	Intensive	Enteritis	4	128	4	0,5	16	2	>128	4
17	Marche	Young	Intensive	Enteritis	16	16	2	1	16	0,5	128	8
18	Veneto	Young	Intensive	Healthy	32	128	8	16	64	4	>128	8
19	Marche	Young	Intensive	Healthy	0,125	16	0,03	2	32	0,03	>128	2
21	Emilia-Romagna	Young	Intensive	Healthy	32	>128	4	2	64	0,5	>128	8
22	Lazio	Young	Intensive	Healthy	0,5	32	0,125	16	32	0,125	>128	8
24	Beira Litoral (PT)	Alduts	Rural	Healthy	0,125	32	0,125	4	8	0,06	1	16
25	Algarve (PT)	Young	Rural	Healthy	0,25	16	0,125	16	4	0,125	0,5	8
26	Emilia-Romagna	Alduts	Rural	Enteritis	0,25	32	0,06	16	8	0,125	1	16
27	Emilia-Romagna	Alduts	Rural	Healthy	0,5	64	0,125	4	4	0,125	2	16
28	Emilia-Romagna	Alduts	Rural	Enteritis	0,25	32	0,06	16	4	0,25	4	16
29	Emilia-Romagna	Alduts	Rural	Healthy	0,125	32	0,06	1	4	0,06	0,5	8
30	Lazio	Alduts	Rural	Healthy	0,25	32	0,125	8	4	0,125	1	16
31	Lazio	Alduts	Rural	Healthy	0,125	32	0,06	2	4	0,03	1	8
32	Lazio	Alduts	Rural	Healthy	0,125	32	0,06	2	4	0,03	1	8

*: * Adults: > 6 months; young: < 6 months.PT: Portugal; CIP: ciprofloxacin; NA: nalidixic acid; ENR: enrofloxacin; AM: ampicillin; TE: tetracycline; GM: gentamycin; E: erytrhomycin; C: Chloramphenicol.

On the basis of the clinical breakpoints indicated by the CSLI for *Enterobacteriaceae* (NCCLS, 2004), (resistant strains according to these breakpoints, are indicated in red on Table 9) we may assume that all *C. cuniculorum* isolates are sensitive to cloramphenicol, gentamycin and ampicillin. However, 25 out of 29 strains tested, resulted resistant to nalidixic acid. Moreover, tetracycline had 16 resistant strains,

enrofloxacin 10 resistant strains, ciprofloxacin 13 and erytromycin 15 resistant strains. Furthermore, in Table 9 based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints for *Campylobacter jeuni* (only available for ciprofloxacin and erythromycin) are indicated the resistant strains in bold and italic.

Table 9. Distribution of MICs for 29 *Campylobacter cuniculorum* isolates.

Antimicrobials	Number of *C.cuniculorum* isolates with MIC of (μg.ml^{-1})														
	≤0,015	0,03	0,06	0,125	0,25	0,5	1	2	4	8	16	32	64	128	>128
Ampicillin						1	2	7	4	9	6				
Chloramphenicol								1	2	12	14				
Ciprofloxacin				7	5	4			***1***	***1***	***2***	***5***	***3***	***1***	
Enrofloxacin		1	9	6			1	2	3	5	2				
Erythromycin						3	9	1	*1*					***1***	***14***
Gentamicin		5	2	7	2	3	3	2	4	1					
Nalidixic Acid											4	9	3	5	8
Tetracycline									9	4	3	10	3		

In what concerns the MIC values for erytromycin, the second peak of that bimodal distribution (≥128 μg.ml^{-1}) had only strains belonging to farms of intensive/industrial production. The same observation is valid for ciprofloxacin and for enrofloxacin.

Regarding the single *C. coli* tested (isolate from farm number 28), the MIC values were: ciprofloxacin 0.25 μg.ml^{-1}, nalidixic acid 8 μg.ml^{-1}, enrofloxacin 0.06 μg.ml^{-1}, ampicillin 16 μg.ml^{-1}, tetracicline 0.25 μg.ml^{-1}, gentamycin 0.25 μg.ml^{-1}, eritromycin 1 μg.ml^{-1} and chloramphenicol 8 μg.ml^{-1}. Following the clinical breakpoints of CLSI for *Enterobacteriaceae*, this strain is susceptible to all antimicrobial tested. According to EUCAST epidemiological cutoff (that are lower than CLSI epidemiological cutoff values), this strain is considered a wild-type.

The isolates from farm 28, *C. cuniculorum* and *C. coli,* had the same MIC values for ciprofloxacin, enrofloxacin, ampicillin and gentamycin (0.25 μg.ml^{-1}, 0.06 μg.ml^{-1}, 16 μg.ml^{-1} and 0.25 μg.ml^{-1}, respectively). However, *C. coli* had for the other four

antibiotics lower MIC values – nalidixic acid, tetracycline, erytromycin and chloramphenicol.

***Escherichia coli* isolation.** Twenty seven farms out of 32 (84.4%) resulted positive for *E. coli* isolation, moreover from a total of 85 rabbits it was possible to isolate *E. coli* only in 55 animals (64.7% of positivities). The rabbits resulting positive for the isolation of this indicator bacteria, showed a low concentration ($\sim 10^2$ CFU.g^{-1}). *E. coli* were not isolated from the animals belonging to farms code 1, 9, 25, 30 and 32.

Disk diffusion test of *E. coli* isolates. Fifty-four rabbit isolates from 27 farms were selected for the disk discussion test. When possible, 2 *E. coli* isolates from different animals within the same farm were analysed; otherwise, 2 *E. coli* isolates from the same animal of each positive farm were picked out. In only one case, 2 isolates from 2 different animals belonging to the same farm were analysed in order to verify in more detail the intrafarm divergence. Briefly, from these 54 *E. coli* isolates, 42 strains were isolated from different animals (2 isolates per farm; 21 farms), 10 strains belonging to the same animal were obtained from 5 farms and in 2 farms a single *E. coli* isolate were tested (Table 10). Overall, 98.1% of the isolates were resistant to at least one of the 17 antimicrobial agents tested. Resistance to streptomycin (88.9%) occurred most commonly, followed by resistance to: tetracycline (83.3%), sulphamethoxazole (81.5%), trimethoprim-sulphamethoxazole (64.8%), ampicillin (37.0%), nalidixic acid (35.2%), kanamycin (31.5%), chloramphenicol and gentamycin (27.8%), enrofloxacin (24.1%), florfenicol (11.1%) and marbofloxacin (7.4%). Moreover, for amoxicillin/clavulanic acid, amikacin, colistin and cephazolin the rate of resistance was 1.9%. All the isolates were sensitive to cefotaxime (Table 11).

Table 10. Antibiotic susceptibility of *E. coli* isolates.

Farm code	S	Te	G	SXT	AM	NA	K	C	GM	ENR	FFC	MAR	CL	CZ	AmC	AN		
2[i]	R	R	R	S	S	S	S	S	R	S	S	S	S	S	S	S		
	R	R	R	R	S	R	R	R	R	R	S	S	S	S	S	S		
3[i]	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S		
4[i]	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S		
	R	R	R	R	R	R	R	R	R	R	S	S	S	R	S	S	*	
5[i]	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S		
	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S		
6[i]	R	R	R	R	R	S	R	R	S	S	S	S	S	S	S	S		
	R	R	R	R	R	S	R	R	S	S	S	S	S	S	S	S		♦
7[i]	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S		
	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S		♦
8[i]	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S		
	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S		
10[i]	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S		
	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S		♦
11[i]	R	R	R	S	R	S	S	S	S	S	S	S	S	S	S	S		
	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	*	
12[i]	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S		
13[i]	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S		
	R	R	R	R	R	S	R	S	S	S	S	S	S	S	S	S		
14[i]	R	R	R	R	S	R	S	R	S	R	S	S	S	S	S	S		
	R	R	R	R	R	R	S	R	R	R	S	S	S	S	R	S		
15[i]	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S		
	R	R	R	R	R	R	S	S	R	S	S	S	S	S	S	S		
16[i]	R	R	R	R	S	R	S	S	R	R	S	S	S	S	S	S		
	R	R	R	R	S	R	S	S	R	R	S	S	S	S	S	S		♦
17[i]	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S		
	R	R	R	R	S	R	S	R	R	S	S	S	S	S	S	S		
18[i]	R	R	R	R	R	R	R	R	S	R	R	S	S	S	S	S		
	R	R	R	R	R	R	R	S	R	S	S	S	R	S	S	S		
19[i]	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S		
	R	R	R	R	S	S	S	S	R	S	S	S	S	S	S	S		
20[i]	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S		
	R	R	R	R	S	S	S	S	R	S	S	S	S	S	S	S		
21[i]	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S		
	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S		
22[i]	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S		
	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	*	♦
23[i]	R	R	R	R	S	R	S	S	S	S	S	S	S	S	S	S		
	R	R	R	R	S	R	S	S	S	S	S	S	S	S	S	S	*	♦
24[r]	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S		
	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S		♦
26[r]	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S		
	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S		
27[r]	R	R	R	R	R	R	S	R	S	R	R	R	S	S	S	S		
	R	R	R	R	R	R	S	R	S	R	R	R	S	S	S	S		♦
28[r]	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S		
	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S		
29[r]	R	S	R	S	S	S	R	R	S	S	S	S	S	S	S	S		
	R	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S		
31[r]	S	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S		
	R	S	R	S	S	S	R	R	S	S	R	S	S	S	S	R	*	
	R	S	R	S	S	S	R	S	S	S	S	S	S	S	S	S		
	R	S	S	S	S	S	R	S	S	S	S	S	S	S	S	S	*	

i: industrial; r: rural; *: Isolates from the same animal; ♦: farms presenting the same resistotype within the isolates. S, streptomycin; Te, tetracycline; G, sulphamethoxazole; SXT, trimethoprim– sulphamethoxazole; AM, ampicillin; NA, nalidixic acid; K, kanamycin; C, chloramphenicol; GM, gentamicin; ENR, enrofloxacin; FFC, florfenicol; MAR, marbofloxacin; CL, colistin; CZ, cephazolin; AmC, amoxicillin–clavulanic acid; AN, amikacin.

Table 11. Antibiotic resistance of 54 *E. coli* isolates recovered from rabbits with different production systems and health status.

Antibiotic	Number (%) of resistant strains					
	Total of isolates(n=54)		System		Health Status	
			Intensive (n=40)	Extensive (n=14)	Diarrhoea (n=21)	Healthy (n=33)
Ampicillin	20	(37,0)	18	2	8	12
Amoxicillin+clavulanic acid	1	(1,9)	1	0	0	1
Amikacin	1	(1,9)	0	1	0	1
Chloramphenicol	15	(27,8)	11	4	9	6
Colistin	1	(1,9)	1	0	0	1
Cefotaxime	0	(0,0)	0	0	0	0
Cephazolin	1	(1,9)	1	0	1	0
Enrofloxacin	13	(24,1)	11	2	8	5
Florfenicol	6	(11,1)	3	3	2	4
Sulphamethoxazole	44	(81,5)	38	6	15	29
Gentamycin	15	(27,8)	15	0	10	5
Kanamycin	17	(31,5)	12	5	8	9
Marbofloxacin	4	(7,4)	2	2	2	2
Nalidixic Acid	19	(35,2)	17	2	9	10
Streptomycin	48	(88,9)	39	9	18	30
Trimethoprim-sulphamethoxazole	35	(64,8)	33	2	13	22
Tetracycline	45	(83,3)	39	6	17	28

Table 12 shows the 30 resistance phenotypes detected among the 54 rabbit isolates. There is a diversity of phenotypes of antimicrobial resistance also related with the farm. The phenotype of resistance most frequently found was S-TE-G-SXT (streptomycin, tetracycline, sulphamethoxazole and trimethoprim–sulphamethoxazole).

In fact, 61.1% (33 out of 54) of the isolates shown at least resistance to streptomycin, tetracycline, sulphamethoxazole and trimethoprim–sulphamethoxazole and 35.2% (19 out of 54) of the strains had at least resistance to streptomycin, tetracycline, sulphamethoxazole, trimethoprim–sulphamethoxazole and ampicillin. Moreover, 21 of the phenotypes corresponded to different farms, by means these farms own their own phenotype. Nevertheless, it was possible to observe 10 intra-farm different resistotypes in which 4 of those had the isolates from the same farm. The isolates from farm number 31 (the only case where a total of 4 strains were tested, (2 from each 2 rabbits), all showed different resistance phenotypes (Table 10).

The EUCAST epidemiological cut-off values available for *E. coli* only contemplate two of the agents used in this study: ampicillin and cefotaxime. When analysing data obtained for these antibiotics, 15 out of 54 strains acquired resistance to ampicillin, while all strains should be considered as wild-type regarding cefotaxime.

Table 12. Phenotypes of resistance detected among the 54 *Escherichia coli* isolates recovered from rabbits. Indication of the farms includes under brackets if more than one isolate of each farm had the same phenotype.

	Phenotype of resistance	n°. isolates	Farms code
1	S	3	26, 28, 5
2	TE	4	24(2), 28, 5
3	TE-G	1	31
4	S-TE-G	3	4, 11, 13
5	S-TE-G-AM	1	11
6	S-TE-G-GM	1	2
7	S-TE-G-SXT	7	8,10(2),15,19,20,21
8	S-TE-G-SXT-AM	4	3, 21, 22(2)
9	S-TE-G-SXT-AM-K	1	13
10	S-TE-G-SXT-AM-K-C	2	6(2)
11	S-TE-G-SXT-AM-NA-K	1	12
12	S-TE-G-SXT-AM-NA-GM	1	15
13	S-TE-G-SXT-AM-NA-K-GM-CL	1	18
14	S-TE-G-SXT-AM-NA-K-C-GM-ENR	2	8, 17
15	S-TE-G-SXT-AM-NA-K-C-GM-ENR-CZ	1	4
16	S-TE-G-SXT-AM-NA-K-C-GM-ENR-FFC-MAR	2	7(2)
17	S-TE-G-SXT-AM-NA-K-C-ENR-FFC	1	18
18	S-TE-G-SXT-AM-NA-C-GM-ENR-AmC	1	14
19	S-TE-G-SXT-AM-NA-C-ENR-FFC-MAR	2	27(2)
20	S-TE-G-SXT-NA	2	23(2)
21	S-TE-G-SXT-NA-C-GM	1	17
22	S-TE-G-SXT-NA-C-ENR	1	14
23	S-TE-G-SXT-NA-K-C-GM-ENR	1	2
24	S-TE-G-SXT-NA-GM-ENR	2	16(2)
25	S-G-K-C	1	29
26	S-G-K	1	31
27	S-G-K-C-FFC-AN	1	31
28	S-TE-G-SXT-GM	2	19, 20
29	S-K	2	29, 31
30	SUSCEPTIBLE	1	26

S, streptomycin; Te, tetracycline; G, sulphamethoxazole; SXT, trimethoprim– sulphamethoxazole; AM, ampicillin; NA, nalidixic acid; K, kanamycin; C, chloramphenicol; GM, gentamicin; ENR, enrofloxacin; FFC, florfenicol; MAR, marbofloxacin; CL, colistin; CZ, cephazolin; AmC, amoxicillin–clavulanic acid; AN, amikacin.

Considering the antimicrobial susceptibility values from *Campylobacter* species and *E. coli* for tetracycline, enrofloxacin and nalidixic acid, it is possible to see that several farms share the same resistances within species (Table 13).

Table 13. Common resistances for tetracycline, enrofloxacin and nalidixic acid in *Campylobacter* species and *E. coli.* In bold are the farms that present resistances to these 3 antimicrobials and are common within bacterial isolates.

Antibiotic	Farms code
Tetracycline	2, 3, 4, 6, **7**, 8, 10, 11, 13, **14**, **16**, 17, **18**, 19, 21, 22
Enrofloxacin	**7, 14**, **16, 18**
Nalidixic acid	2, 4, **7,** 8, 12, **14**, 15, **16,** 17, **18**, 27

Discussion

The values of MICs founded for *C. cuniculorum*, showed monomodal distribution for all antibiotics except for ciprofloxacin, enrofloxacin and erytromycin that had a bimodal frequency distribution which indicates an acquired resistance for these antimicrobial agents with a high level of MIC in the second peak at 4-128 $\mu g.ml^{-1}$, 1-16 $\mu g.ml^{-1}$ and $\geq$ 128 $\mu g.ml^{-1}$, respectively. Based on our knowledge of genetic mechanisms for antimicrobial resistance in *C. jejuni* and *C. coli*, this acquired resistance in fluoroquinolones, e.g. ciprofloxacin and enrofloxacin, is probably mainly with modifications of GyrA subunit of DNA gyrase and/or due to the contribution of an efflux mechanism – *cme*ABC (Alfredson and Korolik, 2007; Moore et al., 2005). Resistance to the fluoroquinolones arises from amino acid(s) substitution(s) in the quinolone resistance-determining region (QRDR) of the corresponding DNA gyrase subunit A (GyrA). Moreover, an increase of expression of CmeABC help to reduce the accumulation of antibiotic in the bacteria (Alfredson and Korolik, 2007). In order to verify if the resistances against fluoroquinolones are linked with mutations in the QRDR of the *gyrA*, it would be necessary to isolate and sequence this gene of *C. cuniculorum* and do further comparison of the sequences in sensible and resistant strains.

In what concerns macrolide resistance, i.e. erytromycin, it is conceivable that not all of the mechanisms contributing in *Campylobacter* have been described (Moore et al., 2005), however modifications of the target (chromosomal mutations in the 23S rRNA gene -*rrn*B operon- and gene encoding ribosomal proteins) and CmeABC have been considered (Alfredson and Korolik, 2007), being the mutations on the *rrn*B operon responsible for the majority of erythromycin resistance in *Campylobacter.* Regarding *C. cuniculorum*, it would be interesting to study further and to understand the molecular

mechanisms that promote their macrolide resistance. However, both resistance to fluoroquinolones and macrolides are not related with mobile elements, thus indicating that *C. cuniculorum* would not have a role in passing these resistances to other *Campylobacter* species, like *C. jejuni* and *C. coli.* Considering that fluoroquinolones and macrolides are the choice antimicrobials for the treatment of campylobacteriosis, these data would have a greater impact if *C. cuniculorum* would be associated with disease, either in humans and/or rabbits.

Regarding MIC values for nalidixic acid, they were quite high (16->128 $\mu g.ml^{-1}$). As in other *Campylobacter* species, such as *C. curvus*, *C. lari* and others, *C. cuniculorum* seems to be intrinsically resistant to nalidixic acid.

On the basis of the clinical breakpoints indicated by the CSLI for *Enterobacteriaceae*, all *C. cuniculorum* isolates resulted susceptible to cloramphenicol, gentamycin and ampicillin.

The single *C. coli* strain resulted susceptible to all the antibiotics tested and based on the epidemiological cutoff values (that are not clinical breakpoints, only record isolates as wild type or non-wild type) available, this isolate is considered to be a wild-type. This isolate origin (farm 28) is from a rural production system, where no antibiotics were used (information given by the owner), indicating the absence of antimicrobials selective pressure. So, we can assume that the *C. cuniculorum* strain isolated in the same farm is as well a wild-type. Indeed, the *C. cuniculorum* strain isolated from farm 28, had same MIC values for ciprofloxacin, enrofloxacin, ampicillin and gentamycin observed in *C. coli* while for the other four antimicrobial agents (nalidixic acid, tetracycline, erythromycin and chloramphenicol), *C. cuniculorum* had only slightly higher MIC values. Comparing the MIC values of other *C. cuniculorum* strains with those of the farm 28 isolate, it was possible to identify strains with acquired resistance to tetracycline. Indeed, although tetracycline showed a monomodal distribution, the MIC values are moderately high (4-64 $\mu g.ml^{-1}$) with the minimum represented by the "wild type" isolated from farm 28. The strains with MIC values >4 $\mu g.ml^{-1}$ might be considered that acquired resistance to tetracycline. Moreover, 83.3% of *Escherichia coli* isolates presented resistance to tetracycline, indicating a selective pressure in the farms. Considering that tetracycline resistance in *Campylobacter* species (*C.jejuni* and

C.coli) is mediated by the ribosomal protection protein Tet(O) on a self-transmissible plasmid, the potential role of *C. cuniculorum* in passing this resistance to other *Campylobacter* species should be considered.

The MIC values of the second peak's for erythromycin, ciprofloxacin and enrofloxacin had only strains belonging to farms of intensive/industrial production. Indeed, the industrial production in order to manage diseases uses antibiotics, contributing for the selection of antimicrobial resistance.

Healthy rabbits usually carry low levels of *E. coli* (Agnoletti et al., 2004; Blanco et al., 1996; Blanco et al., 1994; Camguilhem and Milon, 1989), probably because of the inhibitory influence of the cecal volatile fatty acids (Blanco et al., 1996). In our study we found only 64.7% positivities for *E. coli* isolation in the animals analysed; moreover, the concentration founded of *E. coli* isolation was always quite low ($\sim 10^2$CFU g^{-1}).

To our knowlegde, there are few studies on the antimicrobial susceptibilities of *E. coli* isolated from rabbits in Italy (Camarda et al., 2004; Pisoni et al., 2004). The few reports available found high resistances rates against several antibiotics: trimethoprim–sulphamethoxazole, tetracycline, streptomycin as well as gentamicin. We also observed multiresistances to these antimicrobials. High levels of resistance were observed for streptomycin, tetracycline, sulphamethoxazole and trimethoprim–sulphamethoxazole. Similar results were obtained in a previous study carried out with faecal *E. coli* isolates from food-producing animals in Germany (Guerra et al., 2003). A recent study about antibiotic resistance in *E. coli* strains isolated from rabbits in Egypt (Hassan and Al-Azeem, 2009) described 27.6% of strains that were resistant to at least one of the 8 antibiotics tested; we found 98.1% strains resistant to at least one of the 17 antimicrobial agents tested.

The fact that multiresistances were found represents a serious problem, since it affects clinical treatment in the case of the strains passing from rabbits to humans or even rabbit to rabbit (Poeta et al., 2009).

Multiresistances were noticed mainly in industrial/intensive farming system, however, there is one case, farm 27, where the isolates from a rural farm system showed resistances to 10 antibiotics (streptomycin, tetracycline, sulphamethoxazole, trimethoprim–sulphamethoxazole, ampicillin, nalidixic acid, chloramphenicol,

enrofloxacin, florfenicol and marbofloxacin). It is difficult to speculate about this multiresistance, since we do not have information about the use of antibiotics in this farm and/or the promiscuity with other animals carrying resistances or treated.

Regarding the phenotype of resistance, we found 30 antimicrobial resistance patterns, in which 21 were from different farms. Indeed, a study from Spain (Blanco et al., 1994) referred that strains from different farms usually have a distinct antibiotics resistance pattern. However, one interesting case is the farm 31, where the isolates presented each one their own pattern, indicating that it is advisable to monitor antibiotic resistance in more clones of *E. coli* of the same farm and animals.

Further studies should be done in order to define serotypes and biotypes, and specially to study genetic mechanisms responsable for antibiotic resistance in the *E. coli* isolates.

Considering the antimicrobial susceptibility values from *Campylobacter* species and *E. coli* it was possible to observe that these bacterial species shared resistance profiles.

Conclusion

The aims of this study were to define the antimicrobial susceptibility in *Campylobacter* species and *E. coli*, isolated from rabbits. This is the first time that *C. cuniculorum* antimicrobial susceptibility has been studied. It was possible to find acquired resistance of *C. cuniculorum* to enrofloxacin, ciprofloxacin and erytromycin. High MIC values of *C. cuniculorum* to nalidixic acid can be explained with an intrinsic resistance. In what concerns the *C. coli* isolate, this strain was susceptible to all antimicrobial tested and moreover it is considered as a wild-type strain. Nevertheless, the majority of resistances were found from strains belonging to intensive farming system. The same was verified in the indicator bacteria.

Regarding *E. coli,* rabbits have a low cecal concentration. It is noticeable the antibiotic resistance patterns founded (30 phenotypes of antibiotic resistance) as well as the high rate of resistances to at least one antibiotic - 98.1%.

Further studies should be done in order to define the genetic mechanisms responsible for the antibiotic resistance, and concerning *E. coli* serotypes and biotypes should be determined in order to understand the potential hazard.

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Part 3: General discussion and conclusions

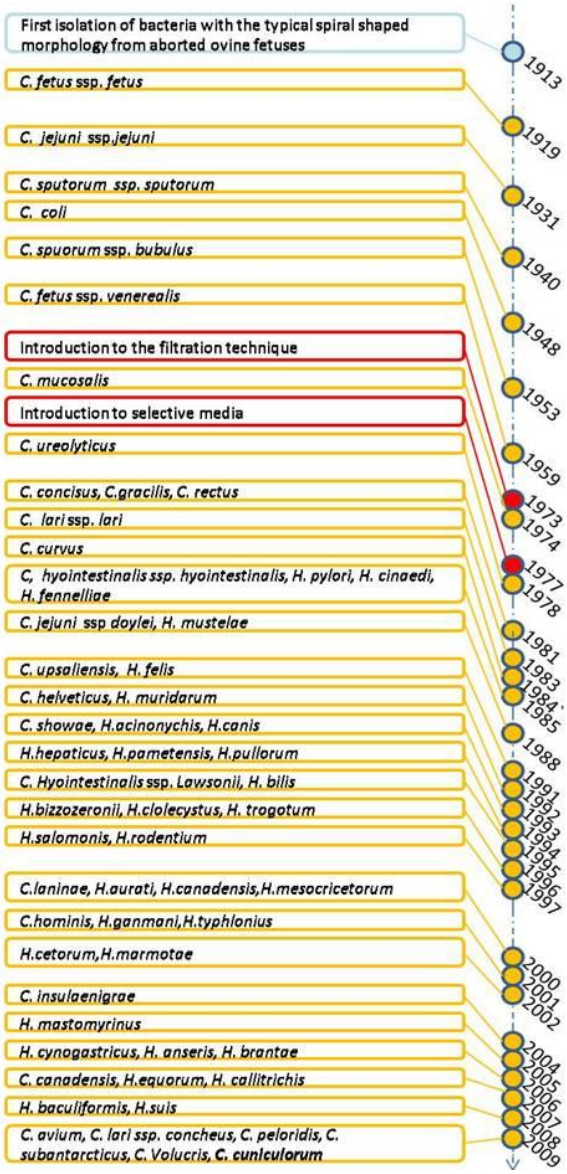

Figure 20. Timeline of the first description of *Campylobacter* and *Helicobacter* species, with the currently accepted names. *Candidatus* species were not included.

The history of *Campylobacter* and *Helicobacter* species is quite fascinating. The first isolation of spiral shaped bacteria occurred in the early of the twenty century, when MacFadyean and Stockman (1913) retrieved this king of bacteria from aborted ovine fetuses. Thereafter, in 1919 occurred the first description by Smith and Taylor (1919) that isolate similar microorganisms from bovine fetuses, for which they proposed the name *Vibrio fetus*, currently known as *Campylobacter fetus* ssp. *fetus.* In the following decades, several *Campylobacter*-like organisms were described, however, only after the introduction of more adequate isolation procedures as well as more rigorous taxonomic approaches, the world of *Campylobacter* and *Helicobacter* was stepping out, with several species blooming afterwards (Figure 20).

Currently, there is no simple gold standard for the routine isolation of all *Campylobacter* and *Helicobacter* species. The optimal solution appears to be the combination of both filtration methods and a selective base medium, as well as the application of a microareobic atmosphere containing hydrogen and longer incubation periods in different temperature ranges. Indeed, the limitation of several diagnostic and research laboratories, in the isolation of these microorganisms, is the restricted methods used, thus leading to an incomplete scenario of the prevalence of these bacteria.

A major aim of the present thesis was to study the enteric spiral microbiota of lagomorphs, since there is a paucity of data regarding the occurrence of Epsilon-

Proteobacteria in these animals. In the course of the present study, by appling several isolation methods, a particular *Campylobacter*-like organisms was retrieved in high concentrations from rabbits epidemiologically not correlated. However, these bacteria were not seen before 6 days of incubation at 37°C. In order to identify this potential new species, a polyphasic approach was followed, and with data of biochemical tests, SDS-PAGE whole protein profile, 16S rRNA, *groEL, rpoB* sequence analysis and G+C% content, it was concluded that the strains isolated from rabbits represented a novel species: *Campylobacter cuniculorum.* Further, in order to understand the occurrence of this new species, as well as other *Campylobacter* and *Helicobacter* species, an higher number of rabbits were sampled (85 animals from 32 farms epidemiologically not correlated, with different production systems – rural or industrial) as well as hares (29 wild animals were sampled). The results showed that all rabbits had *C. cuniculorum*, and only in one farm, with a rural production system, rabbits were co-infected with *Campylobacter coli* (2 animals). Both these 2 animals had enteritis, and in one animal of the same farm, a *Helicobacter* spp. was also detected by PCR. Moreover, in other 2 animals from two distinct rural farms, *Helicobacter* spp. was detected. Although, all three animals probably carried the same *Helicobacter* taxon (due to high 16S rRNA similarity observed), it was not possible to identify it at species level. In addition, all hares resulted negative for the detection of both *Campylobacter* and *Helicobacter* species.

So far, the pathogenicity of this species in human or animals is unknow. Simultaneously of the study ongoing, a research survey on the presence of *Campylobacter* and *Helicobacter* species in 151 human patients with gastroenteric disorders (i.e. diarrhoeae), performed in the same laboratory with the same methodologies, was not able to rivelate *C. cuniculorum*. Even though the sample was limited, these results suggested no role of *C. cuniculorum* in human enteritis. However, it would be favorable to extend this research, in order to understand if this species could really be pathogenic for humans. Nevertheless, determinants of virulence of a discrete number of *C. cuniculorum* strains were evaluated. These strains, isolated from animals epidemiologically not correlated and with or without gastrointestinal disorders, presented a mild Cytolethal Distending Toxic-like effect on different mammalian cells

(HeLa, Vero, Colo-205), and had as well different adhesion and invasion properties towards these cell types. Yet, no statistical significant difference was observed within the different virulence properties of the strains and the health status of the animals, thus it remains inconclusive the pathogenicity of this species.

Antimicrobial susceptibility of several *C. cuniculorum* strains and indicator bacteria *Escherichia coli* was monitored by Minimal Inhibitory Concentration method (MIC) and by Kirby-Bauer method, respectively. From this evaluation, bacteria isolated from industrial/intensive farms, where the use of antimicrobials is almost a necessity, had more resistances than the bacteria isolated from the rural farms. This fact was observed in both bacterial species.

In conclusion, in the present study a new species isolated from rabbits has been described, *C. cuniculorum*. This species has shown a high prevalence in rabbits but was totally absent in hares samples. Although some virulence factors have been identified, further studies are needed to understand the potential pathogenicity and the zoonotic role of *C. cuniculorum*. Moreover, resistance to different antibiotics was found in both *C. cuniculorum* and indicator bacteria, suggesting a possible role of rabbitries in spreading antibiotic resistance.

Part 4: Summaries

Summary

Members of the genera *Campylobacter* and *Helicobacter* have been in the spotlight in recent decades because of their status as animals and/or humans pathogens, both confirmed and emerging, and because of their association with food-borne and zoonotic diseases.
First observations of spiral shaped bacteria or *Campylobacter*-like organisms (CLO) date back to the end of the 19th century, however the lack of adequate isolation methods hampered further research. With the introduction of methods such as selective media and a filtration procedure during the 1970s led to a renewed interest in *Campylobacter*, especially as this enabled elucidation of their role in human hosts. On the other hand the classification and identification of these bacteria was troublesome, mainly because of the biochemical inertness and fastidious growth requirements. In 1991, the taxonomy of *Campylobacter* and related organisms was thoroughly revised, since this revision several new *Campylobacter* and *Helicobacter* species have been described. Moreover, thanks to the introduction of a polyphasic taxonomic practice, the classification of these novel species is well-founded.
Indeed, a polyphasic approach was here followed for characterizing eight isolates obtained from rabbits epidemiologically not correlated and as a result a new *Campylobacter* species was proposed: *Campylobacter cuniculorum* (Chapter 1). Furthermore, there is a paucity of data regarding the occurrence of spiral shaped enteric flora in leporids. In order to define the prevalence both of this new species and other CLO in leporids (chapter 2), a total of 85 whole intestinal tracts of rabbits reared in 32 farms and 29 capture hares, epidemiologically not correlated, were collected just after evisceration at the slaughterhouse or during necroscopy. Examination and isolation methods were varied in order to increase the sensibility level of detection, and 100% of rabbit farms resulted positive for *C. cuniculorum* in high concentrations. Moreover, in 3.53% of the total rabbits examined, a *Helicobacter* species was detected. Nevertheless, all hares resulted negative both for *Campylobacter* or *Helicobacter* species.
High prevalence of *C. cuniculorum* were found in rabbits, and in order to understand if this new species could play a pathological role, a study on some virulence determinants of *C. cuniculorum* was conducted (Chapter 3). Although this new species were able to adhere and invade, exert cytolethal distending toxin-like effects although at a low titre, a *cdtB* was not detected. There was no clear relationship between source of isolation or disease manifestation and possession of statistically significantly levels of particular virulence-associated factors although, cell adhesion and invasion occurred.
Furthermore, antibiotic susceptibility was studied (chapter 4) in *Campylobacter* and in *Escherichia coli* strains, isolated from rabbits. It was possible to find acquired resistance of *C. cuniculorum* to enrofloxacin, ciprofloxacin and erytromycin. *C. coli* isolate was susceptible to all antimicrobial tested and moreover it is considered as a wild-type strain. Moreover, *E. coli* was found at low caecal concentration in rabbits and 30 phenotypes of antibiotic resistance were founded as well as the high rate of resistances to at least one antibiotic (98.1%). The majority of resistances were found from strains belonging to intensive farming system.
In conclusion, in the course of the present study a new species isolated from rabbits was described, *C. cuniculorum,* and its high prevalence was established. Nevertheless, in hare samples no *Campylobacter* and *Helicobacter* species were detected. Some virulence determinants were further analyzed, however further studied are needed to understand the potential pathogenicity of this new species. On the other hand, antimicrobial susceptibility was monitored both in *C. cuniculorum* and indicator bacteria and acquired resistance was observed towards some antibiotics, indicating a possible role of rabbitries in the diffusion of antibiotic resistance. Further studies are necessary to describe and evaluate the eventual zoonotic role of *Campylobacter cuniculorum*.

Sintesi

Membri del genere *Campylobacter* e *Helicobacter* sono stati studiati negli ultimi decenni a causa del loro status di patogeni animali e/o umani, e a causa della loro associazione con tossinfezione alimentare e zoonosi.
Le prime osservazioni di batteri a forma spirillare o organismi simili a *Campylobacter* risalgono alla fine del secolo XIX, tuttavia la mancanza di metodi di isolamento adeguati hanno ostacolato ulteriori ricerche. L'introduzione di innovativi metodi d'isolamento durante gli anni '70, ha portato ad un rinnovato interesse per il genere *Campylobacter*, permettendo di chiarire il suo ruolo nella patologia umana.
In seguito alle difficoltá nel classificare e identificare questi batteri, soprattutto a causa della loro inerzia biochimica ed esigenze colturali, la tassonomia di *Campylobacter* e organismi corelati è stata da sempre motivo di dibattito. I moderni sviluppi della sistematica in batteriologia e l'introduzione nella pratica tassonomica del cosi detto approccio polifasico, che consiste nell'associare alle classiche informazioni fenotipiche quelle di origine genetica e/o genomica, ha permesso di descrivere moltissime nuove species di *Campylobacter* e *Helicobacter*. Un approccio polifasico è stato qui condotto per la caratterizzazione di otto isolati ottenuti da conigli epidemiologicamente non correlati, permettendo la descrizione di una nuova specie: *Campylobacter cuniculorum* (capitolo 1).
Dalla disamina della letteratura, l'isolamento di *Campylobacter* dal coniglio o dalla lepre é sempre descritto come evento sporadico, ma i risultati tra diversi studi appaiono spesso contradditori e un'indagine sistematica sulla prevalenza di questi batteri nel contenuto intestinale di conigli e lepri (*Leporidae*) risulta carente. Con l'obiettivo di definire con maggior precisione la prevalenza di differenti specie di *Campylobacter*, in particolare della nuova specie *C. cuniculorum*, che di *Helicobacter* in *Leporidae*, sono stati campionati un totale di 85 pacchetti intestinali di conigli allevati in 32 aziende e 29 da lepri di cattura (capitolo 2). Tutti i conigli analizzati sono risultati positivi a *C. cuniculorum* e solo in 3 casi é stato possibile determinare la presenza di *Helicobacter* spp., per cui una identificazione di specie non é stata possibile. Soltanto in due casi é stato isolato, insieme ad *C. cuniculorum*, anche *Campylobacter coli*. Diversamente da quanto osservato nei conigli, tutte le lepri sono risultate negative sia per *Campylobacter* che per *Helicobacter*.
Data l'elevata prevalenza di *C. cuniculorum* in conigli allevati a scopo alimentare, al fine di verificare il suo possibile ruolo come agente patogeno, é stato condotto uno studio volto ad analizzare l'eventuale espressione di alcuni fattori di virulenza (capitolo 3): presenza di Cytolethal Distending Toxin (CDT); adesione e invasione in diverse linee cellulari. Sebbene gli isolati di *C. cuniculorum* abbiano mostrato diversi gradi di adesione ed invasione e di esercitare effetti tossici su diverse linee cellulari, la presenza di CDT non è stata rilevata. Inoltre, non é stata riscontrata nessuna relazione tra la presenza di sintomi gastrointestinali nel coniglio e l'espressione dei fattori di virulenza studiati.
Visto la crescente importanza del fenomeno dell'antibiotico resistenza e la sua implicazione in sanitá pubblica, nella presente tesi é stata, inoltre, monitorata la sensibilitá di *Campylobacter* ed *Escherichia coli* isolati da conigli ad un determinato pannello di antibiotici (capitolo 4). É stato possibile determinare acquisita resistenza a enrofloxacina, ciprofloxacina ed eritromicina in *C. cuniculorum*. Diversamente, *C. coli* é risultato sensibile a tutti gli antibiotici testati. Il 98,1% degli isolati di *E. coli* esaminati hanno presentato resistenza ad almeno un antibiotico (98,1%), per un totale di 30 fenotipi di resistenza riscontrati. La maggior parte delle resistenze osservate sono state riscontrate in ceppi isolati da animali allevati con sistema intensivo.
In conclusione, nel corso del presente studio una nuova specie isolata da conigli è stata descritta, *C. cuniculorum*. Questa specie ha mostrato un'elevata prevalenza in conigli ma é risultata totalmente assente nei campioni di lepre. Sebbene alcuni fattori di virulenza siano stati riscontrati, ulteriori studi sono necessari per capire la potenziale patogenicità ed il ruolo zoonosico di *C. cuniculorum*. Tuttavia, resistenza a differenti antibiotici é stata riscontrata sia in *C. cuniculorum* che in batteri indicatori, suggerendo il possibile ruolo dell'allevamento cunicolo nella diffusione dell'antibiotico resistenza.

Sumário

Membros dos géneros *Campylobacter* e *Helicobacter* têm sido o centro das atenções nas últimas décadas tanto devido à importância como agentes patogénicos (confirmados e emergentes) de animais e/ou humanos, como devido à sua associação com zoonoses.

As primeiras observações de bactérias de forma espiralar ou organismos *Campylobacter*-like (OCL) remontam ao final do século XIX. Porém, a falta de métodos de isolamento adequados prejudicou a investigação destes microorganismos. Com a introdução de métodos durante a década de 1970, como meios selectivos e processos de filtração, um renovado interesse em *Campylobacter* surgiu, especialmente porque este permitiu a elucidação do seu papel em hospedes humanos. Por outro lado, devido à sua bioquímica inerte e requisitos de crescimento fastidioso, a classificação e identificação dessas bactérias foi verdadeiramente problemática. Em 1991, a taxonomia de *Campylobacter* e organismos relacionados foi completamente revista, e a partir desta revisão diversas novas espécies de *Campylobacter* e *Helicobacter* têm sido descritas. Além disso, a classificação destas novas espécies está bem fundamentada devido à introdução de uma prática taxonómica polifásica.

Com efeito, uma abordagem polifásica foi aqui utilizada para caracterizar oito estirpes obtidas de coelhos epidemiologicamente não correlacionados, e como resultado uma espécie nova de *Campylobacter* foi proposta: *Campylobacter cuniculorum* (Capítulo 1). Sabendo que existe uma grande escassez de dados sobre a ocorrência de bactérias de forma espiralar na flora entérica de leporídeos, e a fim de definir a prevalência tanto desta nova espécie como de outros OCL em coelhos e lebres (capítulo 2), um total de 85 tractos intestinais de coelhos de 32 explorações (industriais e rurais), epidemiologicamente não correlacionadas, e 29 tractos intestinais de lebres de captura, foram amostrados no matadouro ou durante necroscopia. Os métodos de isolamento e detecção utilizados foram variados, com o objectivo de aumentar o nível de sensibilidade, e 100% das explorações de coelhos resultaram positivas para *C. cuniculorum*. Além disso, em 3.53% do total de coelhos analisados uma espécie de *Helicobacter* foi detectada, porém não identificada. No entanto, em todas as lebres analisadas a detecção de *Campylobacter* e *Helicobacter* foi negativa.

Dada a alta prevalência de *C. cuniculorum* encontrada em coelhos, e com o intuito de compreender se esta nova espécie poderia desempenhar um papel patogénico, um estudo sobre alguns factores de virulência foi realizado em 13 estirpes de *C. cuniculorum* (Capítulo 3). Embora esta nova espécie seja capaz de aderir, invadir, e exercer efeitos semelhantes à toxina de distensão citoletal (embora a um baixo título), a *cdtB* não foi detectada. Não houve uma relação estatisticamente significativa entre a fonte de isolamento ou manifestação da doença e os factores de virulência estudados.

A susceptibilidade aos antibióticos foi igualmente estudada, nas seguintes estirpes isoladas de coelhos: 29 de *C. cuniculorum*, ums de *Campylobacter coli* e 54 ide *Escherichia coli* (capítulo 4). Foi possível encontrar a resistência adquirida de *C. cuniculorum* à enrofloxacina, ciprofloxacina e eritromicina. O isolado de *C. coli* resultou susceptível a todos os agentes antimicrobianos testados e é considerada como uma estirpe de tipo selvagem. No entanto, a maioria das resistências foram encontrados em estirpes originárias do sistema intensivo/industrial. O mesmo foi verificado nas bactérias indicadoras (*E. coli)*. Além disso, os isolados de *E. coli* mostraram uma elevada taxa de resistência a pelo menos um antibiótico (98.1%), e 30 fenótipos de antibiótico-resistência foram encontrados.

Em conclusão, no decurso da presente tese uma nova espécie isolada de coelhos foi descrita, *C. cuniculorum*, e a respectiva prevalência foi estabelecida. Se bem que alguns factores de virulência foram analisados, são necessários mais estudos para compreender a potencial patogenicidade desta nova espécie. No entanto, a susceptibilidade antimicrobiana foi monitorada, tanto em *C. cuniculorum* como em bactérias indicadoras e a aquisição de resistência foi observada para alguns antibióticos, sugerindo um potencial papel na disseminação de antibiotico-resistência por parte das explorações de coelhos. Mais estudos são necessários para descrever e avaliar o eventual papel zoonótico de *C. cuniculorum*.

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