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Biodiversity Dynamics of *Campylobacter* Species in Chicken Tissues in Rural Households in Region Epirus, Greece

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Abstract: The *Campylobacter* species is considered as an emerging zoonotic threat to public health. *C. jejuni* and *C. coli* are the most studied species, yet a variety of other species of the same genus were found to be the causative agents of other diseases. Chicken meat has been described as an excellent vehicle for the transmission of some *Campylobacter* species but most of the relevant research has been conducted in urban populations and concerned meat of industrial-grade birds. To investigate the abundance and prevalence of the *Campylobacter* genus in rural free-grazing chicken, quantitative and qualitative methods at 37 and at 42 °C were employed. The possible correlation of the prevalence with certain epidemiological factors (size of the flock, presence of other poultry species, presence of small ruminants, feeding concentrates, or leftovers) has been also investigated. In total, 242–249 strains (depending on the method) belonging to the following 18 different *Campylobacter* species have been isolated: *C. coli*, *C. rectus*, *C. hominis*, *C. helveticus*, *C. upsaliensis*, *C. jejuni*, *C. avium*, *C. fetus*, *C. hepaticus.*, *C. lari*, *C. sputorum*, *C. mucosalis*, *C. gracilis*, *C. showae*, *C. hyointestinalis*, *C. concisus*, *C. cuniculorum*, and *C. ureolyticus*. The size of the flock and the presence of small ruminants in the same household were the most influential factors affecting the prevalence of most species. *Campylobacter* species biodiversity can be attributed to environmental, zoonotic, or anthropogenic contamination. Rural populations should be educated about the importance of self-protection measures during their contact with their poultry and the necessity to cook sufficiently the meat.

Keywords: *Campylobacter*; biodiversity; chicken

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1. Introduction

Backyard poultry breeding is very popular among the rural population in Greece as it is internationally. It is the easiest and cheapest form of animal breeding, it is accepted by all religions andsupplies the household with proteins of high nutritional value [1] and therefore it is not a surprise that chicken meat is combined into many traditional culinary dishes around the world. On the other hand, chicken and chicken meat can be easily contaminated from the stage of production and all along the logistic chain [2,3] let alone in the rural environment where the hygienic conditions are seriously compromised. The visceral organs of the birds, their skin, and their feathers, all harbor bacteria which can contaminate the meat during plucking and mainly during evisceration due to the rupture of the intestine [4–8].

Large populations of *Campylobacter jejuni* and *Campylobacter coli* live in the intestines of various animals such as sheep, cattle, and poultry, and can contaminate food and water resulting in human disease [9–11]. These species are considered as a leading cause of infection of the digestive tract ranging from self-limiting gastroenteritis to life-threatening bacteremia and enterocolitis [10,12]. Other clinical entities associated with the *Campylobacter*

species such as Guillain–Barre syndrome (one-third of the cases are due to *C. jejuni*) and Miller–Fisher syndrome are of autoimmune origin [9,10]. *C. jejuni* is the prime concern for public health, being responsible for 90% of the campylobacteriosis cases encountered internationally [11,12]. Chicken meat consumption when raw or undercooked is the most frequent source of foodborne disease in humans [13,14]. From 2010 to 2017, there have been 236 foodborne *Campylobacter* outbreaks reported in the USA [15]. Campylobacteriosis is the most accounted gastrointestinal illness in the EU/EEA. In 2017, 250,161 cases of campylobacteriosis were checked in the 29 EU/EEA countries, while the overall EU/EEA report rate was 44.4 cases per 100.000 population [16].

C. jejuni and *C. coli* are perhaps the most investigated *Campylobacter* species due to their impact on public health, but other species of this genus are also detrimental to human health causing serious and—occasionally—life-threatening disease. To mention a few, *C. concisus* has been linked to cases of enterocolitis, periodontal disease, and inflammatory bowel disease (IBD) [17], *C. fetus* has been described as the causative agent of cervical osteomyelitis [18], and bacteremia [19], while *C. upsaliensis* and *C. ureolyticus* have been isolated from patients undergoing periodontitis, IBD, and gastroenteritis [20]. The list is much longer of course and still growing, raising alarm with respect to the genus Campylobacter.

Most of the research concerning *Campylobacter* in poultry in Greece and internationally is limited to industrial broilers and poultry slaughterhouses and retail establishments. EU legislation (Greek law complies) by Regulation 1495/2017 states that sampling once per week is obligatory in slaughterhouses and other establishments and that samples should be analyzed according to EN ISO 10272-2. This kind of priority is quite understandable since industrial poultry meat feeds most of the urban population worldwide and to a degree, it is expected that from this research the backyard household chicken has been excluded. Yet this subject is equally important from the perspective of public health, since practically all rural populations breed and consume backyard chicken meat.

The purpose of this study is to investigate the prevalence of the species of the *Campylobacter* genus in rural households' chicken and its possible correlation to various epidemiological factors.

2. Materials and Methods

2.1. Samples Collection

Lists of registered rural households were provided by the municipal agencies of Arta, Epirus, Greece. From these lists and with the aid of random numbers a series of households were located as sampling points. In every household the aim of the study was explained to the owner through a telephone conversation the previous day and on the day of the visit he was asked to sacrifice between one and three birds depending on the size of the flock (3–15 chickens, 15–40 chickens, and more than 40). In case of absence or denial, the team contacted and visited the next listed household. The days chosen for visits were always Thursdays and Fridays because according to the local culinary tradition of the area, these are the days for slaughtering in order for the bird's meat to season until Sunday, the day of its consumption in the family table. We decided that a maximum of 20 households for every group of combined criteria (see below) would be sufficient. All owners sacrificed the birds by cervical dislocation and immediately afterward our team took the necessary samples aseptically using sterile forceps and scissors as follows: (a) approximately 30 g of chicken skin, (b) 100 g of the pectoralis muscle, and (c) one swab of the visceral cavity and the matching liver. The samples were placed in a sterile polyethylene bag or small bottles containing a small quantity of 0.1% peptone water (Oxoid, Basingstoke, England) just to avoid dryness, and the samples were transported to the laboratory in insulated boxes with ice packs and processed within 1 h. A consent document was obtained from the head of each household for participation in this study.

Classification

The samples were classified with respect to the following four criteria (see Discussion):

(A) The size of the flock: up to 15 birds (*Gallus domesticus*), 15–40 birds, and more than 40 birds to 60;
(B) The presence or not in the same household of other poultry species like turkeys, ducks, etc;
(C) The presence or not in the same household of small ruminants (sheep and goats) and pigs;
(D) The administration of households' leftovers of plant origin (potatoes, tomatoes etc.) or the administration of industrial-grade concentrated feeds (corn, barley, etc.).

From these 4 criteria, 15 study groups emerged (Table 1).

Table 1. Sampling size and groups formed by the four criteria of the study.

Study Groups	Households (*n*)	Number of sampled birds per household (Includes 3 sub-samples: pectoral muscle; neck skin, and; liver with one swab of visceral cavity)
(1) A1, B(no), C(no), D(a)	20	1
(2) A1, B(yes), C(no), D(a)	20	1
(3) A1, B(no), C(yes), D(a)	20	1
(4) A1, B(yes), C(yes), D(a)	20	1
(5) A1, B(yes), C(yes), D(b)	20	1
Total	100	100
(6) A2, B(no), C(no), D(a)	20	2
(7) A2, B(yes), C(no), D(a)	20	2
(8) A2, B(no), C(yes), D(a)	20	2
(9) A2, B(yes), C(yes), D(a)	20	2
(10) A2, B(yes), C(yes), D(b)	20	2
Total	100	200
(11) A3, B(no), C(no), D(a)	20	3
(12) A3, B(yes), C(no), D(a)	20	3
(13) A3, B(no), C(yes), D(a)	20	3
(14) A3, B(yes), C(yes), D(a)	20	3
(15) A3, B(yes), C(yes), D(b)	20	3
Total	100	300
Total	**300**	**600 birds**

A: Size of the flock (1) up to 15 birds, (2) 15–40 birds, (3) more than 40 birds to 60; B: presence of other poultry species in the household (yes/no); C: presence of small ruminants and pigs in the household (yes/no); D: feeding practice (a) mainly, leftovers of vegetable origin, (b) concentrated feeds.

2.2. Recovery of Presumptive Campylobacter spp. Isolates

All samples were analyzed in parallel by two assays, a quantitative (enumeration of total *Campylobacter* colony counts) and qualitative (presence/absence) analysis of the samples.

2.2.1. Quantitative Analysis

To investigate and quantify the presence of *Campylobacter* isolates in the stored samples, each sample was weighed, collected in an aseptic way by using sterile forceps and scissors and put in a sterile Stomacher blender bag under completely sterile conditions, and homogenized slowly for 2 min using a Stomacher 400 Circulator. Two (2) g samples (mate-

rial: muscle, skin, or content from visceral cavity/liver) were placed into 18 mL diluting liquid in a sterile plastic bag and were again homogenized for 1 min using a stomacher. In the present study, the following materials were used as diluting agents: (a) 0.1% buffered peptone water-BPW (Oxoid, Basingstoke, England; (b) maximum recovery diluent (Oxoid Deutschland GmbH, Wesel, Germany); (c) Bolton Broth base (Oxoid, Basingstoke, England) containing *Campylobacter* selective supplement, with 5% lysed horse blood and (d) Bolton broth base, with 5% lysed horse blood (Oxoid) and rifampin (Sigma-Aldrich) concentrations 12.5 mg/L (R-BB). Before being added to the Bolton broth, a stock solution of the antibiotic Rifampin was prepared by dissolving it in dimethyl sulfoxide (Sigma-Aldrich) and was filtered through a 0.2-lm syringe filter (MACHEREY-NAGEL, Duren, Germany).

A volume of 1 mL from the sample (10^{-1}), in triplicate, was spread-plated (0.3, 0.3, 0.3, and 0.1 mL) over four [21]: (a) modified charcoal cefoperazone deoxycholate agar plates (mCCDA) (Oxoid, Basingstoke, England); (b) CampyFood agar plates (CFA; Biomerieux, l'Etoile, France); and (c) Karmali Agar plates (Oxoid) with *Campylobacter* Selective Supplement (Karmali) SR0167. The first dilution (10^{-1}) was followed by 10-fold dilutions in 0.1% buffered peptone water -BPW, until reaching a dilution of 10^{-5}. Aliquots (0.1 mL) of these dilutions were spread using the direct plating method in triplicate on the surface of mCCDA, CampyFood agar, and Karmali agar (with supplement) plates. The first series of Petri dishes were incubated at 37 °C for 48 h under micro-aerobic conditions and the second series was incubated at 42 °C for 48 h under micro-aerobic conditions. Microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) were achieved by introducing sachets of CampyGen (Oxoid) in a rectangular jar (2.5 L capacity). Excess moisture during microaerobic incubation can lead to undesirable confluent or swarming growth of *Campylobacter* owing to its high motility. Accordingly, excess moisture was avoided by the addition of 4–5 drops of glycerol onto a piece of filter paper in an uncovered petri dish along with the plates in the chamber.

For each positive plate, and if necessary, up to five typical *Campylobacter* colonies were then subcultured onto plates of Columbia Blood Agar (Oxoid) for further characterization, in accordance with the standard procedure of the International Organization for Standardization (ISO) 10272 [22–24].

2.2.2. Qualitative Analysis

For the enrichment culture method, 10 mL from the initial homogenate sample were put into 90 mL: (a) Bolton Broth Base, with 5% lysed horse blood, (b) Bolton Broth Base, with 5% lysed horse blood (Oxoid) and rifampin concentrations 12.5 mg/L (R-BB), (c) Bolton broth (Oxoid, Basingstoke, Hampshire, UK), containing *Campylobacter* selective supplement, supplemented with 5% lysed horse blood, and (d) Preston broth (Nutrient broth No.2 CM0067 plus modified Preston *Campylobacter* selective supplement SR0204E, Oxoid, Basingstoke, England) with 5% sterile defibrinated sheep blood. The falcon tubes were placed in respective jars with CampyGen (Oxoid, Basingstoke, England) sachets to create a microaerobic atmosphere and were sealed. The falcon tubes were incubated at 37 °C for 4 to 6 h and then at 42 °C for 48 h. Enriched samples were plated onto (a) modified charcoal–cefoperazone–deoxycholate mCCD) agar (Oxoid) supplemented with CCDA Selective Supplement; (b) Preston agar (Oxoid) supplemented Modified Preston *Campylobacter* Selective Supplement (Oxoid™), and (c) CampyFood agar. Plates were incubated at 37 and 42 °C for 44 h. After microscopy, at least five typical colony, were taken from each selective agar and subcultured onto Columbia Blood Agar (Oxoid) to further analyze for oxidase, Hippurate hydrolysis test, and indoxyl acetate [22].

2.2.3. Species Identification

Isolates which presumptively were characterized as *Campylobacter* spp. were cultured on sheep Columbia Blood Agar, incubated in microaerobic conditions as described above at 42 °C for 24 h and subsequently through the methodology of Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS; Bruker Biotyper

Microflex; Bremen Germany), were identified at the species level. Analysis of the obtained spectra was performed by the MALDI Biotyper software package (MBT Compass version 4.1) and the reference database version (BDAL Rev. No 11, 10,833 database entries) (Bruker Daltonik GmbH, Bremen, Germany). The entire process, including MALDI-TOF mass spectrometry measurement to strain identification, was performed automatically without interference from any researcher. Although about 100 peaks were generated by the software, the threshold for peak acceptance was at a signal-to-noise ratio of 3. However and after alignment of the peaks, a mass-to-charge ratio difference of 2.00, was considered correct for the identification at the species level while a ratio between 1.7 and 1.999, was considered correct for the identification at the genus level and these differences were applied in the current study [25,26].

2.3. Statistical Analysis

Multilinear regression models were used to infer the impact of the four criteria (epidemiological factors) on the biodiversity of the *Campylobacter* species with a significance level of $p < 0.05$. The Chi-square test of independence was applied wherever necessary with a significance level $p < 0.05$.

3. Results

In general, and for both quantitative methods (incubation conditions: 42 and 37 °C) the counts ranged from 1.20 log CFU to 3.50 log CFU. In most cases where a species was isolated from more than one tissue, the counts in skin samples were slightly higher (but not significantly) than the counts from pectoral muscle samples which were also slightly higher than the ones in the liver and the visceral cavity.

In total 18 *Campylobacter* species were isolated by the four different analytical methods, 16 being common for all methods, while *C. rectus* was isolated at 42 °C by both isolation methods (quantitative and qualitative characterization) and *C. hominis* was isolated only by the qualitative method at 37 °C. By far, *C. jejuni* was the most abundant species, followed by *C. coli*. These two species accounted for more than 61% of the total isolated *Campylobacter* strains (Tables 2 and 3).

Table 2. Number of strains for every *Campylobacter* species isolated by the four different methods.

Species		Method			
		Quantitative 42 °C	**Qualitative 42 °C**	**Quantitative 37 °C**	**Qualitative 37 °C**
1	*C. coli*	64	64	63	63
2	*C. rectus*	1	1	-	-
3	*C. hominis*	-	-	-	1
4	*C. helveticus*	1	1	1	1
5	*C. upsaliensis*	2	2	2	2
6	*C. jejeuni*	97	98	96	97
7	*C. avium*	17	21	18	21
8	*C. fetus*	19	19	19	19
9	*C. hepaticus*	10	11	10	11
10	*C. lari*	23	24	24	24
11	*C. sputorum*	4	4	4	4
12	*C. mucosalis*	1	1	1	1
13	*C. gracilis*	2	2	2	2
14	*C. showae*	1	1	1	1
15	*C. hyointestinalis*	4	4	4	4
16	*C. concisus*	2	2	2	2
17	*C. cuniculorum*	1	1	1	1
18	*C. ureolyticus*	5	6	6	6
	Total	256	264	256	262

Table 3. Isolations of *Campylobacter* strains from the skin, the pectoral muscle and the visceral cavity and liver of the birds (qualitative method at 42 °C).

Species		Tissue			
		Skin	Pectoral Muscle	Visceral Cavity/Liver	Total
1	*C. coli*	57	21	-	78
2	*C. rectus*	1	1	1	3
3	*C. helveticus*	1	-	2	3
4	*C. upsaliensis*	3	1	-	4
5	*C. jejeuni*	92	49	2	143
6	*C. avium*	14	4	2	20
7	*C. fetus*	18	9	-	27
8	*C. hepaticus*	5	3	8	16
9	*C. lari*	18	9	1	28
10	*C. sputorum*	2	2	-	4
11	*C. mucosalis*	-	1	1	2
12	*C. gracilis*	2	-	-	2
13	*C. showae*	1	-	-	1
14	*C. hyointestinalis*	2	3	2	7
15	*C. concisus*	1	1	-	2
16	*C. cuniculorum*	-	1	1	2
17	*C. ureolyticus*	5	1	3	9
	Total	222	106	23	351

Table 4 presents the birds (*Gallus domesticus*) found positive for any *Campylobacter* strain in any of the examined tissues. From a total of 600 birds, the positivity score varied between 40.33% and 41.50% depending on the method, with the qualitative method at 42 °C detecting the highest prevalence rate (41.50%). Interestingly, only 6 birds (1%) showed mixed infection by two *Campylobacter* species. Multilinear regression revealed that for all methods the size of the flock and the presence of small ruminants were decisive factors very strongly correlated to the prevalence of *Campylobacter* spp. ($R > 0.943$, $p < 0.001$). Particularly, the small size of the flock (3–15 birds) acted as a protective factor, while the larger size (more than 40 birds) was correlated with increased prevalence.

Table 4. Number of positive birds per experimental group for every different method.

Group		Method			
		Qt 42 °C	Ql 42 °C	Qt 37 °C	Ql 37 °C
1	A (1), B(no), C(no), D (a)	5	5	3	2
2	A (1), B (yes), C (no), D (a)	9	9	9	8
3	A (1), B (no), C (yes), D (a)	9	9	9	9
4	A (1), B (yes), C (yes), D (a)	9	9	9	9
5	A (1), B (yes), C (yes), D (b)	7	7	7	7
6	A (2), B (no), C (no), D (a)	13	13	15	12
7	A (2), B (yes), C (no), D (a)	12	12	12	12
8	A (2), B (no), C (yes), D (a)	14	16	14	17
9	A (2), B (yes), C (yes), D (a)	22	22	22	22
10	A (2), B (yes), C (yes), D (b)	18	18	18	18
11	A (3), B (no), C (no), D (a)	18	18	18	18
12	A (3), B (yes), C (no), D (a)	21	27	27	27
13	A (3), B (no), C (yes), D (a)	26	25	26	26

Table 4. *Cont.*

Group		Method			
		Qt 42 °C	Ql 42 °C	Qt 37 °C	Ql 37 °C
14	A (3), B (yes), C (yes), D (a)	30	30	30	30
15	A (3), B (yes), C (yes), D (b)	29	29	29	29
Total		242	249	248	246

A: number of birds per household (1) 3–15, (2) 15–40, (3) more than 40. B: rearing other poultry specie besides chicken in the same backyard. C: rearing mammalian animal species (small ruminants, pigs etc.) besides chicken in the same backyard. D: mainly feeding leftovers (a), or mainly concentrates (b).

Table 5 shows the number of *Campylobacter* strains isolated with respect to the different epidemiological groups and the different methods applied. With the aid of the qualitative method at 42 °C more strains (n = 264) were isolated in comparison to the strains isolated by the other methods. For all methods multilinear regression points at the size of the flock as the sole factor affecting the overall abundance of the genus Campylobacter. The small size (3–15 birds) being reductive and the large size of the flock (more than 40 birds) increasing the number of isolations, regardless of the other factors.

Table 5. Number of *Campylobacter* spp. strains isolated from every group by different methods.

Group		Method			
		Quantitative 42 °C	Qualitative 42 °C	Quantitative 37 °C	Qualitative 37 °C
1	A (1), B(no), C(no), D (a)	6	5	4	4
2	A (1), B (yes), C (no), D (a)	9	10	8	8
3	A (1), B (no), C (yes), D (a)	9	9	9	9
4	A (1), B (yes), C (yes), D (a)	7	8	7	8
5	A (1), B (yes), C (yes), D (b)	7	8	7	8
6	A (2), B (no), C (no), D (a)	13	15	15	15
7	A (2), B (yes), C (no), D (a)	11	12	12	12
8	A (2), B (no), C (yes), D (a)	14	16	14	17
9	A (2), B (yes), C (yes), D (a)	22	22	22	22
10	A (2), B (yes), C (yes), D (b)	18	18	18	18
11	A (3), B (no), C (no), D (a)	27	28	27	28
12	A (3), B (yes), C (no), D (a)	27	27	27	27
13	A (3), B (no), C (yes), D (a)	26	26	26	26
14	A (3), B (yes), C (yes), D (a)	30	30	30	30
15	A (3), B (yes), C (yes), D (b)	30	30	30	30
	Total	256	264	256	262

A: number of birds per household (1) 3–15, (2) 15–40, (3) more than 40. B: rearing other poultry specie besides chicken in the same backyard. C: rearing mammalian animal species (small ruminants, pigs etc.) besides chicken in the same backyard. D: mainly feeding leftovers (a), or mainly concentrates (b).

Table 6 and Figures 1–4 present the frequencies of isolations of *Campylobacter* spp. from the tissues of the birds with respect to the different epidemiological groups and the different methods. The number of isolations is different from the number of strains (Table 4) because a strain could be present at the same time in the skin and/or in the pectoral muscle and/or in the visceral cavity and the liver. Both qualitative methods (at 42 °C and at 37 °C) managed to produce more isolations than the quantitative ones. Yet for all methods, the skin was the tissue with most isolations and then followed the pectoral muscle and finally the visceral cavity and the liver. Figures 5–7 show in a comparative manner the isolations per tissue for all the epidemiological groups. These tables and figures demonstrate that the discrepancies between the different analytical methods are more intense in groups where the *Campylobacter* strains are more abundant.

Table 6. Isolations of *Campylobacter* spp. strains from the different tissues with respect to the group and to the method.

Method	**Quantitative 42 °C**			**Qualitative 42 °C**			**Quantitative 37 °C**			**Qualitative 37 °C**		
Tissue	**S** [a]	**P** [b]	**V** [c]	**S**	**P**	**V**	**S**	**P**	**V**	**S**	**P**	**V**
Group	*Campylobacter* strains (n)											
1 *	4	0	1	4	0	0	3	0	1	4	0	1
2	4	4	2	6	5	2	3	4	2	5	6	2
3	7	6	1	8	6	2	6	6	1	8	6	2
4	8	2	2	8	3	2	8	3	2	8	3	2
5	7	3	0	8	4	0	8	2	0	8	4	0
6	10	2	5	11	5	7	10	3	2	13	7	5
7	11	2	1	10	4	1	8	3	3	11	5	2
8	10	6	1	14	9	1	9	6	1	14	9	1
9	14	7	1	21	13	1	13	6	0	20	14	1
10	8	5	0	17	5	0	9	5	0	15	5	0
11	10	3	1	18	12	3	9	2	1	16	10	2
12	11	4	1	20	6	2	10	4	1	20	6	2
13	12	5	0	21	11	0	10	4	0	20	12	0
14	12	7	1	29	11	1	12	7	1	28	11	1
15	12	8	0	27	12	1	12	8	0	27	13	1
Total	140	64	17	222	106	23	130	63	15	217	111	22

[a]: skin; [b]: pectoral muscle; [c]: visceral cavity/liver. *: Group 1, 2, 3 . . . 15 as they appear in Tables 4 and 5.

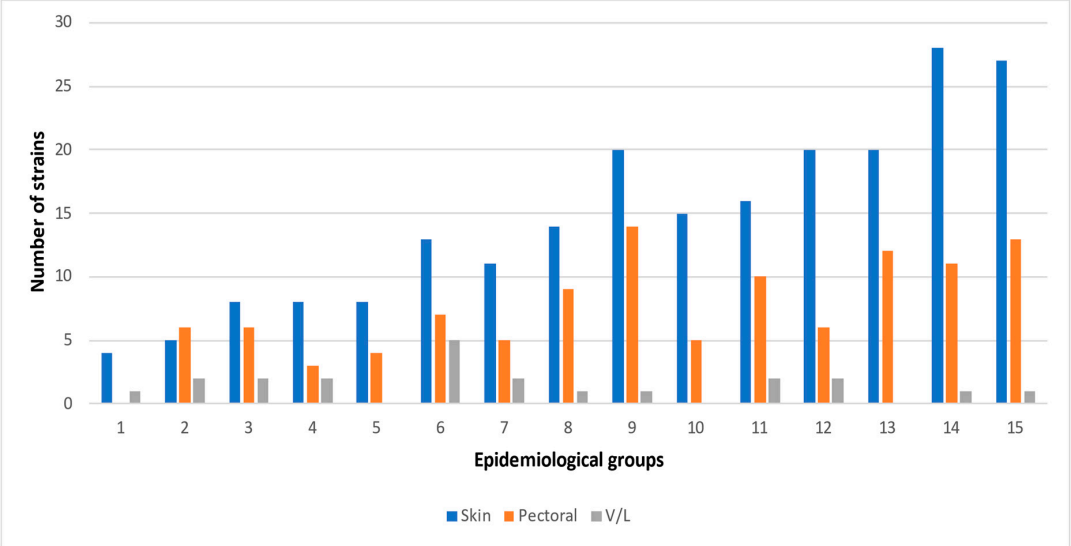


Figure 1. *Campylobacter* spp. strains isolated by the qualitative method at 37 °C from the skin, the pectoral muscle and the visceral cavity, and the liver and their distribution in the epidemiological groups. (Enumeration of the epidemiological groups as in Tables 3–6); V/L: visceral cavity/liver.

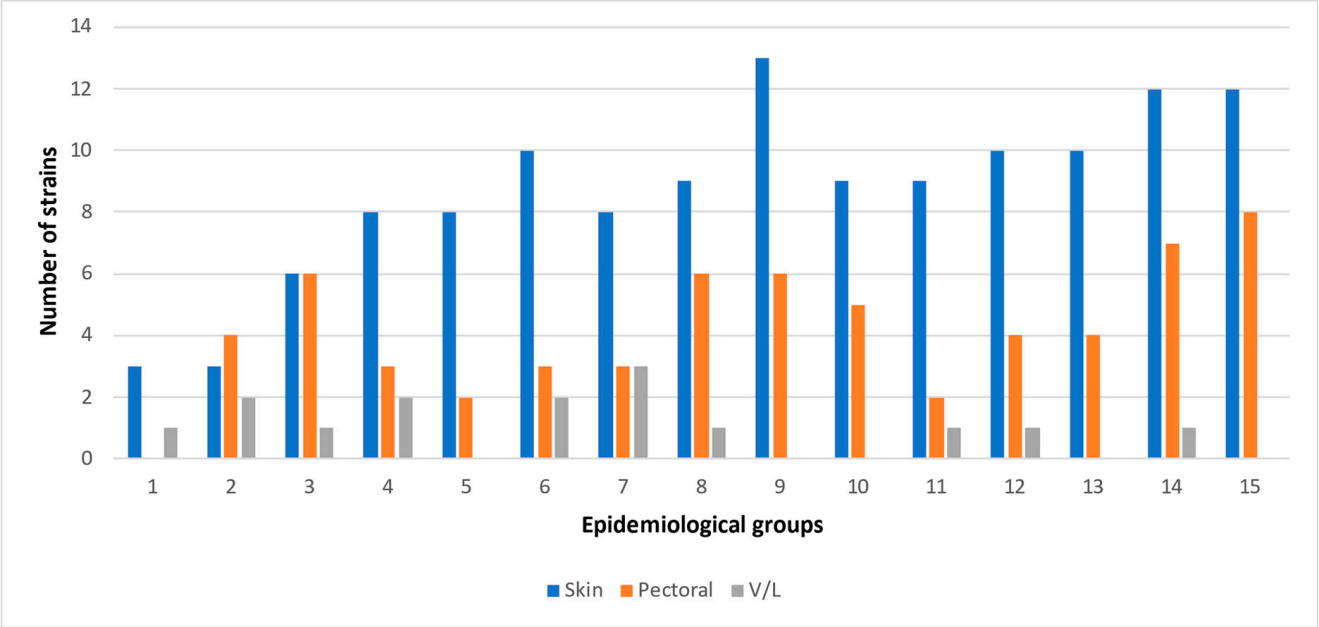


Figure 2. *Campylobacter* spp. strains isolated by the quantitative method at 37 °C from the skin, the pectoral muscle and the visceral cavity, and the liver and their distribution in the epidemiological groups. (Enumeration of the epidemiological groups as in Tables 3–6); V/L: visceral cavity/liver.

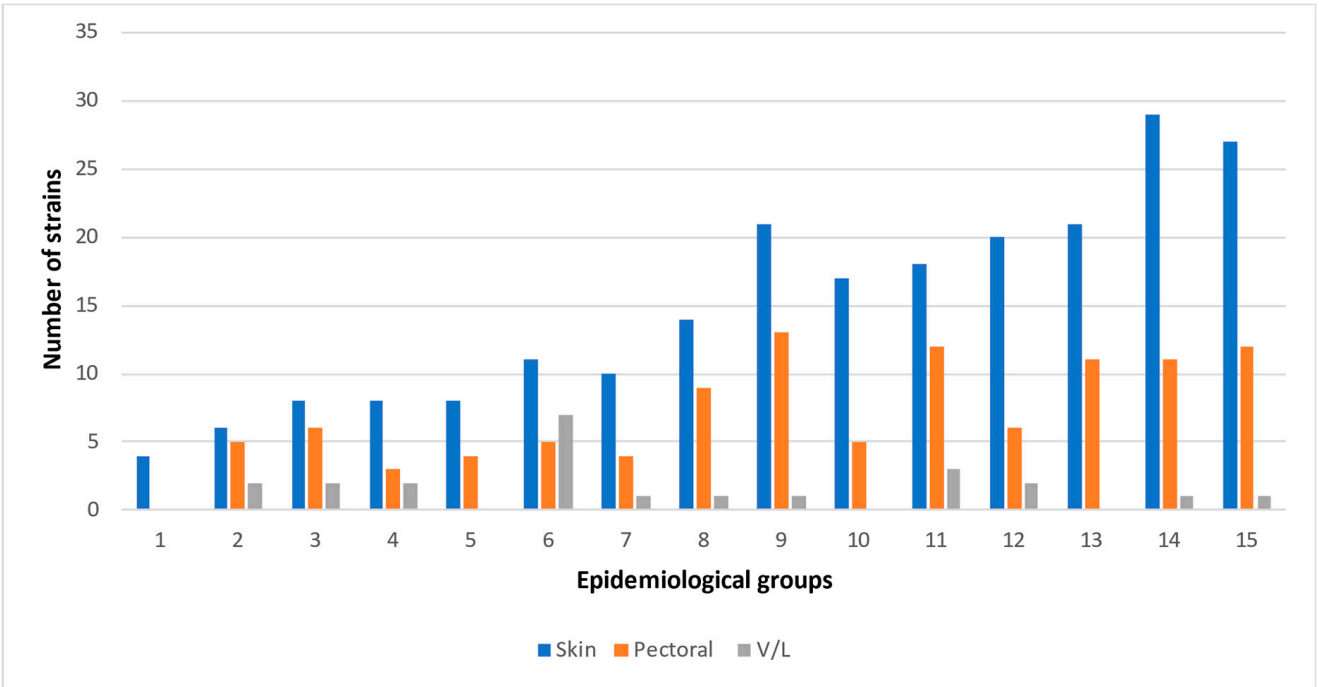


Figure 3. *Campylobacter* spp. strains isolated by the qualitative method at 42 °C from the skin, the pectoral muscle and the visceral cavity, and the liver and their distribution in the epidemiological groups. (Enumeration of the epidemiological groups as in Tables 3–6); V/L: visceral cavity/liver.

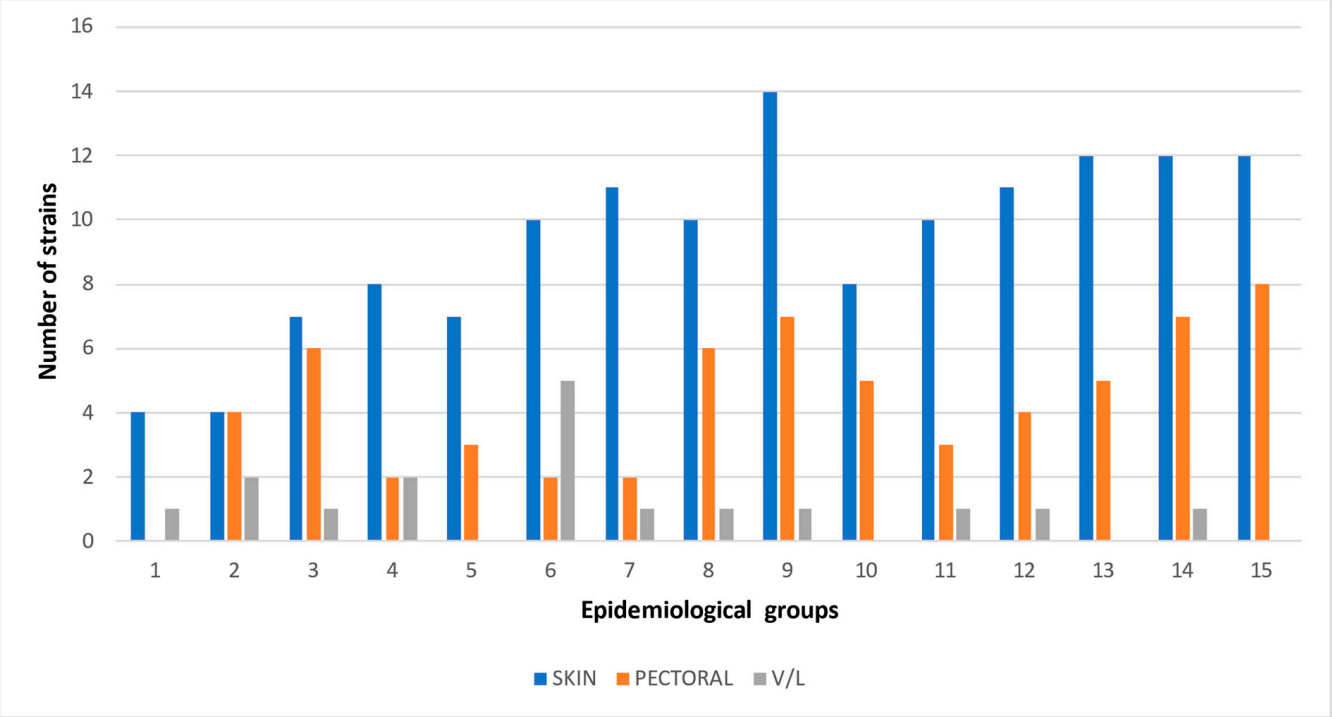


Figure 4. *Campylobacter* spp. strains isolated by the quantitative method at 42 °C from the skin, the pectoral muscle and the visceral cavity, and the liver and their distribution in the epidemiological groups. (Enumeration of the epidemiological groups as in Tables 3–6); V/L: visceral cavity/liver.

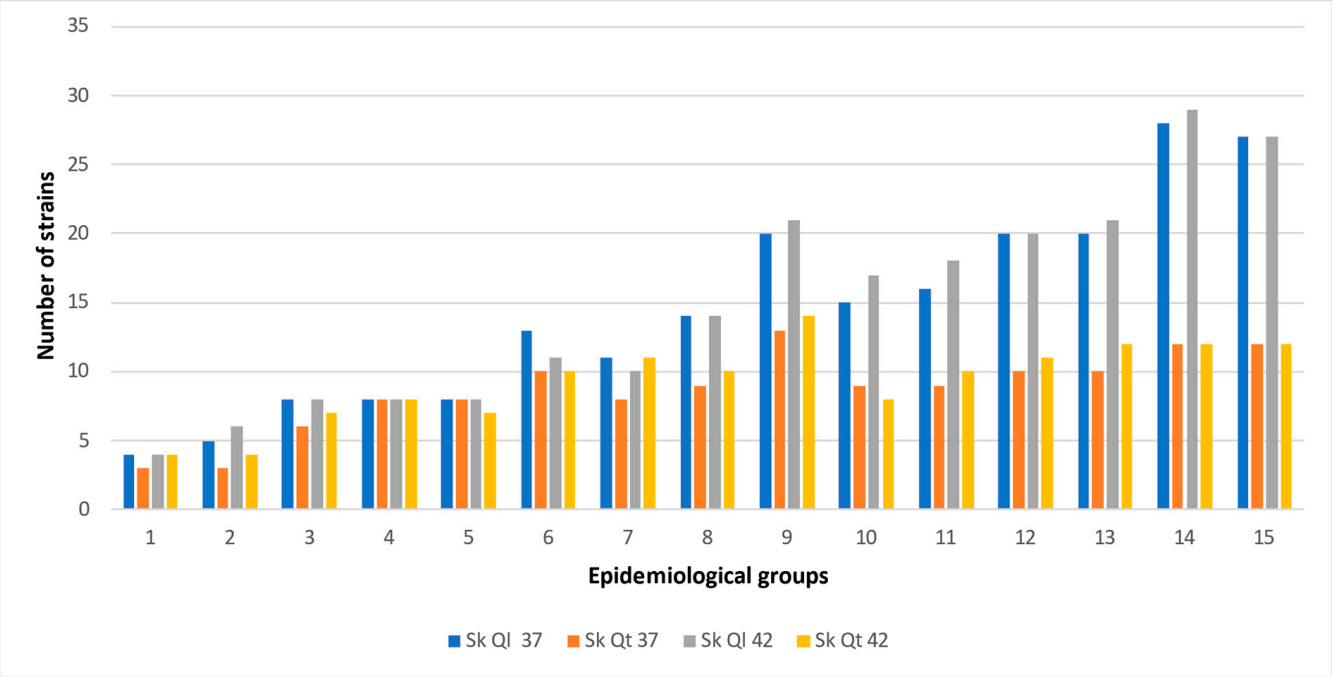


Figure 5. *Campylobacter* spp. strains isolated from the skin and their distribution in the epidemiological groups. (Enumeration of the epidemiological groups as in Tables 3–6). Sk Ql 37 °C: Skin qualitative method at 37 °C, Sk Qt 37 °C: Skin quantitative method at 37 °C, Sk Ql 42 °C: Skin qualitative method at 42 °C, Sk Qt 42 °C: Skin quantitative method at 42 °C.

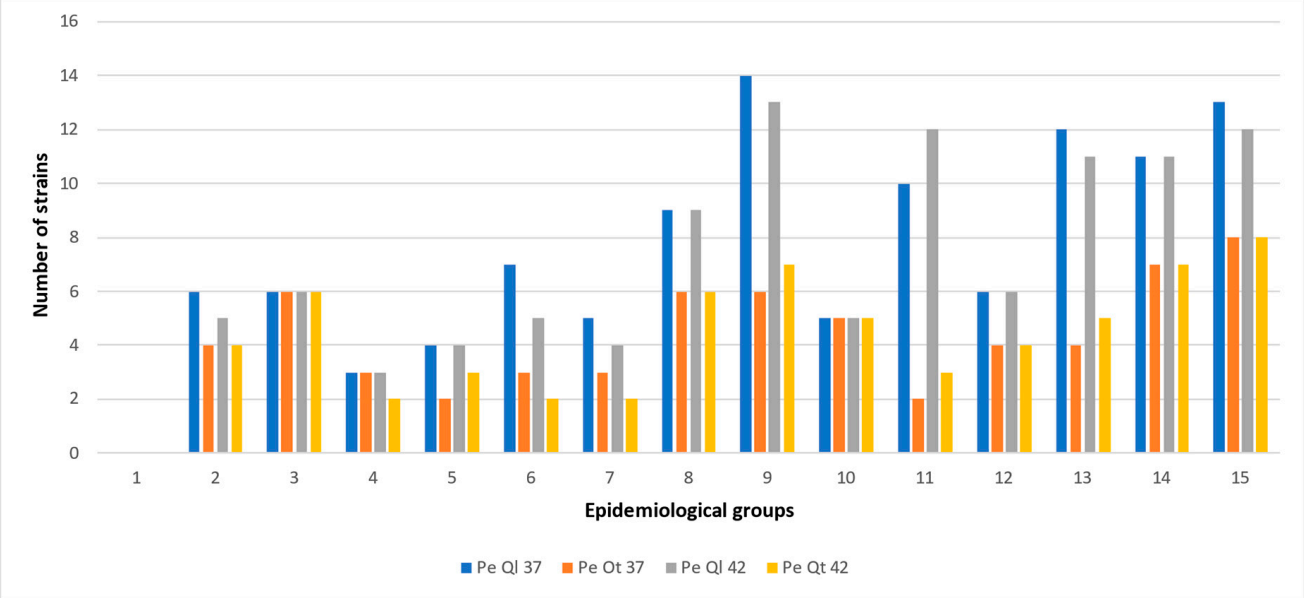


Figure 6. *Campylobacter* spp. strains isolated from the pectoral muscle and their distribution in the epidemiological groups. (Enumeration of the epidemiological groups as in Tables 3–6). Pe Ql 37 °C: Pectoral muscle qualitative method at 37 °C, Pe Qt 37 °C: Pectoral muscle quantitative method at 37 °C, Pe Ql 42 °C: Pectoral muscle qualitative method at 42 °C, Pe Qt 42 °C: Pectoral muscle quantitative method at 42 °C.

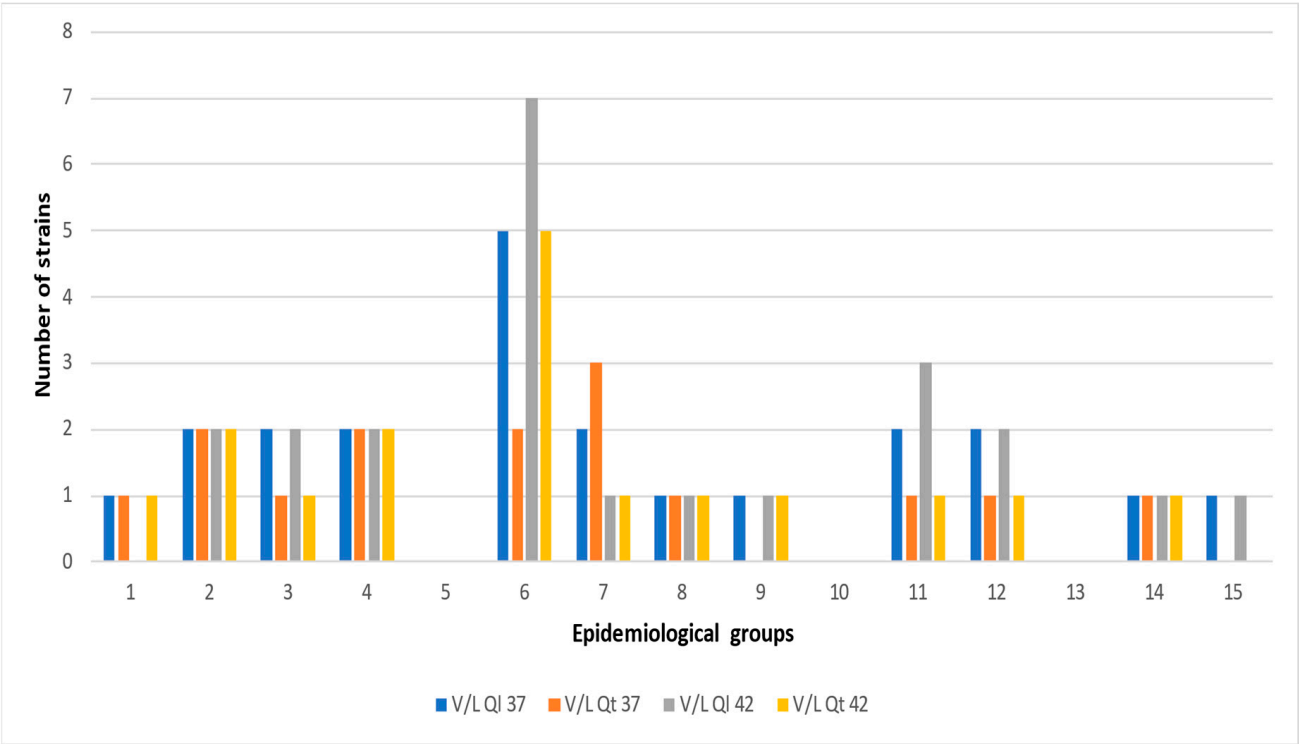


Figure 7. *Campylobacter* spp. strains isolated from the visceral cavity and the liver and their distribution in the epidemiological groups. (Enumeration of the epidemiological groups as in Tables 3–6). **V/L Ql 37 °C**: Visceral cavity/liver qualitative method at 37 °C, **V/L Qt 37 °C**: Visceral cavity/liver quantitative method at 37 °C, **V/L Ql 42 °C**: Visceral cavity/liver Qualitative method at 42 °C, **V/L Qt 42 °C**: Visceral cavity/liver Quantitative method at 42 °C.

Regression analysis showed that where different analytical methods produced different results, the statistical inference of the impact of the four criteria on the number of isolations could also be different. In the skin, the number of isolated *Campylobacter* strains was negatively affected by the small size of the flock (3–15 birds) for all methods. According to the qualitative method at 37 °C, the quantitative method at 37 °C, and the qualitative at 42 °C (but not the quantitative at 42 °C), the presence of small ruminants was very strongly positively correlated to the number of isolated *Campylobacter* ($R > 0.883$, $p < 0.001$). Likewise, for all methods except for the quantitative at 37 °C, the number of isolations from the pectoral muscle was strongly negatively correlated to the small size of the flock (3–15 birds) while for both quantitative methods, the presence of small ruminants was also strongly positively correlated ($R > 0.604$, $p < 0.05$). Regression analysis showed no significant correlations for the isolations by both qualitative and quantitative methods at 42 °C from the visceral cavity and the liver but moderate negative ($R = 0.588$, $p = 0.02176$) and strong negative ($R = 0.608$, $p = 0.01606$) correlations with the presence of small ruminants for the qualitative method at 37 °C and for the quantitative method at 37 °C, respectively.

Since the qualitative method at 42 °C was the most prolific and revealed the wider range of *Campylobacter* biodiversity, a further analysis was performed in the most abundant species as they are shown in Table 7. *C. jejuni* isolated from skin was very strongly positively correlated to the small size of the flock (3–15 birds) and to the presence of small ruminants ($R = 0.811$, $p = 0.001586$) while for *C. jejuni* isolated from the pectoral muscle, a strong positive correlation to the large size of the flock (more than 40 birds) and to the presence of small ruminants was observed ($R = 0.739$, $p = 0.008737$). *C. coli* isolated from skin showed a very strong negative correlation to the small size of the flock (3–15 birds) and a very strong positive correlation to the large size of the flock (more than 40 birds) ($R = 0.846$, $p = 0.000535$), while *C. coli* isolated from the pectoral muscle did not show any significant correlation to any of the four criteria. *C. avium* isolated from the skin showed a strong negative correlation to the presence of small ruminants ($R = 0.654$, $p = 0.008164$), *C. fetus* showed a strong positive correlation to the presence of small ruminants, and *C. lari* showed a moderate positive correlation to the large size of the flock ($R = 0.533$, $p = 0.04077$).

Table 7. Distribution of the most abundant *Campylobacter* species with respect to the various groups and tissues.

	Group	c/s[a]	j/s[b]	a/s[c]	f/s[d]	l/s[e]	c/p[f]	j/p[g]
1	A (1), B(no), C(no), D (a)	3	0	0	0	0	0	1
2	A (1), B (yes), C (no), D (a)	2	2	2	0	0	3	1
3	A (1), B (no), C (yes), D (a)	1	5	0	0	0	2	3
4	A (1), B (yes), C (yes), D (a)	1	3	0	2	0	2	1
5	A (1), B (yes), C (yes), D (b)	1	3	1	1	2	1	1
6	A (2), B (no), C (no), D (a)	2	3	3	0	0	1	1
7	A (2), B (yes), C (no), D (a)	4	2	2	0	1	2	0
8	A (2), B (no), C (yes), D (a)	3	5	0	4	0	1	5
9	A (2), B (yes), C (yes), D (a)	4	9	0	5	3	1	8
10	A (2), B (yes), C (yes), D (b)	5	7	0	2	1	1	2
11	A (3), B (no), C (no), D (a)	8	0	3	0	0	3	0
12	A (3), B (yes), C (no), D (a)	8	9	1	0	4	0	6
13	A (3), B (no), C (yes), D (a)	5	10	0	2	2	1	6

Table 7. *Cont.*

	Group	c/s[a]	j/s[b]	a/s[c]	f/s[d]	l/s[e]	c/p[f]	j/p[g]
14	A (3), B (yes), C (yes), D (a)	6	17	0	2	2	2	7
15	A (3), B (yes), C (yes), D (b)	4	17	2	0	3	1	8
Total		57	92	14	18	18	21	49

c/s[a]: *C. coli*, skin; j/s[b]: *C. jejuni*, skin; a/s[c]: *C. avium*, skin; f/s[d]: *C. fetus*, skin; l/s[e]: *C. lari*, skin; c/p[f]: *C. coli*, pectoral muscle; j/p[g]: *C. jejuni*, pectoral muscle. *A: number of birds per household (1) 3–15; (2) 15–40; (3) more than 40. B: rearing other poultry species besides chicken in the same backyard. C: rearing mammalian animal species (small ruminants, pigs, etc.) besides chicken in the same backyard. D: mainly feeding leftovers; (a) or mainly concentrates (b).*

The distribution of the isolated strains by the qualitative method at 42 °C among the three tissues was significantly different only for *C. hepaticus* ($x^2 = 7.616$, $p = 0.02219$), *C. coli* ($x^2 = 5.384$, $p = 0.02032$) and *C. jejuni* ($x^2 = 10.616$, $p = 0.00495$) (Table 6).

4. Discussion

In this study, four criteria were used to assess the prevalence of *Campylobacter* species. Given that the research on this topic focuses almost entirely on industrial broilers and not on rural backyard chickens, the design of this study was similar to a navigation in uncharted waters. Rural husbandry and slaughter practices may vary significantly among different households. It is practically impossible to classify in detail all the different factors involved, so we formulated these four criteria with deductive logic. In Criterion A, the number of chickens in the household referred to the density of the birds and intraspecies transmission. Yet, one household may rear few birds but in close confinement while another household may rear more birds but in a larger space. In Criteria B and C, the presence in the same household of other species of poultry and of small ruminants respectively, were used to investigate possible interspecies transmission of *Campylobacter* strains. Of course, here too other factors are involved such as the size of the flock of the birds or of the small ruminants, the husbandry practices, or the degree of the contact. Finally, in Criterion D, the feeding practice (leftovers or concentrates) is important not only for the possibility of *Campylobacter* transmission through feeds, but also by affecting the general condition of the chicken. A further analysis of this criterion could involve factors such as the amount and the composition of concentrates fed. It is needless to say that further research is required and that the present research has also the character of a pilot study.

It seems that humans and chickens, despite their immense biological differences, share many *Campylobacter* species. The latter have specific growth requirements, regarding the temperature and this fact can be a limiting factor for their survival outside warm-blooded animals, but not an eliminating one. Their optimal growth is at either 42 °C (body temperature of chicken) or at 37 °C (human body temperature). A known epidemiological problem is the simultaneous presence of various *Campylobacter* species or types in human infections. What happens in such cases is that the diagnostic laboratories usually isolate and identify a single colony, missing thus the whole etiological picture of an outbreak. Likewise, when isolating *Campylobacter* from foods, enrichment should be performed at both temperatures, otherwise some strains present will not be identified.

Isolating *Campylobacter* spp. from samples such as raw food and environmental samples, which contain a plethora of species can be compromised by the antagonistic and inhibitory presence of more dominant species. Although the *Campylobacter* species have specific requirements for growth (such as temperature, pH, and microaerophile conditions) which make their survival not an easy task, yet they survive in environments where they are not expected, such as in refrigerated food. The *Campylobacter* species have been characterized as thermotolerant [27], or even thermophile due to its optimum growth at 41.5 °C. They cannot grow at temperatures lower than 30° [28]. Actually, the term "the *Campylobacter* conundrum" refers to this paradox. The very basic purpose of this study was to investigate the *Campylobacter* populations in free-grazing chicken in the rural

environment. The skin of birds living outdoors is not the ideal niche for the *Campylobacter* species and this is the main reason that all accepted standard methods [24], both qualitative and quantitative were employed. The possibility of isolation of rare species in low counts or the existence of *Campylobacter* traumatized bacterial cells, underlined the necessity of an enrichment step before plating on solid agar [29,30]. A single ideal approach for the isolation of all *Campylobacter* species does not exist since some less-common species are inhibited by the selective media or at the incubation at 42 °C [7,31,32]. Therefore, where the occurrence of such organisms is expected or investigated, suitable cultivation conditions are required, such as membrane filtration, special atmospheric and temperature conditions, prolonged incubation, and subsequent plating on non-selective media [33,34].

Any references to skin or to meat contamination by *Campylobacter* spp. found in literature, refer to skin samples or meat samples originating from slaughter facilities or retail establishments but none from samples of rural origin. The reasons are understandable since (a) *Campylobacter* infections are among the principal causes of foodborne illnesses in urban areas where people consume industrial broilers and (b) industrial poultry meat targets the global food market with an annual turnover of billions of US$, while the rural poultry is consumed by local populations and visitors affecting much less both public health and economy. This may well be true, but the backyard chicken is remaining a global culinary practice, and the rural populations are still in danger of *Campylobacter* infections not only through ingestion of contaminated meat but also through direct contact with the birds or from the environment [35].

The occurrence rates of *Campylobacter* spp. in the birds of the present study varied depending on the analytical method and were calculated 40.33% for the quantitative method at 42 °C, 41.50% for the qualitative method at 42%, 41.33% for the quantitative method at 37 °C, and 41.00% for the qualitative method at 37 °C. These rates are far below the nearly 100% prevalence rate reported in the European industrial broiler farms [36,37] and USA broiler farms [38–40], or even from 60.30% in Sub-Saharan Africa [41], but higher than 0.6–13.1% reported in Northern European countries [42]. However, all these studies refer to samples from caeca or to fecal samples where the conditions are ideal for the main thermophilic *Campylobacter* species, and not to skin or to meat samples taken in the field as in our study [43,44].

A most obvious observation concerning our study is that three criteria out of the four, i.e., the presence of other species of poultry (criterion B), the presence of small ruminants (criterion C), and the feeding practice (criterion D) had no effect on the prevalence and on the distribution of the overall *Campylobacter* genus among the different epidemiological groups (Table 3). On a species level, however, some species were significantly affected by the presence of small ruminants. Criterion D (feeding practice), besides the possibility of transmission of *Campylobacter* from the environment through animal feed to poultry farms [13] was theoretically expected to affect indirectly the general condition of the birds since the concentrates provide more nutrients than the household's leftovers of plant origin and consequently the immune system would function more efficiently keeping at bay at least some *Campylobacter* species. However, in rural areas, the birds graze freely and acquire whatever they need from the natural environment of the estate they live, and this appears to equalize the differences in the feeding practices. As for the presence of other species of poultry, it seems that birds that share the same habitat regardless of their species, carry similar microbiomes and so the same microorganisms circulate among them. Ethology factors perhaps could limit the close contact of birds of different species creating thus a sort of intraspecies barrier.

Small ruminants are a source of various *Campylobacter* species, shedding strains in their environment [45,46]. Possible routes of transmission are considered the pests (rodents mainly) and the personnel. Other authors however, like Ridley et al. (2011) propose a two-way direction of the *Campylobacter* contamination and they report an interaction between broilers and ruminants [47].

C. fetus colonizes the intestines of cattle and sheep which act as the reservoir of the microorganism [48,49]. It is a well-known pathogen in these species causing abortion and infertility [50,51]. Regression analysis confirmed the very strong correlation of *C. fetus* to the presence of small ruminants among the epidemiological groups (R = 0.814, $p = 0.0059$), a rather expected finding. In fact, *C. fetus* strains were only isolated from epidemiological groups where small ruminants were present in the same household as chicken. Yet in the same regression model, the large and the small number of birds were strongly negatively correlated to the number of strains of *C. fetus*, a finding implying that the size of the flock creates an ecosystem where the dynamics of antagonistic species regulate the prevalence rates of *C. fetus*. According to all four methods, 19 strains were isolated in total. Most of the isolations were from the skin and none from the visceral cavity and the liver. It is possible that this increased skin infection is due to environmental contact with small ruminants' feces. By the qualitative method at 42 °C distribution (Table 6), the correlation to the presence of the small ruminants was strong (R = 0.629, $p = 0.012$).

The overall *C. jejuni* prevalence among the different groups was very strongly positively correlated to the large size of the flock (more than 30 birds) and to the presence of small ruminants (R = 0.832, $p < 0.001$). *C. jejuni* is known to colonize the digestive tract of warm-blooded hosts [48] infecting thus the poultry. Depending on the method, 96 to 98 strains were isolated making *C. jejuni* the most prevalent of all *Campylobacter* species (Table 2), a finding agreeing with similar findings of other authors who reported that this species is the most dominant and poultry is considered as its primary reservoir [49,50]. The distribution of *C. jejuni* strains was found significantly increased in skin as compared to the one in the pectoral muscle by the qualitative method at 42 °C ($x^2 = 10.6162$, $p = 0.004851$) implying environmental contamination.

C. coli was the second dominant species in this study (Table 2) with 64 strains (both methods at 42 °C) or 63 strains (both methods at 37 °C). None of these strains was isolated from the visceral cavity and the liver. Most of the isolations were from the skin ($x^2 = 5.3841$, $p = 0.0203$) as in the case of all other *Campylobacter* species. Interestingly, the quantitative methods at both temperatures (42 °C and 37 °C) revealed a strong positive correlation (R = 0.789, $p = 0.003$) to the the large size of the flock (more than 30 birds) while the qualitative methods (42 and 37 °C) showed a strong negative correlation (R = 0.725, $p = 0.002$) to the small size of the flock (3–10 birds). These differences can be attributed to the different distribution of strains according to the different analytical methods, but what these findings have in common is that the multitude of chickens in the flock plays an important role.

Although *C. coli* and *C. jejuni* are reported to survive in the guts of various mammals as well as in other poultry species caeca, our results do not show any increased prevalence in the epidemiological groups where small ruminants and/or other poultry species were present, implying that other environmental sources of contamination like rodents or the water.

C. avium has been isolated from chicken and turkeys [43,51–54] yet our results do not show any increased prevalence in the groups where other poultry species shared the same environment. All four analytical methods we used, concur that the presence of small ruminants was strongly negatively correlated with its prevalence rates ($R > 0.721$, $p < 0.002$), suggesting intense antagonistic dynamics from other *Campylobacter* strains shed by these animals.

C. lari was third after *C. jejuni* and *C. coli* prevalent species with twenty-four (24) isolated strains by three of the four methods (Table 2). This species is usually isolated from various sea birds like seagulls, from marine mammals and has been also isolated from domestic animals [55]. In our study, its isolations have been positively strongly correlated (R = 0.702, $p = 0.00284$) to the large size of the flock (more than 30 birds). As previously discussed for other species, the large size of the flock creates perhaps more opportunities for certain species to find niches.

C. hepaticus is a causative agent of Spotty Liver Syndrome (SLD), a disease of free-range laying hens [56,57]. All birds in our study were clinically healthy and obviously, the

ones from which *C. hepaticus* was isolated were asymptomatic carriers. Most isolations occurred from liver samples. *C. hepaticus* shows lower growth rates in iron-depleted environments and this explains its tissue tropism [58]. Regression analysis showed a strong positive correlation to the large size of the flock (more than 30 birds) and a strong negative correlation to the presence of small ruminants ($R = 0.742$, $p = 0.00831$).

Six species were singletons since only one strain of them was isolated (Table 2). From these *C. cuniculorum* has been isolated from rabbits [59] and probably its source in our study was rabbits kept in the same household. Since *C. rectus*, *C. helveticus*, *C. mucosalis*, and *C. showae* originate from humans and other animals such as dogs, cats, and pigs, while *C. hominis* originates from humans [51], it is quite possible that the contamination of the birds in our study was either anthropogenic or coming from the household's dog or from the omnipresent cats. Anthropogenic transmission seems likely since *C. rectus* and *C. showae* are involved in the pathogenesis of gingivitis and periodontitis [60,61]. It is interesting that in *C. rectus*, *C. hominis* and *C. helveticus*, isolated exclusively from the epidemiological group 1, where the flock was small (3–15 birds), there were neither other poultry species nor small ruminants present, and the birds were fed with leftovers. Possibly these households are small estates, and the birds live in close contact with humans and pets.

Two of the species from which two strains were isolated (Table 3), *C. gracilis* and *C. concisus*, are found in the oral cavity of humans in health and disease, e.g., periodontitis [62–64], so an anthropogenic contamination to the birds is possible, while *C. upsaliensis* has been isolated from dogs' and cats' healthy and diarrheic feces [65,66].

C. sputorum has been isolated from cattle, sheep, humans, and dogs [67], providing plenty of opportunities for transmission to birds, particularly in a rural environment. In our study, all four strains (Table 3) have been isolated from groups 3, 8, and 13, in which small ruminants were present. Similarly, *C. hyointestinalis* has been isolated from cattle, sheep, and deer [68,69] and in the present study, all four strains (Table 3) were isolated from groups 8, 13, and 14, where small ruminants are present. On the contrary, *C. ureolyticus* was isolated from groups 6 and 11 where other poultry species and ruminants were not present. This species has been isolated from cattle and horses [54] as well as from humans suffering from periodontitis and other diseases [70].

5. Conclusions

Chicken-rearing practices in rural households are very different and adapt to different environments in comparison to the more steadfast industrial ones. Hence, the research is more complicated, since there are many factors involved. From the present study, the following results were obtained:

1. Eighteen species of the *Campylobacter* genus have been isolated from the free grazing chicken in the rural environment, an impressive abundance that suggests that this genus can survive in more environmental niches than has been thought.
2. The isolation of *Campylobacter* strains raises public health issues as well as animal health issues, concerning rural nonindustrial environments.
3. The multitude of birds in the flock of chickens was the most decisive factor which affected the prevalence of most *Campylobacter* species.
4. The presence of small ruminants in the same household significantly affected the prevalence of certain species such as *C. fetus*.
5. The presence of other species of poultry and the feeding practice (leftovers or concentrates) did not affect the prevalence of *Campylobacter* strains.
6. Although the danger to human health is possible, the anthropogenic contamination of the birds cannot be excluded, particularly for some *Campylobacter* species involved in some human oral cavity ailments such as periodontitis.
7. The qualitative methods were more proliferative in isolating the *Campylobacter* species, especially at 42 °C.
8. Rural populations must be educated on the necessity to cook chicken meat well enough and maintain good personal hygiene practices.

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