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Short communication

Wild boars as reservoir for *Campylobacter* and *Arcobacter*

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ABSTRACT

Campylobacteriosis is a significant public health concern with *Campylobacter jejuni* and *Campylobacter coli* as main causative agents. Moreover, there is an increasing recognition of other pathogenic *Campylobacter* species and *Campylobacter*-like organisms as *Arcobacter*. However, current knowledge on presence of *Arcobacter* species in wild boars (*Sus scrofa*) is lacking, and knowledge on *Campylobacter* species is based on methods favoring growth of thermotolerant species. In this study, fecal samples originating from 76 wild boars hunted in Campania region (Italy) were examined for the presence of *Campylobacter*(-like) organisms by a culture dependent approach. Three isolation protocols were performed in parallel: *Arcobacter*-selective agar plates, mCCDA plates and isolation by passive filtration onto non-selective blood agar plates were used as quantitative isolation methods. Enrichment broths, i.e. *Arcobacter* selective enrichment broth, Preston broth and CAT broth were used for qualitative detection of low levels or stressed *Campylobacter*(-like) organisms. The *Arcobacter* and *Campylobacter* isolates were identified at species level using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and 16S ribosomal RNA (rRNA) sequence analysis. Overall, 41 (53.9%) of the animals excreted *Arcobacter* or *Campylobacter* while 38 (50.0%) shed *Campylobacter* and 8 (10.5%) *Arcobacter*. *Campylobacter lanienae* predominated and was isolated from 31 (40.8%) animals. No statistical difference between the age groups or gender with regard to the fecal excretion of *Campylobacter*(-like) organisms was observed. Thirty animals (39.5%) shed *Campylobacter* spp. exceeding levels of 10^3 CFU g^{-1} feces. As samples were obtained from hunted wild boars intended for consumption, a potential contamination of meat with these bacterial pathogens must be considered.

1. Introduction

The genera *Campylobacter* and *Arcobacter* are closely related Gram-negative bacteria comprising several pathogenic species. *Campylobacter* is recognized as a major global public health concern representing 50% of all reported cases of zoonotic diseases (EFSA and ECDC, 2021). The genus *Campylobacter* currently contains 39 species, with five further divided into 11 subspecies. The main causative species of campylobacteriosis are *Campylobacter jejuni* subsp. *jejuni* (hereafter referred to as *Campylobacter jejuni*) and *Campylobacter coli* (EFSA and ECDC, 2021). *Arcobacter* species were originally classified as aerotolerant campylobacters, but later allocated to a separate genus (Vandamme et al., 1991). A division of the genus into six genera was proposed (Perez-Cataluna et al., 2018), though thereafter refuted (On et al., 2021). Therefore, the classical nomenclature is used in the present study. Of the 31 *Arcobacter* species currently described, both *A. butzleri* and *A. cryaerophilus*, are classified as emerging foodborne pathogens, but other species have also been documented in human infections (Kerkhof et al., 2021b).

A wide range of natural hosts often harbor *Arcobacter* and *Campylobacter* spp., but isolation from their feces requires specialized selective media and optimized incubation conditions. It also depends on the levels of *Arcobacter* or *Campylobacter* spp. and other microbiota present in the sample (Biesta-Peters et al., 2019). Limited information is presently available concerning the presence of *Arcobacter* and *Campylobacter* spp. in game, more specific in wild boar (*Sus scrofa*). In Italy, wild boar is the most widespread wild ungulate, and meat thereof is the main game food consumed (Peruzy et al., 2019). In the present study, the aim was to assess if Italian wild boar act as possible reservoir

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of *Campylobacter* and *Arcobacter*, using three culture-dependent isolation methods followed by identification using matrix-assisted laser desorption/ionization time-of-flight mass (MALDI-TOF MS) spectrometry analysis.

2. Material and methods

2.1. Geographical study area and sample collection

From October to December 2019, fresh fecal samples of 76 wild boars were collected in different municipalities of four provinces (Avellino, Benevento, Caserta, and Salerno) of the region Campania, southern Italy (Fig. 1). On several occasions, wild boars were shot by certified hunters and immediately bled in the field. The boars were collected in accordance with Italian and EU legislation within the routine sanitary surveillance project "*Wild Boar Emergency Plan in Campania* – 2016–2019". The information about sex, relative age, and weight of each animal were recorded and shown in Supplementary Table 1. The boars were classified into yearlings (1–2 years)i ($n = 42$, 55.3%); and adults (>2 years) ($n = 34$, 44.7%), with a mean age of 2.6 years (± 1.6 SD). The animals were brought to collection places where the evisceration and skinning were performed. Subsequently, rectal fecal samples were collected using sterile gloves and individually placed in sterile stomacher blender bags or sample containers. The samples were sent to the Laboratory of Microbiology, Ghent University within a maximum of 24 h after collection, and processed immediately after being received via post. Due to lack of data in literature about the presence of *Arcobacter* in wild boar feces, on each delivery, a positive controle sample was included by spiking one additional fecal subsample with *Arcobacter butzleri* LMG 10828^T at a final concentration of about 10^3 to 10^4 CFU g^{-1}, to evaluate the impact of shipping.

2.2. Isolation of Campylobacter and Arcobacter spp

Per sample, 3 times 5 g was aseptically added to sterile stomacher bags, ten-fold diluted in 45 ml of the following three broths: *Arcobacter* selective enrichment broth (ASB) for the isolation of *Arcobacter* spp., and Preston and CAT (Cefoperazone, Amphotericin B and Teicoplanin) broth for the isolation of the thermotolerant and non-thermotolerant *Campylobacter* spp., respectively. The *Arcobacter* selective enrichment broth contained (l^{-1}) 24 g *Arcobacter* broth (CM 0965, Oxoid, Basingstoke, United Kingdom), 50 ml lysed defibrinated horse blood (International Medical Products, Brussels, Belgium), and a selective supplement, previously developed by Van Driessche et al. (2003), comprising (l^{-1}) 100 mg cycloheximide (C7698, Sigma, St. Louis, USA), 100 mg 5-fluorouracil (F6627, Sigma), 10 mg amphotericin B (A4888, Sigma), 16 mg cefoperazone (C4292, Sigma), 32 mg novobiocin (N1628, Sigma) and 64 mg trimethoprim (T0667,Sigma). Preston broth contained Nutrient Broth No.2 (CM0067, Oxoid), *Campylobacter* selective supplements (polymyxin B, rifampicin, trimethoprim, and cycloheximide) (SR0117, Oxoid) and 5% lysed defibrinated horse blood. CAT broth consisted of Tryptone Soy Broth (CM0129, Oxoid) supplemented with (l^{-1}) 50 ml lysed defibrinated horse blood and C.A.T. antibiotic supplement (SR0174, Oxoid), i.e., (l^{-1}) 8.0 mg cefoperazone, 10.0 mg amphotericin B, and 4.0 mg teicoplanin. All subsamples were homogenized for 3 min at 230 rpm using a peristaltic homogenizer (Stomacher®

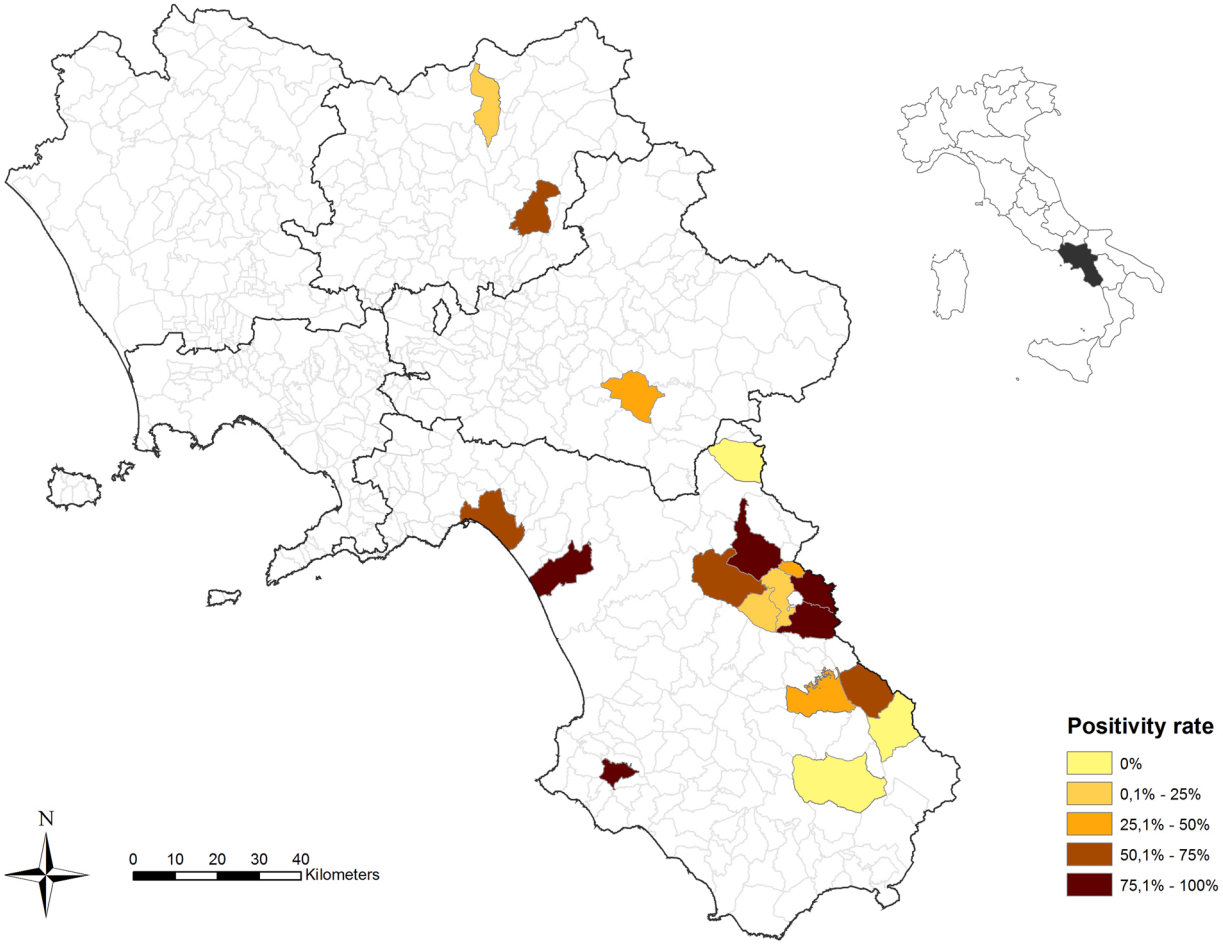

Fig. 1. Map of sampling locations and distribution of animals excreting *Campylobacter*(-like) organisms in the provinces of Campania, Italy. The map was generated in ARCGIS 10.8 software ARCGIS® (ESRI).

400 Circulator machine, Seward, UK).

To assess the level of *Campylobacter*(-like) organisms, quantitative analyses were performed. For this, 100 µl of each ASB homogenate was directly inoculated onto two *Arcobacter*-selective agar (ASA) plates by streak plating, and a loopful (10 µl) of each Preston broth homogenate was spread onto modified cefoperazone charcoal deoxycholate agar plates (mCCDA; CM0739, Oxoid) supplemented with the corresponding supplement (SR0155E, Oxoid). The ASA plates were incubated for 48 h up to 72 h at 28 °C while mCCDA plates were incubated at 37 °C for 24–48 h. Furthermore, six drops of each CAT broth homogenate were transferred onto the surface of a 47 mm and 0.6 µm pore size Whatman Nuclepore polycarbonate filter (GE Healthcare, Chicago, United States), placed on a blood agar plate (CM0331, Oxoid), and allowed to passively pass through the filter at 35 °C for one hour under microaerobic atmosphere (Steele and McDermott, 1984). Care was taken not to let the drops spill over the edge of the membrane. Then, the filter was removed and plates incubated further for up to 48 h. The detection limits were 10^2 CFU g^{-1} for ASA, and 10^3 CFU g^{-1} for both mCCDA and CAT. All plates were incubated in a jar under microaerobic atmosphere by evacuating 80% of the normal atmosphere and introducing a gas mixture consisting of 8% CO_2, 8% H_2, and 84% N_2 into each jar. Each time, reference strains of *C. jejuni* R-30538, *C. coli* R-30532, and *A. butzleri* LMG 10828^T were included as positive controls.

To detect low numbers of *Campylobacter*(-like) bacteria, and to allow resuscitation of stressed cells, additional qualitative analyses were performed by which the remaining ASB, Preston, and CAT homogenates were incubated at 28 °C for 48 h, 37 °C for 48 h, and 35 °C for 24 h, respectively. The enriched homogenates were then plated onto agar media and incubated as described above.

After incubation, plates were examined for typical or morphologically suspected colonies without delay to avoid cultivability loss. Morphologically suspected *Campylobacter* and *Arcobacter* colonies, characterized as being small, translucent, greyish, flat, and moist, sometimes with metallic sheen and tendency to spread, were picked and subcultivated on blood agar plates for further identification as described below.

2.3. Identification of the isolates

Identification of up to 10 morphologically suspected colonies per isolation agar plate was performed using MALDI-TOF MS by ethanol-formic acid extraction according to the manufacturer's instructions. Cell extracts (1 µl) were spotted in duplicate on a 96-well stainless-steel target plate (Bruker Daltonics, Bremen, Germany). Next, air-dried sample spots were overlaid with 1 µl of matrix solution (10 mg/ml α-cyano-4-hydroxycinnamic acid in acetonitrile: trifluoroacetic acid: MilliQ [50:2.5:47.5] water-solvent) and again left to air-dry at room temperature. Mass spectra were generated on a Microflex LT/SH smart MALDI-TOF MS platform (Bruker Daltonics) operating in linear positive ion detection mode under the Bruker FlexControl 3.4 software (Bruker Daltonics) according to the manufacturer's recommended settings. Prior to analysis, the mass spectrometer was calibrated using the Bacterial Test Standard (Bruker Daltonics). The obtained mass spectra were compared to those in the Bruker MBT Compass Library DB-7854 using MTB Compass explorer software according to the manufacturer's settings (Bruker Daltonics). The library comprised reference spectra of 91 *Campylobacter* isolates representing 20 species (i.e. *C. avium*, *C. canadensis*, *C. coli*, *C. concisus*, *C. curvus*, *C. fetus*, *C. gracilis*, *C. helveticus*, *C. hominis*, *C. hyointestinalis*, *C. insulaenigrae*, *C. jejuni*, *C. lanienae*, *C. lari*, *C. peloridis*, *C. rectus*, *C. showae*, *C. sputorum*, *C. upsaliensis*, and *C. ureolyticus*). In addition to this commercial database that contains only 13 *Arcobacter* strains, a previously published in-house developed MALDI-TOF MS database for the identification of *Arcobacter* spp. was also used. This in-house database has been validated and allows higher confidence species level identifications and significantly improved sensitivity compared to the commercial database (Kerkhof et al., 2021b). The resulting log scores indicated a high-confidence identification (a log score >2.0), low-confidence identification (a log score of 1.70–1.99), or no identification possible (log score <1.70). Spectra of morphologically suspected colonies without reliable identification by the MALDI-TOF MS analysis, were imported into BioNumerics 7.2.6 software (Applied Maths, Sint-Martens-Latem, Belgium) in order to select representatives for further analysis using 16 S ribosomal RNA (rRNA) sequencing. For this, Pearson correlation coefficient was applied and curve-based analysis was performed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering algorithm. Based on dendrogram distance level settings and best matches ranking, representative isolates were selected for subsequent 16 S rRNA gene amplification and sequencing. Genomic DNA was prepared by heating 1 µl loop of cell material at 95 °C for 15 min in 20 µl lysis buffer (0.25% (w/v) SDS and 0.05 M NaOH). Following lysis, 180 µl distilled water was added to the lysate. The 16 S rRNA gene sequence was amplified using the forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ (hybridizing at positions 8–27 according to the *Escherichia coli* numbering system) and the reverse primer 5′-AAGGAGGTGATCCAGCCGCA-3′ (positions 1541–1522). PCR products were purified using a NucleoFast 96 PCR clean-up kit (Macherey-Nagel, Eupen, Belgium). Amplicons were collected and submitted for Sanger sequencing (Eurofins, Ebersberg, Germany) with sequencing primers described by Coenye et al. (1999). Sequence assembly was performed using BioNumerics version 7.6 (Applied Maths). Taxonomic identity was assessed using the EzBioCloud identification service (Yoon et al., 2017). Confirmed colonies were stored in whole horse blood at − 80 °C.

2.4. Statistical analysis

For each studied area (municipalities), the positivity (n° positive/n° examined) was calculated and subdivided into five classes, with percentages ranging from 0 to 100. Data were imported into ESRI ArcGIS 10.8 (ESRI, Redlands, CA, USA) for mapping. A chi-squared test of independence was used to assess the differences between age and gender excretion prevalence. A value of $p < 0.05$ was considered significant.

3. Results

A total of 76 fecal samples obtained from 39 male and 37 female wild free-living boars, aged between 1 and 7 years were analyzed for the presence of *Campylobacter* spp. and *Arcobacter* spp. (Supplementary Table 1). Isolation from subsamples spiked to evaluate shipping conditions was concordant with the initial number added.

A total of 190 typical *Campylobacter* or *Arcobacter* colonies on the agar plates were subjected to identification by MALDI-TOF MS. For 155 isolates, identification at species-level was obtained. Of the remaining 35 isolates examined by 16 S rRNA gene sequencing, 33 were identified as *C. lanienae*. Two isolates with a sequence similarity of 99.69%, had a sequence similarity of 97.81% to *Campylobacter hyointestinalis* subsp. *lawsonii* CHY5^T (AF097685).

In general, 41 animals excreted at least one *Campylobacter* or *Arcobacter* species, detected by at least one of the three isolation methods applied, leading to an overall prevalence of 53.9% (Table 1). Distribution and positivity rate of *Campylobacter*(-like) excreting animals are shown in Fig. 1. A chi-square test of independence revealed no significant association between the age classes and excretion of *Campylobacter* (-like) organisms, $\chi 2$ (1, $n = 76$) 1.79; $p = 0.18$. Besides, there was no significant statistical difference in prevalence between genders $\chi 2$ (1, $n = 76$) = 0.49, $p = 0.48$.

Fecal excretion of *Campylobacter* spp. was detected in 38 (50.0%) animals of which eight excreted more then one *Campylobacter* species simultaneously. *Campylobacter lanienae* (40.8%) was observed as predominant species, followed by *C. hyointestinalis* (14.5%), *C. coli* (7.9%), *C. jejuni* (1.3%), and *C. fetus* (1.3%). In total, 30 animals had a

Table 1
Isolation rates of *Campylobacter* and *Arcobacter* spp. from wild boar fecal samples ($n = 76$) by different methods.

Media	Direct	Enrichment	Direct and/or Enrichment
Positive samples			
ASA	0 (0.0%)	8 (10.53%)	8 (10.53%)
mCCDA	22 (28.95%)	14 (18.42%)	26 (34.21%)
BA	20 (26.32%)	15 (19.74%)	27 (35.53%)
Total[a]	**30 (39.47%)**	**27 (35.53)%**	**41 (53.95%)**
***Campylobacter* spp.**			
C. lanienae			
ASA	ND	ND	ND
mCCDA	20	4	21
BA	14	10	20
Total[a]	28	13	**31 (40.79%)**
C. hyointestinalis			
ASA	ND	ND	ND
mCCDA	1	6	6
Filter	2	4	6
Total[a]	3	9	**11 (14.47%)**
C. coli			
ASA	ND	ND	ND
mCCDA	1	2	3
BA	3	1	4
Total[a]	4	3	**6 (7.89%)**
C. jejuni			
ASA	ND	ND	ND
mCCDA	1	1	1
BA	1	1	1
Total[a]	1	1	**1 (1.32%)**
C. fetus			
ASA	ND	ND	ND
mCCDA	1	1	1
BA	1	1	1
Total[a]	1	1	**1 (1.32%)**
***Arcobacter* spp.**			
A. cryaerophilus			
ASA	ND	7	**7**
mCCDA	ND	ND	ND
BA	ND	ND	ND
Total[a]	ND	7	**7 (9.21%)**
A. butzleri			
ASA	ND	1	1
mCCDA	ND	ND	ND
BA	ND	ND	ND
Total[a]	ND	1	**1 (1.32%)**
A. skirrowii			
ASA	ND	1	**1**
mCCDA	ND	ND	ND
BA	ND	ND	ND
Total[a]	ND	1	**1 (1.32%)**

ASA: Arcobacter-selective agar plates
mCCDA: modified cefoperazone charcoal deoxycholate agar plates
BA: membrane filtration on blood agar plates
ND: not detected
[a] number of positive samples with at least one isolation method

Campylobacter bacterial load of more than 10^3 CFU g^{-1} feces, detected on 22 (29.0%) and 20 (26.3%) of the directly inoculated mCCDA and blood agar plates, respectively. Plating after enrichment increased the number of positive samples by the two isolation methods to 26 (34.2%) and 27 (35.5%), respectively.

No animals excreted arcobacters above 10^2 CFU g^{-1} feces, and *Arcobacter* excretion was detected in 8 (10.5%) animals only after plating the enriched homogenates onto the ASA medium. Five of those animals excreted *Arcobacter* and *Campylobacter* simultaneously, and one animal excreted two *Arcobacter* species. The most frequently isolated species was *A. cryaerophilus* (9.2%), and the species *A. butzleri* and *A. skirrowii* were both present in 1.3% of the samples.

4. Discussion

The overall *Campylobacter* prevalence (50.0%) and dominant *Campylobacter* species reported in the present study are in accordance with a Japanese and Spanish study in which *Campylobacter* spp. were isolated from 30.0% to 40.0% of wild boar rectal samples (Carbonero et al., 2014; Sasaki et al., 2013). In another study conducted in Spain, *C. lanienae* was the most frequently detected species in urban wild boar, although at lower prevalence of 10.0% (Navarro-Gonzalez et al., 2014). One study, by Diaz et al., reported a higher overall sample prevalence (66.0%) (Diaz-Sanchez et al., 2013). In contrast, Wacheck et al. could not detect *Campylobacter* species while screening 73 wild boar fecal samples with an immunoassay in Geneva, Switzerland (Wacheck et al., 2010), and a study in Swedish wildlife only detected thermotolerant *Campylobacter* spp. in 12.0% of wild boars (Wahlstrom et al., 2003). Although the prevalence of *Campylobacter* spp. in wild boars varies among studies, it is clear that wild boars can be carriers of these bacteria.

Remarkably, the overall prevalence reported in the present study is inline with what is detected in farm-raised pigs. Nevertheless, unlike in wild boars, the dominant species in pigs is usually *C. coli* (Kempf et al., 2017).

Previous studies reporting on occurrence of *Campylobacter* species in wild boars (Carbonero et al., 2014; Diaz-Sanchez et al., 2013; Navarro-Gonzalez et al., 2014; Sasaki et al., 2013; Wacheck et al., 2010; Wahlstrom et al., 2003) primarily use methods favoring growth of thermotolerant *Campylobacter* species. By contrast, the present study applied a lower incubation temperature (35–37 °C), a hydrogen-rich microaerobic atmosphere, and a membrane filtration method to also improve isolation of other *Campylobacter* species. The lower incubation temperature permitted isolation of non-thermotolerant campylobacters, such as *C. fetus, C. hyointestinalis*, and *C. lanienae*. Microaerobic atmosphere was enriched with hydrogen needed for growth of several other *Campylobacter* species (e.g., *C. consisus, C. curvus, C. gracilis, C. mucosalis, C. rectus,* and *C. showae*). However, none of the latter species were isolated. Incubation of the isolation plates for an additional 2–4 days showed to be important as often more than one species of *Campylobacter* was present. Nevertheless, bacteria-rich matrices such as feces complicate prolonged incubation at temperatures below 42 °C by the occurrence of almost inevitable overgrowth of competitive fecal microorganisms.

All *Campylobacter* species isolated in the present study, i.e. *C. lanienae* (40.8%) *C. hyointestinalis* (14.5%), *C. coli* (7.9%), *C. jejuni* (1.3%) and *C. fetus* (1.3%), are of public health concern. Together with *C. jejuni*, *C. coli* is the common cause of bacterial diarrhoeal illness in humans (EFSA and ECDC, 2021). Both *C. hyointestinalis* and *C. fetus* may also cause gastroenteritis in humans. However, for the latter two species additional subspecies-level classification is desired as human infection is most frequently attributed to certain subspecies only. Finally, the most frequently isolated species in this study, *C. lanienae*, was initially considered to be a non-pathogenic commensal in humans, but case reports of *C. lanienae*-associated enteritis indicated its pathogenic potential (Fornefett et al., 2021).

Arcobacters have been isolated from healthy farm animals (pigs, cattle, sheep, and horses) (Ramees et al., 2017), and *Arcobacter* excretion in domestic pigs can be as high as $\geq 10^4$ CFU g^{-1} feces (Van Driessche et al., 2004). In the present study, three *Arcobacter* species; *A. cryaerophilus* (9.2%), *A. butzleri* (1.3%), and *A. skirrowii* (1.3%) were isolated from wild boar feces resulting in a total prevalence of 10.5% ($n = 8$). Generally, the same three species (*A. butzleri*, *A. cryaerophilus*, and *A. skirrowii*) are frequently isolated throughout the conventional pig production chain where, in contrast to our findings, *A. butzleri* is the dominant species (Van Driessche et al., 2004). However, there is evidence that pigs carry other *Arcobacter* species such as *A. cibarius*, *A. lanthieri*, *A. thereius*, *A. trophiarum*, and *A. vandammei* (Kerkhof et al., 2021a), which were not isolated in this study. Nevertheless, a bias of the study outcome due to the isolation method implemented was

minimalized as both direct isolation and isolation after enrichment of a previously validated *Arcobacter* isolation method were applied (Van Driessche et al., 2003). The presence of *Arcobacter* spp. in the feces of apparent healthy wild boars show that these can act as a potential reservoir and indicate the possible role played by these animals in transmission. Further research is necessary to explore the detailed epidemiology and source of origin of arcobacters in wild boar.

Previous assessment of initial bacterial contamination on wild boar meat already revealed that consumption of wild boar meat may be of public health risk, since wild boar carried a higher bacterial contamination level compared to pork (Peruzy et al., 2019). This is further supported by the results in the present study, which point out that wild boar excrete also zoonotic bacterial pathogens, such as *Arcobacter* and *Campylobacter* spp. Moreover, *Campylobacter* are assumed to display a moderate-to-high transmissibility potential from wild swine to humans (Ruiz-Fons, 2017). Atanassova et al. isolated *Campylobacter* from wild boar meat indicating its potential to enter the food chain (Atanassova et al., 2008). Seasonality, previously observed for *Arcobacter* and *Campylobacter* (Frosth et al., 2020; Van Driessche et al., 2003), could not be ascertained for in the present study as samples were collected in one hunting season only. The high prevalence of pathogenic *Campylobacter* spp. in the present study suggests that appropriate control measures during eviscerating, handling carcasses of wild boars, and meat processing are required to reduce contamination by these foodborne bacteria. In addition, due to their mobility, wild boars may play a direct or indirect role in the zoonotic transmission of *Campylobacter* through the potential of spill-over to livestock, fecal contamination of the environment, or via surface water especially where water is not treated before consumption or is used for recreational purposes (Navarro-Gonzalez et al., 2014).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2022.109462.

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