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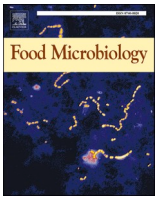

Foodborne and waterborne *Arcobacter* species exhibit a high virulent activity in Caco-2

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ABSTRACT

Infection mechanisms of *Arcobacter* remain uncertain. This study aimed to determine whether 65 food and waterborne isolates of at least six species were able to adhere and invade Caco-2 cells; and whether this ability could be related to *cadF*, *cj1349*, *ciaB,* and/or *hecA*, specific genetic markers related to host cell adhesion and invasion. All adhered and invaded the cells, and harboured at least two virulence markers. The mean virulent activity shown by *A. butzleri* was superior to that of *A. cryaerophilus* ($p < 0.05$); but the mean adhesion and invasion values of *A. lanthieri*, *A. skirrowii,* and *A. vitoriensis* were even higher. Sewage isolates were significantly ($p < 0.05$) more adherent and invasive than the rest, and their associated gene content was higher ($p < 0.05$). For the first time, an association between *cadF* and *hecA* and a high adhesion capability was identified ($p < 0.05$). The results provide new data on the pathogenic potential of *Arcobacter* species present in food and water by highlighting the superiority of *A. butzleri* over *A. cryarophilus*; providing evidence on the virulence of minority species as *A. lanthieri* and *A. vitoriensis;* and confirming sewage as an important source of potentially more virulent arcobacters.

1. Introduction

The genus *Arcobacter* is considered a group of emerging food and waterborne pathogens (Hsu and Lee, 2015), associated with human intestinal and extraintestinal infections (Barboza et al., 2017; Kerkhof et al., 2020; Lau et al., 2002; Simaluiza et al., 2021; Soelberg et al., 2020; Woo et al., 2001). These bacteria are frequently isolated from a wide variety of food animals and products, but also from fresh and brackish waters (Iwu et al., 2021). Due to their broad distribution, several ways of *Arcobacter* transmission to humans have been suggested (Fernández et al., 2004; Vandamme et al., 1992; Vasiljevic et al., 2019); the main one being the consumption of faecally contaminated food products and water (Chieffi et al., 2020).

When infecting humans, these bacteria are mainly associated with symptomatic enteritis (Ramees et al., 2017) usually characterized by diarrhoea, abdominal cramps, nausea, vomiting, and fever (Snelling et al., 2006). In these cases, *A. butzleri* and *A. cryaerophilus* are the major species isolated (Ferreira et al., 2016; Ruiz de Alegría Puig et al., 2023). However, although less frequently, *A. skirrowii*, *A. thereius,* and *A. lanthieri* have also provoked gastrointestinal symptoms in humans (Kerkhof et al., 2020; Van den Abeele et al., 2014; Wybo et al., 2004). In fact, *Arcobacter* spp. has been the only pathogen detected in clinical samples from patients with symptoms on several occasions (Barboza et al., 2017; Fernández et al., 2004; Figueras et al., 2014; Kayman et al., 2012; Kerkhof et al., 2020; Lappi et al., 2013; Lerner et al., 1994; Marinescu et al., 1996; Ruiz de Alegría Puig et al., 2021, 2023; Simaluiza et al., 2021; Tang et al., 2023; Wang et al., 2021; Wybo et al., 2004). Moreover, cases of asymptomatic infections, peritonitis or bacteraemia have also been reported associated with some species of the genus, including *A. cryaerophilus*, *A. butzleri,* and *A. mytili* (Arguello et al., 2015; Jiménez-Guerra et al., 2020; Vasiljevic et al., 2019; Yap et al., 2013). In addition, even without having been associated with human pathology,

Abbreviations: cMEM, complete Minimum Essential Medium; BHI, Brain Heart Infusion; FBS, Foetal Bovine Serum; NEAA, non-essential amino acids; PBS, Phosphate Buffered Saline.

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various studies have also pointed to the pathogenic potential of other *Arcobacter* species as *A. lacus* (Barel and Yildirim, 2023), *A. faecis* (Zambri et al., 2019), and *A. vitoriensis* (Alonso et al., 2020).

As derived from *in vitro* studies, *Arcobacter* shows resistance to several antimicrobial agents; impairs intestinal epithelial barrier; adheres to and invades different extra-intestinal (Hep-2, HeLa, INT407) and intestinal cell lines (Caco-2, IPI-2I, HT29, Caco-2/HT29-MTX, HT29/B6, and IPEC-J2); and provokes cytotoxic effects on some of them (Brückner et al., 2020; Buzzanca et al., 2021; Isidro et al., 2020; Karadas et al., 2016; Levican et al., 2013). Next generation sequencing and comparative genomic analyses have made it possible to identify, in the different sequenced genomes of *Arcobacter*, numerous virulence genes that could be responsible for these virulent properties (Chieffi et al., 2020; Hänel et al., 2021; Müller et al., 2020). Ten of them (*cadF*, *cj1349*, *pldA*, *tlyA*, *mviN*, *iroE*, *irgA*, *hecA,* and *hecB)* stand out for being commonly used as virulence markers in *Arcobacter* species (Girbau et al., 2015; Martinez-Malaxetxebarria et al., 2022; Rathlavath et al., 2017). Indeed, transcriptomic has recently highlighted the importance of *cadF* (cell adhesion), *irgA* (iron metabolism), and *iroE* (iron acquisition), along with that of other genes related to TonB complex and iron assimilation, by confirming their upregulation during the process of an *in vitro A. butzleri* infection (Buzzanca et al., 2023). However, despite the advances of recent years, the role of many of those putative virulence genes or their combinations in *Arcobacter* is still unknown. In spite of the efforts made through *in vitro* infections and the application of molecular techniques (Buzzanca et al., 2021; Carbone et al., 2003; Fernández et al., 2010; Ferreira et al., 2014; Gugliandolo et al., 2008; Ho et al., 2007; Ho et al., 2007; Isidro et al., 2020; Karadas et al., 2013; Levican et al., 2013; Musmanno et al., 1997), no mechanism of pathogenesis has been well characterized yet in this bacterial genus. Many of the previous studies carried out with *Arcobacter* failed on this purpose for any of these three general reasons: limited number of strains, few species and/or low diversity in the origin of the isolates. In fact, most of the *in vitro* infection assays on culture cells have been carried out with clinical isolates of *A. butzleri* and/or *A. cryaerophilus*. All have suggested that *A. cryaerophilus* is more virulent than *A. butzleri*, that the adhesion and invasion capabilities are strain-specific, and that no association appears to exist between pathogenic potential and origin of the strains. As far as we know, only one study combines food and water derived *Arcobacter* isolates (Levican et al., 2013). Considering that these are the main routes of transmission of these bacteria to humans, such studies are of great interest.

Further knowledge on the infection mechanisms of the different *Arcobacter* species is of essential need, and the combination of *in vitro* infection assays and gene occurrence analysis may help to address this challenge. Although the presence of a gene does not necessarily indicate that its host has a certain capability, the correlation between a gene pattern and a specific phenotype may be indicative. Therefore, the present study aimed to evaluate the adherent and invasive capability of a set of 65 potentially pathogenic isolates of six different *Arcobacter* species isolated from a variety of food products and water samples; and to correlate those capabilities with four specific virulence markers related to host cell attachment and invasion: *cadF* and *cj1349*, that code for fibronectin-binding proteins that promote the binding of bacteria to intestinal cells; *ciaB,* which encodes the invasive antigen B; and *hecA,* involved in attachment, aggregation and epidermal cell killing.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A total of 65 *Arcobacter* strains (Table 1) were studied. Sixty belonged to six of the 34 accepted species of the genus (https://lpsn.dsmz.de/genus/arcobacter), namely *A. butzleri* (n = 35), *A. cryaerophilus* (n = 16), *A. skirrowii* (n = 2), *A. thereius* (n = 3), *A. lanthieri* (n = 2), and *A. vitoriensis* (n = 2); and the other five were *Arcobacter* spp. isolates that could not be identified to the species level. All of them had previously been isolated from different food and water sources: cockle (n = 9), squid (n = 6), shrimp (n = 6), turkey (n = 4), carrot (n = 3), rabbit (n = 3), quail (n = 3), lettuce (n = 1), fresh cheese (n = 1), spinach (n = 1), clam (n = 1), mussel (n = 1), sewage (n = 15), and river water (n = 11) (Alonso et al., 2018, 2020; Martinez-Malaxetxebarria et al., 2022; Nieva-echevarria et al., 2013). For the adhesion and invasion assays, *Salmonella enterica* serovar Typhimurium LT2 CECT 722 (Spanish Culture Cell Type) and *Escherichia coli* DH5α NCCB 2955 (Netherlands Culture Collection of Bacteria) were included as positive control for the adhesion and invasion assays the first, and as positive control for adhesion and negative for invasion the latter.

Arcobacter strains were routinely grown aerobically at 30 °C for 12–16 h in Brain Heart Infusion (BHI) broth (Oxoid) or for 24–48 h on blood agar base (Oxoid) supplemented with 5% defibrinated sheep blood (Liofilchem). *Salmonella* Typhimurium LT2 and *Escherichia coli* DH5α were routinely grown at 37 °C for 12–16 h in BHI broth (Oxoid) or on Muller-Hinton agar (Oxoid) in an aerobic environment. Shaking (150 rpm) was applied when necessary.

2.2. Cell culture

The human colorectal cell line Caco-2 was obtained from the American Type Culture Collection (ATCC HTB-37; LGC Standards). Cells were routinely cultured in 75 cm^2 tissue culture flasks (Corning) in complete Minimum Essential Medium [cMEM; MEM 1X (Thermo Fisher Scientific) with 10% foetal bovine serum (FBS; Thermo Fisher Scientific), 2 mM L-glutamine (Thermo Fisher Scientific), 1% non-essential amino acids (NEAA; Sigma), penicillin 10,000 U and streptomycin 10,000 μg mL^{-1} (Sigma)]. Cultures were incubated at 37 °C under a humidified atmosphere of 5% CO_2 until a confluence of 80% was reached, when they were washed twice with 10 mL of 1X phosphate buffered saline (PBS) and detached by a solution of 3 mL trypsin–EDTA. For adhesion and invasion assays, confluent monolayers were formed in 24-well-plates by adding 2×10^4 cells mL^{-1} to each well and subsequently incubating for approximately 48 h under the above-mentioned conditions.

2.3. Bacterial adhesion and invasion assays

Caco-2 cells were infected following a previously described method (Levican et al., 2013) with slight modifications. Briefly, overnight bacterial liquid cultures were diluted to an OD_{600} of 0.08 (approximately 10^9 CFU mL^{-1}) for *Arcobacter* strains and 0.05 (approximately 10^8 CFU mL^{-1}) for the control strains; harvested by centrifugation (900×*g* for 5 min); and suspended in the same volume with tempered cMEM without antibiotics (37 °C). For the adhesion assays, cell monolayers were inoculated with 0.5 mL of bacterial suspension (approximately 5×10^7 CFU) and incubated at 37 °C for 2 h. Cells were then washed twice with 1X PBS and lysed with 1% Triton X-100 (Sigma) by adding 0.5 mL per well and letting it act for 10 min. The total number of Caco-2 associated bacteria was then calculated. For the invasion assays the plates were inoculated and incubated as same as the adhesion assays conditions and subsequently treated with gentamicin (Thermo Fisher Scientific) during 1 h, in order to kill extracellular bacteria (0.5 mL of MEM 1X containing 125 μg mL^{-1} was added to each well). Cells were then washed and lysed as above-mentioned and the number of intracellular bacteria was calculated. The total numbers of cell-associated bacteria and intracellular bacteria were determined by plating the respective lysates on BHI agar plates (Oxoid) supplemented with 5% defibrinated sheep blood (Oxoid). The number of adhered bacteria was calculated as the difference between the total number of bacteria associated with the Caco-2 cells and the number of intracellular bacteria. Per experiment, each strain was studied in triplicate (three wells were inoculated) and the experiments were repeated on three independent occasions. The results were expressed as the mean number of bacteria (log_{10} CFU mL^{-1}) that

Table 1
Virulence genotypes of 65 *Arcobacter* strains and their adhesion and invasion capabilities to Caco-2 cells [a].

Species and strain	Source of isolation	Adhesion [b]		Invasion [b]		Virulence genotype [c]
		log_{10} CFU ml^{-1} ± standard error	Categorization	log_{10} CFU ml^{-1} ± standard error	Categorization	
Controls						
Salmonella Typhimurium LT2		6,96 ± 0,03		5,22 ± 0		ND
Escherichia coli DH5α		6,39 ± 0,02		NI		ND
Tested strains						
A. butzleri						
Ab-LCH1	Lettuce	5,94 ± 0,13	High	4,62 ± 0,11	High	*cadF, cj1349, ciaB*
Ab-Z1	Carrot	4,95 ± 0,12	Low	4,22 ± 0,07	Good	*cadF, cj1349, ciaB*
Ab-Z3	Carrot	5,41 ± 0,07	Good	4,43 ± 0,15	High	*cadF, cj1349, ciaB*
Ab-PV1	Turkey	5,78 ± 0,10	Good	5,09 ± 0,07	High	*cadF, cj1349, ciaB*
Ab-PV2	Turkey	**7,38 ± 0,13** [d]	High	**5,30 ± 0.00**	High	*cadF, cj1349, ciaB,* ***hecA***
Ab-PV16	Turkey	**7,01 ± 0,03**	High	5,17 ± 0,08	High	*cadF, cj1349, ciaB, hecA*
Ab-CH1	Squid	6,09 ± 0,07	High	5,13 ± 0,03	High	*cadF, cj1349, ciaB*
Ab-CH2	Squid	6,09 ± 0,12	High	**5,28 ± 0,08**	High	*cadF, cj1349, ciaB*
Ab-G1	Shrimp	6,43 ± 0,09	High	4,59 ± 0,16	High	*cadF, cj1349, ciaB, hecA*
Ab-G2	Shrimp	6,35 ± 0,05	High	4,80 ± 0,13	High	*cadF, cj1349, ciaB,* ***hecA***
Ab-CN1	Rabbit	6,43 ± 0,04	High	4,46 ± 0,07	High	*cadF, cj1349, ciaB, hecA*
Ab-CN2	Rabbit	6,30 ± 0.00	High	4,18 ± 0.00	Good	*cadF, cj1349, ciaB*
Ab-CZ5	Quail	**6,91 ± 0,14**	High	4,42 ± 0,10	High	*cadF, cj1349, ciaB*
Ab-CZ6	Quail	6,45 ± 0,06	High	4,42 ± 0,10	High	*cadF, cj1349, ciaB*
Ab-BER4	Cockle	6,54 ± 0.00	High	2,61 ± 0,08	Low	*cadF, cj1349, ciaB*
Ab-BER7	Cockle	6,07 ± 0,03	High	3,52 ± 0,03	Good	*cadF, cj1349, ciaB*
Ab-QS1	Cheese	4,63 ± 0,12	Low	**5,50 ± 0,30** [d]	High	*cadF, cj1349, ciaB,* ***hecA***
Ab-E1	Spinach	**6,76 ± 0,17**	High	4,40 ± 0.00	High	*cadF, cj1349, ciaB*
RW1	River water	6,30 ± 0.00	High	4,13 ± 0,05	Good	*cadF, cj1349, ciaB*
RW2	River water	5,79 ± 0,04	Good	4,25 ± 0,28	High	*cadF, cj1349, ciaB*
RW3	River water	6,40 ± 0.00	High	3,93 ± 0.00	Good	*cadF, cj1349, ciaB*
RW4	River water	6,54 ± 0.00	High	4,28 ± 0,13	High	*cadF, cj1349, ciaB*
RW5	River water	6,46 ± 0,18	High	4,30 ± 0.00	High	*cadF, cj1349, ciaB*
RW6	River water	6,35 ± 0,05	High	4,39 ± 0,19	High	*cadF, cj1349, ciaB*
RW7	River water	6,17 ± 0,10	High	4,52 ± 0,05	High	*cadF, cj1349, ciaB, hecA*
RW8	River water	6,33 ± 0,05	High	4,64 ± 0,07	High	*cadF, cj1349, ciaB,* ***hecA***
RW9	River water	**6,82 ± 0,05**	High	4,8 ± 0,06	High	*cadF, cj1349, ciaB, hecA*
RW10	River water	5,63 ± 0,34	Good	3,92 ± 0,20	Good	*cadF, cj1349, ciaB*
FW2	Sewage	**6,92 ± 0,16**	High	4,04 ± 0,25	Good	*cadF, cj1349, ciaB, hecA*
FW6	Sewage	6,44 ± 0,18	High	4,54 ± 0,36	High	*cadF, cj1349, ciaB, hecA*
FW7	Sewage	6,10 ± 0,06	High	4,00 ± 0,14	Good	*cadF, cj1349, ciaB, hecA*
FW10	Sewage	6,48 ± 0,15	High	4,47 ± 0,13	High	*cadF,* ***cj1349****, ciaB, hecA*
FW11	Sewage	6,27 ± 0,12	High	4,95 ± 0,08	High	*cadF, cj1349, ciaB, hecA*
FW12	Sewage	6,22 ± 0,17	High	4,56 ± 0,14	High	*cadF, cj1349, ciaB, hecA*
FW14	Sewage	6,29 ± 0,16	High	4,94 ± 0,07	High	*cadF, cj1349, ciaB*
A. cryaerophilus						
Ac-BER1	Cockle	5,75 ± 0,10	Good	4,79 ± 0,06	High	*cadF,* ***cj1349****, ciaB*
Ac-BER2	Cockle	6,37 ± 0,06	High	4,40 ± 0,10	High	*cadF,* ***cj1349****, ciaB*
Ac-BER5	Cockle	6,47 ± 0,07	High	4,46 ± 0,10	High	***cadF****, ciaB*
Ac-BER6	Cockle	6,47 ± 0,09	High	4,09 ± 0,12	Good	***cadF****,* ***cj1349****, ciaB*
Ac-BER7	Cockle	6,18 ± 0,35	High	4,18 ± 0.00	Good	*cadF,* ***cj1349****,* ***ciaB***
Ac-BER8	Cockle	6,47 ± 0,24	High	4,60 ± 0,15	High	***cadF****,* ***cj1349****, ciaB*
Ac-BER10	Cockle	6,59 ± 0,07	High	3,44 ± 0,09	Good	*cadF,* ***cj1349****, ciaB*
Ac-PV1	Turkey	5,27 ± 0,08	Good	3,50 ± 0,12	Good	*cadF,* ***cj1349****, ciaB*
Ac-CZ1	Quail	6,07 ± 0,07	High	3,71 ± 0,10	Good	*cadF, ciaB, hecA*
Ac-CH1	Squid	**6,78 ± 0,04**	High	4,67 ± 0,08	High	***cadF****,* ***cj1349****, ciaB*
Ac-A2	Clam	6,38 ± 0.00	High	5,08 ± 0,08	High	***cadF****,* ***cj1349****, ciaB*
Ac-CN1	Rabbit	5,84 ± 0.00	Good	4,18 ± 0.00	Good	***cj1349****, ciaB, hecA*
Ac-M1	Mussel	5,69 ± 0,10	Good	5,10 ± 0,05	High	***cadF****,* ***cj1349****, ciaB*
Ac-G2	Shrimp	5,70 ± 0,05	Good	4,42 ± 0,08	High	***cj1349****, ciaB*
Ac-G8	Shrimp	5,45 ± 0,05	Good	4,43 ± 0,08	High	***cadF****,* ***cj1349****, ciaB*

(*continued on next page*)

Table 1 (*continued*)

Species and strain	Source of isolation	Adhesion [b]		Invasion [b]		Virulence genotype [c]
		log_{10} CFU ml^{-1} ± standard error	Categorization	log_{10} CFU ml^{-1} ± standard error	Categorization	
Ac-RW1	River water	5,98 ± 0,21	High	4,25 ± 0,08	High	*ciaB*, ***hecA***
A. skirrowii						
As–CH1	Squid	6,51 ± 0,22	High	5,05 ± 0,02	High	***cj1349***, *ciaB*
As-G1	Shrimp	6,58 ± 0,05	High	**5,30 ± 0.00**	High	***cj1349***, *ciaB*
A. thereius						
At-CH1	Squid	5,17 ± 0.00	Good	3,09 ± 0,03	Low	***cj1349***, *ciaB*
At-CH2	Squid	5,17 ± 0.00	Good	3,04 ± 0,29	Low	***cj1349***, *ciaB*
At-G1	Shrimp	5,29 ± 0,42	Good	3,8 ± 0,11	Good	***cj1349***, *ciaB*
A. lanthieri						
FW34	Sewage	**6,91 ± 0,13**	High	4,71 ± 0,06	High	***cadF***, ***cj1349***, *ciaB*, *hecA*
FW40	Sewage	**7,11 ± 0,10**	High	4,85 ± 0,11	High	***cadF***, ***cj1349***, *ciaB*, *hecA*
A. vitoriensis						
F199	Carrot	6,43 ± 0,08	High	4,78 ± 0,06	High	***cadF***, ***cj1349***, *ciaB*, ***hecA***
FW-59	Sewage	6,29 ± 0,02	High	4,5 ± 0,09	High	***cadF***, ***cj1349***, *ciaB*, *hecA*
***Arcobacter* spp.**						
FW-4	Sewage	6,06 ± 0,14	High	4,72 ± 0,34	High	***cadF***, ***cj1349***, *ciaB*, ***hecA***
FW-8	Sewage	6,29 ± 0,24	High	4,90 ± 0,04	High	***cadF***, ***cj1349***, *ciaB*, *hecA*
FW-53	Sewage	5,88 ± 0,08	High	4,66 ± 0,26	High	***cadF***, ***cj1349***, *ciaB*, *hecA*
FW-54	Sewage	6,13 ± 0,10	High	4,68 ± 0,27	High	***cadF***, ***cj1349***, *ciaB*
FW-61	Sewage	**7,18 ± 0,12**	High	4,55 ± 0,13	High	***cadF***, ***cj1349***, *ciaB*

[a] Abbreviations: NI, no invasion detected; ND, not determined; The values for adhesion and invasion were proportionally calculated to an inoculum of 10^8 CFU mL^{-1} (8.0 log_{10} CFU ml^{-1}) for each strain.

[b] Results equal to or higher than that for the mean of *S.* Typhimurium LT2 and *E. coli* for adhesion and *S.* Thypimurium LT2 for invasion are shown in bold.

[c] Genes detected in this study that were previously reported to be absent in the same strains are shown in bold and underlined.

[d] Kruskal-Wallis test based statistically significant differences ($p < 0.001$) for the adhesion and invasion capabilities among isolates.

adhered to or invaded cells ± standard error (SE), and subsequently categorized as low, good, or high adhesion and/or invasion according to Levican et al. (2013), who divided the range of results between the detection limit and the mean value obtained for *S. enterica* (the positive control) into the three mentioned categories.

2.4. PCR detection of adhesion and invasion related genes

The occurrence of *cadF, cj1349, ciaB,* and *hecA* had been previously determined in the set of *Arcobacter* strains studied here (Alonso et al., 2018; Girbau et al., 2015; Martinez-Malaxetxebarria et al., 2022), except for *A. butzleri* RW1, RW2 and FW7. However, the procedures employed in the above-mentioned studies can lead to false negative results for some of the genes in *Arcobacter* species other than *A. butzleri* due to the limitations of the techniques (Douidah et al., 2012). Therefore, all *A. cryaerophilus, A. skirrowii, A. thereius, A. lanthieri, A. vitoriensis,* and *Arcobacter* spp. isolates were re-subjected to PCR detection of *cadF*, *cj1349,* and *hecA* using new primers designed in this study (Table S1). Those primers were designed with the Clone Manager 9 Professional Edition software (Sci Ed Software LLC), based on comprehensive analyses and alignments of the published genome sequences of *A. butzleri* RM4018^T (CP000361.1), *A. cryaerophilus* ATCC 43158^T (NZ_CP032823.1), *A. skirrowii* CCUG 10374^T (NZ_CP032099.1), *A. thereius* LMG 24486^T (NZ_CP035926.1), *A. lanthieri* LMG 28516^T (NZ_CP053839.1), *A. vitoriensis* CECT 9230^T (NZ_PDKB00000000.1), and '*(Ali)arcobacter hispanicus*' CECT 9188^T (NZ_PDKI00000000). Additionally, all the *A. butzleri* isolates were also subjected to PCR detection of *hecA* using the newly designed corresponding primers. DNA from the isolates was extracted from overnight liquid cultures using PrepMan® Ultra Reagent (Applied Biosystems); and all PCRs were carried out in final volumes of 25 μl containing 1X PCR reaction buffer, 2.5 mM of $MgCl_2$, 0.2 μM of each primer, 0.4 mM of each dNTP (Bioline), 1 U of Supreme NZYtaq II DNA polymerase (NZYTech), and 50 ng of DNA. Genomic DNA from *A. butzleri* RM4018^T, *A. cryaerophilus* CCUG 17801^T, *A. skirrowii* CECT 8223^T, *A. thereius* CCUG 56902^T, *A. lanthieri* LMG 28516^T and '*(Ali)arcobacter hispanicus*' CECT 9188^T were used as positive controls, and deionized water as negative one.

2.5. Data analysis

The statistical analyses were carried out using the SPSS Statistics 26 software (SPSS Inc., Chicago, 221 IL, USA). Kruskal-Wallis and Mann-Whitney U tests were used to compare the values obtained in the adhesion and invasion assays among isolates, sources of isolation, and species. Taking into account the number of representatives of each species, two groups were stablished when those values were compared among species: i) *A. butzleri* (n = 35) vs *A. cryaerophilus* (n = 16); ii) *A. skirrowii* (n = 2) vs *A. lanthieri* (n = 2) vs *A. thereius* (n = 3) vs *A. vitoriensis* (n = 2) vs *Arcobacter* spp. (n = 5). On the other hand, Pearson's Chi-square and Fisher's exact tests were used to identify possible associations between variables. The isolates identified as *A. skirrowii* (n = 2), *A. thereius* (n = 3), *A. lanthieri* (n = 2), *A. vitoriensis* (n = 2), and *Arcobacter* spp. (n = 5), along with those showing low adhesion (n = 2) and invasion (n = 3) capabilities, were excluded from the association analyses based on their low representation. Significance was established at p level of <0.05.

3. Results

3.1. Adhesion to and invasion of Caco-2 cells by Arcobacter isolates

The capability of *Arcobacter* to adhere to and invade Caco-2 cells is detailed in Table 1. All the isolates adhered to and invaded Caco-2 cells, showing adhesion values ranging from 4.63 ± 0.12 to 7.38 ± 0.13 log_{10}

CFU mL^{-1} and invasion values ranging from 2.61 ± 0.08 to 5.50 ± 0.30 log_{10} CFU mL^{-1}. Fifty (76.9%) isolates were highly adherent and 47 (72.3%) highly invasive. Among them, ten isolates (15.4%) showed higher adhesiveness than the mean value of both *S.* Typhimurium LT2 and *E. coli* DH5α; and four (6.2%) higher invasiveness than *S.* Typhimurium LT2. Ab-PV2 and Ab-QS1, both representatives of *A. butzleri*, were significantly ($p < 0.001$) the most highly adherent (7.38 ± 0.13 log_{10} CFU mL^{-1}) and invasive (5.50 ± 0.30 log_{10} CFU mL^{-1}) isolates, respectively. On the other hand, *A. lanthieri* FW40 and *A. skirrowii* As-G1 showed, respectively, the second highest values for adherence (7.11 ± 0.10 log_{10} CFU mL^{-1}) and invasion (5.30 ± 0.00 log_{10} CFU mL^{-1}).

The average adhesion and invasion values shown by the isolates varied according to the species to which they belong (Fig. 1A) and to the origin (Fig. 1B). When comparing the mean values shown by the two main species associated with human infection, *A. butzleri* was significantly ($p < 0.05$) more adherent and invasive than *A. cryaerophilus* (6.26

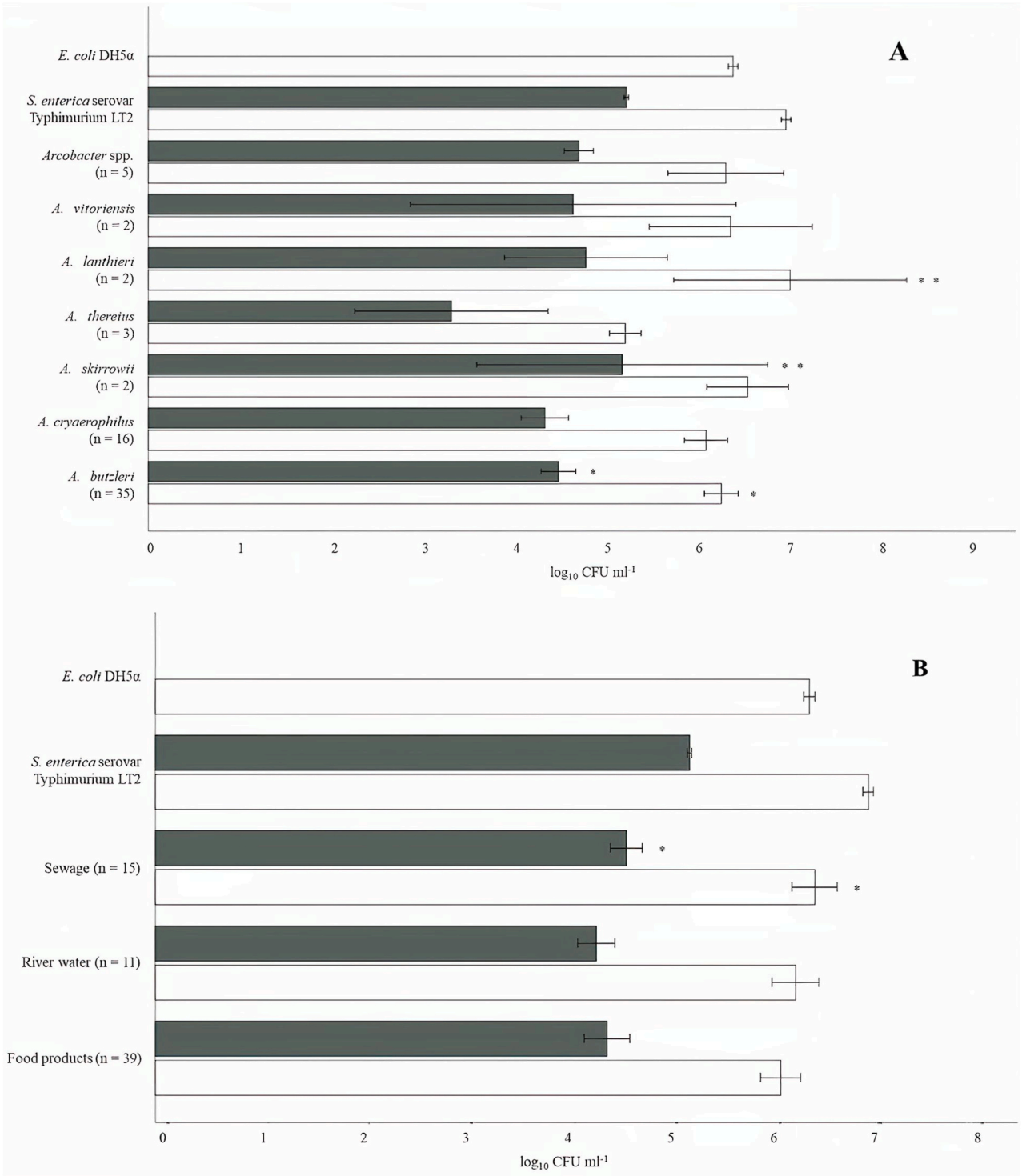

Fig. 1. Bars representing the adhesion to (white) and invasion of (grey) Caco-2 cells shown by the 65 *Arcobacter* strains grouped by species (A) and isolation source (B). Control strains are also represented. * and ** Kruskal-Wallis test based statistically significant differences ($p < 0.05$ and $p < 0.001$, respectively). In A, significance values are according to the two groups that were established (majority species and minority species).

± 0.04 and 4.47 ± 0.04 $\log_{10}$ CFU mL^{-1}, respectively, for *A. butzleri* vs 6.11 ± 0.05 and 4.33 ± 0.05 $\log_{10}$ CFU mL^{-1} for *A. cryaerophilus*). Significant differences ($p < 0.001$) were also detected among the mean values observed for the minority species: *A. lanthieri* presented the highest adhesion (7.01 ± 0.04 $\log_{10}$ CFU mL^{-1}), *A. skirrowii* the highest invasion (5.18 ± 0.04 $\log_{10}$ CFU mL^{-1}), and *A. thereius* the lowest adhesion (5.21 ± 0.06 $\log_{10}$ CFU mL^{-1}) and invasion (3.31 ± 0.09 $\log_{10}$ CFU mL^{-1}). Regarding the origin of the species, the strains isolated from sewage were significantly ($p < 0.05$) more adherent (6.44 ± 0.00 $\log_{10}$ CFU mL^{-1}) and invasive (4.60 ± 0.04 $\log_{10}$ CFU mL^{-1}) than those isolated from river water (6.25 ± 0.04 and 4.30 ± 0.03 $\log_{10}$ CFU mL^{-1}, respectively) or food products (6.12 ± 0.04 and 4.42 ± 0.05 $\log_{10}$ CFU mL^{-1}, respectively).

The Fisher exact test established an association ($p < 0.05$) between sewage strains and a high adhesion capability; and between strains isolated from food products and a good adhesion capability (Table S2).

3.2. Detection and prevalence of cadF, cj1349, ciaB, and hecA

The primer pairs designed for the partial amplification of the genes *cadF*, *cj1349,* and *hecA* in *Arcobacter* species other than *A. butzleri,* generated, in each case, a single band of the expected size. Specifically, *cadF* primers generated an amplicon of 887 bp in *A. cryaerophilus* and a 775 bp one in *A. lanthieri*, *A. vitoriensis,* and *A. hispanicus*; *cj1349* primers a 721 bp one in *A. cryaerophilus* and *A. vitoriensis,* another one of 742 bp in *A. skirrowii* and *A. thereius,* and a third one of 711 bp in *A. lanthieri* and *A. hispanicus*; and *hecA* primers generated an amplicon of 659 bp in all the species except in *A. thereius,* for which it was impossible to design *hecA* specific primers.

Overall, the four genes were detected among all the analysed isolates being *ciaB* the most prevalent (100%), followed by *cj1349* (95.4%), *cadF* (87.7%) and *hecA* (38.5%). Table 1 shows the detailed gene content of each isolate and Table S3 the presence and distribution of the four genes investigated in the *Arcobacter* isolates analysed. Among the seven different gene patterns identified, the combination of *cadF*, *cj1349,* and *ciaB* was the most prevalent (50.8%), followed by that of *cadF*, *cj1349*, *ciaB,* and *hecA* (33.8%).

The genes were differently distributed among species and sources. 42.9% of the *A. butzleri* isolates showed the four genes. *cadF*, *cj1349,* and *ciaB* were detected in all the *A. butzleri* isolates, while the detection-rate of *hecA* was of 42.9%. The virulence gene content of *A. cryaerophilus* was lower: none of the isolates showed all four genes. The most prevalent gene in this species was *ciaB*, which was detected in all isolates. The genes *cadF* and *cj1349* were detected in 81.3% of the isolates and *hecA* was the least detected, occurring in 18.8% of the isolates. Among the less represented species, *cj1349* and *ciaB* were detected in all the isolates; *cadF* was present in all *A. lanthieri*, *A. vitoriensis,* and *Arcobacter* spp. isolates; and *hecA* was in all *A. lanthieri* and *A. vitoriensis* and in the 60% of *Arcobacter* spp. isolates. Statistical analyses showed that *cadF* and *cj1349* were significantly ($p < 0.05$) more prevalent in *A. butzleri* than in *A. cryaerophilus,* while for minority species no significant differences were observed. Depending on the source of isolation, *hecA* was significantly ($p < 0.001$) more frequently detected in strains isolated from sewage. The combination of the four genes analysed was also significantly ($p < 0.05$) more prevalent in these isolates.

Regarding possible associations between certain gene patterns and the ability of the strains to adhere to and/or invade cells (Fig. 2; Tables S2 and S3), the Fisher exact test detected a significant association ($p < 0.05$) between the presence of *cadF* or *hecA* and a high adhesion capability. By contrast, no association was identified between any of the four genes or their combinations and a level of categorization of invasiveness.

4. Discussion

A number of species of the genus *Arcobacter* have been associated with several human diseases, mainly with diarrhoea causing enteritis (Ramees et al., 2017), but the infection mechanisms of these bacteria remain uncertain. Bacterial adhesion to epithelial cells is a necessary

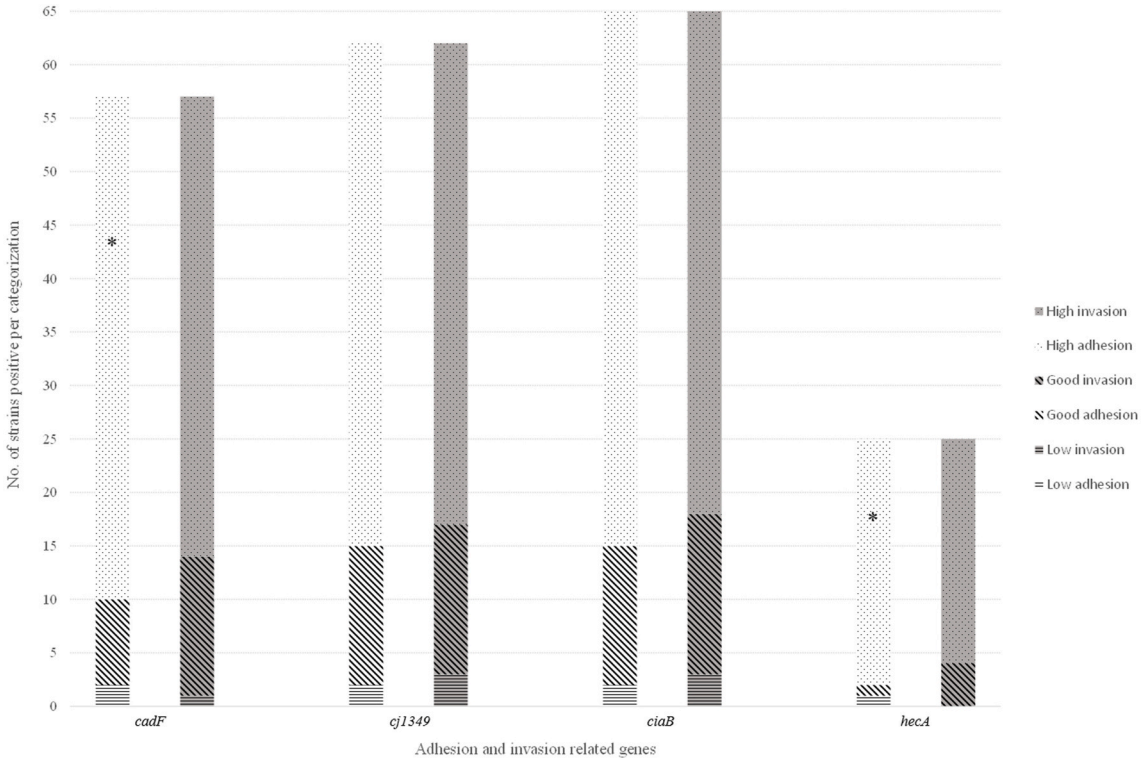

Fig. 2. Bars representing the number of strains in each of the adherence (white) and invasion (grey) categories for each of the genes studied. * Fisher exact test based statistically significant differences ($p < 0.05$).

step for any infection to occur as may be followed by tissue colonization and subsequent invasion of host cells, intracellular proliferation, spread to other tissues, or persistence (Pizarro-Cerdá and Cossart 2006). Therefore, the adhesion and invasion capabilities of 65 food and waterborne *Arcobacter* isolates were investigated here. Additionally, possible correlations between infection phenotypes and the number of virulence markers associated with host cell adhesion (*cadF*, *cj1349*, and *hecA*) and/or invasion (*ciaB*) were analysed.

Caco-2 cells were used for adhesion and invasion assays. This line was selected for being human colorectal adenocarcinoma cells that can mimic enterocytes (Fedi et al., 2021), which makes it suitable for the study of pathogens that mainly provoke enteritis with diarrhoea, as *Arcobacter* (Chieffi et al., 2020; Kietsiri et al., 2021). It is also a well stablished model that has been used previously with *Arcobacter*, on which variable adhesion and invasion capabilities have been reported (Buzzanca et al., 2021; Ferreira et al., 2014; Ho et al., 2007; Ho et al., 2007; Karadas et al., 2013; Levican et al., 2013). Surprisingly, all the 65 isolates studied here adhered to and invaded Caco-2 cells. Similar results have been reported previously for *A. butzleri* (Buzzanca et al., 2021; Ferreira et al., 2014; Karadas et al., 2013; Levican et al., 2013) and *A. cryaerophilus* (Ho et al., 2007), but, to our knowledge, this is the first time that all the tested *A. skirrowii* and *A. thereius* isolates show both capabilities. It is also the first time, as far as we know, that cell infection assays are hold with *A. lanthieri* and *A. vitoriensis*.

Several authors have previously highlighted the high virulent activity of *A. butzleri* and *A. cryaerophilus in vitro* (Buzzanca et al., 2021; Fernández et al., 2010; Ferreira et al., 2014; Gugliandolo et al., 2008; Ho et al., 2007; Karadas et al., 2013; 2016; Levican et al., 2013). However, to our knowledge, only two studies have compared this ability between the two species (Ho et al., 2007; Levican et al., 2013) and the results of both point out *A. cryaerophilus* as the most virulent. By contrast, according to the data from the present study *A. butzleri* is significantly superior to *A. cryaerophilus* ($p < 0.05$). This would support that *A. butzleri* is the main species of the genus associated with human infection. However, our data also highlight the high pathogenic potential of other species such as *A. lanthieri*, *A. skirrowii*, and *A. vitoriensis*, for which, surprisingly, mean adherence and invasion values are higher than those obtained for *A. butzleri* and *A. cryaerophilus*. In fact, the mean adhesion values shown by *A. lanthieri* (7.01 ± 0.04 $\log_{10}$ CFU mL^{-1}) are similar to the highest values observed in the *A. butzleri* isolates; and *A. skirrowii* stands out for its high invasiveness, showing one of the two strains analysed the second highest value among the 65 total strains (5.30 ± 0.00 $\log_{10}$ CFU mL^{-1}). Levican et al. (2013) also detected that the most potentially virulent strain among the 60 strains tested on Caco-2 cells was a representative of *A. skirrowii*. However, none of the two isolates of *A. skirrowii* tested by Ho et al. (2007) was able to invade Caco-2 or IPI-2I cells, which leads us to think that the variability of results among studies may be due either to methodological differences (e.g. different infection times or growth media) or to the origin and/or intrinsic characteristics of the strains, as previously proposed elsewhere (Karadas et al., 2013). Indifferently, the low number of representatives of *A. lanthieri*, *A. skirrowii*, and *A. vitoriensis* analysed in this study (two of each) does not allow us to compare them with *A. butzleri* or *A. cryaerophilus*, but it would be of great interest to conduct future studies in order to determine whether these minority species are really so virulent. The superior virulence of a minority species such as *A. trophiarum* over *A. butzleri* has been previously highlighted (Levican et al., 2013). On the other hand, the high adhesion and invasion capabilities of FW4, FW8, FW53, FW54, and FW61 are also remarkable. These are sewage isolates, potentially representative of new species of the genus, which may pose a considerable risk for human health based on the results of our study.

While there are some studies on strains from diverse origins such as food, surface and marine waters, and livestock faeces (Carbone et al., 2003; Fernández et al., 2010; Gugliandolo et al., 2008; Musmanno et al., 1997); the majority of the previous studies have focused on exploring the virulent potential of strains isolated from humans compared to non-human ones (Buzzanca et al., 2021; Ferreira et al., 2014; Ho et al., 2007; Karadas et al., 2013, 2016). In the basis of those results, the virulent potential appears to be strain dependant rather than origin dependant, as some strains adhered and/or invaded and others did not, irrespective of their origin. Even though consumption of contaminated food and/or water is the main route of human *Arcobacter* infection, there are hardly any studies analysing possible differences between isolates from those sources. To date, the only study that could resemble ours is that of Levican et al. (2013), which combines isolates derived from sewage and food, among other sources. In fact, that is the only study in which having analysed strains of different origin, an association between isolation source and virulent capability was established. Our findings confirm the reported association between sewage and a higher virulent potential of the strains ($p < 0.001$), and could suggest that *Arcobacter* pathogenity is related to source rather than strain. To our knowledge, these are the only two studies carried out to date with sewage *Arcobacter* strains. However, some other studies have been conducted with isolates derived from river and brackish water (Carbone et al., 2003; Musmanno et al., 1997). Not in line with our observations, only 35.3% and 5.6% of the strains tested in the aforementioned studies were able to adhere to epithelial cells. The use of different cell lines in each study [Caco-2 here; HEp-2 and HeLa in the study of Carbone et al. (2003); and Vero and CHO in that of Musmanno et al. (1997)] may be the reason for the differences observed between them.

The virulent potential of bacterial strains is determined, in part, by their gene content. Interestingly, the PCR analyses held here show that sewage strains have a significantly higher gene content associated with adhesive and invasive capabilities than the strains isolated from other sources ($p < 0.001$), which goes in line with that observed by Levican et al. (2013), who also detected a higher proportion of virulence genes in strains from faecal samples. Our results also show, for the first time, an association ($p < 0.05$) between the presence of *cadF* and *hecA* and a high adhesion capability in *Arcobacter* spp. As far as we know, none of the previous studies attempting this item could stablish any association between virulence gene patterns and the adherence and/or invasiveness of *Arcobacter* spp. (Ferreira et al., 2014; Karadas et al., 2013; Levican et al., 2013). It should be noted that the primers used here are not the same as those used in the aforementioned studies, at least for *A. cryaerophilus, A. skirrowii, A. thereius, A. lanthieri,* and *A. vitoriensis.* The design of species-specific primers has allowed us to detect *cadF, cj1349,* and *hecA* in a more efficient way (see Table 1) in view of the results previously obtained for the majority of the strains studied here (Alonso et al., 2018; Girbau et al., 2015; Martinez-Malaxetxebarria et al., 2022). Specifically, we were able to detect *cadF* in 16 strains in which the gene had not previously been detected, *cj1349* in 27, and *hecA* in seven. Therefore, results of previous studies carried out with non-*A. butzleri* species may have been false negatives, and this may be the underlying reason for the lack of associations detected to date.

5. Conclusions

This study provides new data on the pathogenic potential of different *Arcobacter* species present in food and water. For the first time, it is shown that the virulent activity of *A. butzleri* is superior to that of *A. cryaerophilus*, thus supporting *A. butzleri* as the main species of the genus associated with human pathology. Our results also provide evidence on the virulence potential of *A. lanthieri* and *A. vitoriensis,* setting the basis for further studies. Additionally, our results confirm sewage to be an important source of potentially more virulent arcobacters, and let us hypothesize that rather than strain-dependant, their virulent potential is origin-dependant. This finding should be taken into consideration, as sewage can be a source of environmentally transmitted pathogens that can reach the population through different routes. Finally, the species-specific primers designed here, which have been shown to be useful in detecting four of the ten classical virulence markers of

Arcobacter in species other than *A. butzleri*.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2023.104424.

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