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Isolation and characterization of the emerging foodborn pathogen *Arcobacter* from human stool

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Abstract

At present, isolation of arcobacters from human specimens is performed by slightly of not modified *Campylobacter*, *Yersinia* or *Leptospira* isolation techniques, and knowledge if arcobacters are part of the human commensal flora is lacking. Therefore, an *Arcobacter* selective isolation procedure was validated for the examination of human fecal specimens, and the presence and characteristics of *Arcobacter* in feces of asymptomatic humans was examined in order to assess the clinical relevance of arcobacters in diarrheal stool. With this method, *Arcobacter* was isolated from seven of 500 (1.4%) stool samples of healthy people with *Arcobacter cryaerophilus* as the only species present. Seven *A. cryaerophilus* genotypes were detected and only one genotype was found per person. Neither *A. butzleri* nor *A. skirrowii* were isolated, therefore the presence of those latter species in clinical samples requires further attention. Though the pathogenic role and potential virulence factors of arcobacters have to be further examined, the current status of arcobacters as emerging pathogens remains justified.
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Keywords: *Arcobacter*; Carrier; Human; Isolation; Stool; Healthy

1. Introduction

Arcobacters are considered as emerging foodborne pathogens. These Gram-negative slender curved bacteria differ from the closely related campylobacters by their ability to grow at lower temperatures and in air (Vandamme and De Ley, 1991). Since the creation of *Arcobacter* as a second genus within the family *Campylobacteraceae,* six species have been characterized (Houf et al., 2005). *Arcobacter nitrofigilis*, *Arcobacter halophilus* and a number of not yet established species as candidatus *Arcobacter sulfidicus* are environmental-related species(Donachie et al., 2005; McClung et al., 1983; Wirsen et al., 2002). No association with human or animal infection has been reported. The other species, *Arcobacter butzleri*, *Arcobacter cryaerophilus*, *Arcobacter skirrowii* and *Arcobacter cibarius* have all been isolated from poultry products (Houf et al., 2005). They have also been isolated from the feces of healthy farm animals (Van Driessche et al., 2003). Diseases as mastitis in cattle (Logan et al., 1982), gastric ulcers in swine (Suarez et al., 1997), and reproductive disorders in both animal species have also been reported (On et al., 2002).

In humans, predominantly *A. butzleri* has been associated with enteritis and occasionally septicemia, but also *A. cryaerophilus* and recently *A. skirrowii* have been isolated from stool of diarrheic patients (Vandenberghe et al., 2004; Wybo et al., 2004). Clinical symptoms are similar to a *Campylobacter jejuni* infection though a higher association with persistent and watery diarrhea has been reported (Vandenberghe et al., 2004). Infection may occur by handling or consumption of contaminated water and food or by direct contact with infected humans (Vandamme et al., 1992). Arcobacters are commonly present on food of animal origin with the highest prevalence for poultry, followed by pork and beef (Houf et al., 2001a). Contamination of pork and beef probably occurs during slaughter by fecal contamination in contrast to poultry, for which no unequivocal evidence that arcobacters are present in the intestinal track is available (Houf et al., 2002a,b). Contaminated water has been identified as a major source of infection in developing countries with

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insufficient water supplies, though *A. butzleri* and *A. cryaerophilus* have also been isolated from river water and water drinking reservoirs in industrialized countries all over the world (Jacob et al., 1993). Outbreaks have rarely been reported so far, but enteritis due to *A. butzleri* with several patients infected has been recorded in Italy and Canada (Rice et al., 1999; Vandamme et al., 1992).

To date, knowledge on the pathogenicity of *Arcobacter* is very limited and no information is available of the occurrence of arcobacters in healthy humans. Furthermore, at present, there is no standard protocol for the selective isolation and quantification of arcobacters. Isolation from human specimens has been performed by slightly or not modified *Campylobacter*, *Yersinia* or even *Leptospira* isolation techniques of which we previously reported the lack in their ability to isolate all *Arcobacter* species and strains (Houf et al., 2001b). The first aim of this study was to evaluate a previous developed *Arcobacter* selective procedure (Houf et al., 2001a) already applied in veterinary and food microbiology, for the examination of human stool. Furthermore, the presence and level of shedding as well as characteristics of *Arcobacter* in feces of asymptomatic humans was examined in order to assess the clinical relevance of arcobacters in diarrheal stool.

2. Materials and methods

2.1. Validation of the isolation procedure

Eleven *Arcobacter* strains were obtained from the Belgian coordinated collections of micro-organisms, Laboratory for Microbiology (BCCM/LMG) Ghent University (Ghent, Belgium) or from previous clinical studies (Vandenberghe et al., 2004; Wybo et al., 2004) and used in the validation of the *Arcobacter* selective isolation procedure (Table 1). All strains were grown on blood agar plates (Mueller Hinton, CM 337, Oxoid-, Basingstoke, UK and 50 ml l^{-1} lysed defibrinated horse blood [E and O Laboratories Ltd., Bonnybridge, Scotland]) and incubated for 48 h at 37 °C under microaerobic conditions by evacuating 80% of the normal atmosphere and introducing a gas mixture of 8% CO_2, 8% H_2 and 84% N_2 into a jar. This incubation atmosphere was used for all further *Arcobacter* cultures and isolations. From each strain, a bacterial suspension was prepared in 10 ml of sterile *Arcobacter* enrichment broth (containing 24 g l^{-1} *Arcobacter* broth [CM 965-Oxoid, Basingstoke, UK], and the selective supplement (100 mg l^{-1} 5-fuorouracil, 10 mg l^{-1} amphotericine B, 16 mg l^{-1} cefoperazone, 32 mg l^{-1} novobiocine and 64 mg l^{-1} trimethoprim)) with an optical density of 0.9 (measured at 660 nm) which corresponded with a bacterial concentration of approximately 10^9 cfu ml^{-1}(Houf et al., 2001a). Serial 10-fold dilutions in sterile *Arcobacter* enrichment broth were prepared for the use in the validation process. Counts of the expected 10^3 and 10^4 cfu ml^{-1} bacterial suspensions were performed in 10-fold by inoculating 100 µl by the spiral plating method onto *Arcobacter* selective agar plates (containing 24 g l^{-1} *Arcobacter* broth, 12 g l^{-1} Agar Technical No.3 [L13-Oxoid] and the selective supplement described above) (Houf et al., 2001a). Following incubation at 28 °C for 24–72 h, the average and the standard deviation of the logarithm of the colony counts were calculated.

Table 1
Arcobacter strains used for validation

Organisms	Strain number	Source	Origin
Arcobacter butzleri	LMG 10828[a]	Man, feces	U.S.A.
	LMG 10240	Horse feces	Canada
	Clinical isolate	Man, feces	Belgium
Arcobacter cryaerophilus	LMG 9904[a]	Bovine fetus, brain	Northern Ireland
	LMG 9908	Porcine fetus	Northern Ireland
	Clinical isolate	Man, feces	Belgium
Arcobacter skirrowii	LMG 6621[a]	Lamb, feces	U.K.
	LMG 14985	Bull, preputial wash	U.S.A.
	Clinical isolate	Man, feces	Belgium
Arcobacter cibarius	LMG 21996[a]	Chicken, skin	Belgium
	LMG 21997	Chicken, skin	Belgium

[a] Type strain.

For the examination of the natural *Arcobacter* contamination of stool used in the validation, 1 g feces from five persons were transferred into small sterile stomacher bags in duplicate. Next, 9 ml *Arcobacter* enrichment broth supplemented with 50 ml l^{-1} lysed defibrinated horse blood was added. The mixtures were homogenized for 1 min with a stomacher blender at normal speed. After homogenization, 100 µl of each homogenate was inoculated onto two *Arcobacter* selective agar plates by spiral plating. Plates were incubated for 24–72 h at 28 °C. The remaining homogenates were incubated for 48 h at 28 °C. Following incubation, 50 µl of the homogenates was streaked onto two *Arcobacter* selective agar plates. Plates were incubated for 24–72 h at 28 °C and checked every 24 h for bacterial growth. All colonies were counted and subcultured onto blood agar plates. *Arcobacter* identification was performed at species level by a previously developed multiplex-PCR (m-PCR) (Houf et al., 2000). For the isolates tested negative in the m-PCR, an *Arcobacter*-genus-specific PCR assay using the primers Arco I and Arco II was applied (Harmon and Wesley, 1996). Negative cultures were further examined by classical microbiological and biochemical tests. For the direct isolation procedure, repeatability, in-lab reproducibility, sensitivity and specificity were evaluated. From each fecal sample, 45 subsamples of 1± 0.05 g were taken and transferred into sterile stomacher bags. Each subsample was then spiked with 1 ml of a single *Arcobacter* collection strain at a concentration of 10^4 and 10^3 cfu ml^{-1}. This was performed in duplicate. One subsample was spiked with 1 ml sterile *Arcobacter* enrichment broth and acted as blank. Then, 8 ml *Arcobacter* enrichment broth was added. After homogenization, ten times 100 µl was transferred onto ten *Arcobacter* selective agar plates by means of the spiral plater. The remaining inoculated homogenates were stored for 5 days at 4 °C. After chill-storage, 100 µl per homogenate was transferred 10 times onto *Arcobacter* selective agar plates. All agar plates were incubated for up to 5 days at 28 °C and checked every 24 h for the presence of bacterial growth.

All colonies were counted and further tested by m-PCR and when necessary by genus-PCR. The average and standard deviation of the logarithm of the 10 colony counts were calculated.

For the enrichment method, the detection limit was determined. From each fecal sample, ten times 1 g was taken and transferred into sterile stomacher bags. Eight milliliters *Arcobacter* enrichment broth was added. Samples were respectively spiked with 1 ml of the bacterial suspensions ranging from 10^3 to 10^0 cfu ml^{-1} in duplicate. Two samples were spiked with 1 ml sterile *Arcobacter* enrichment broth and acted as blank. After homogenization, all samples were incubated at 28 °C. After 24 h, 48 h and 72 h of incubation, 50 µl of the homogenates was streaked onto *Arcobacter* selective agar plates. The plates were incubated and checked every 24 h for bacterial growth up to 3 days.

Results were analyzed using univariate analysis of variance ($P<0.05$).

2.2. Healthy human Arcobacter carriers

In a prospective study of stool samples from healthy staff members of slaughterhouses, cutting and meat processing plants, logistics and butcher shops for the occurrence of *Salmonella*, 500 specimens of different persons from different parts of Switzerland were additionally examined in October, November and December 2005 for the presence of *Arcobacter* species. All samples were collected in urban areas and each person was tested only once. The population consisted of healthy adults without diarrhea aged between 20 and 60 years, a quarter being female. Samples were stored under cooled condition and examined within 72 h. A maximum of 1 g feces per sample was homogenized with 1:10 ml *Arcobacter* selective isolation broth, and twice 100 µl of each homogenate was brought onto two *Arcobacter* selective agar plate by the spiral plating method. All incubations were performed at 28 °C under microaerobic conditions as described above. After incubation for 48 h, twice 50 µl of the enriched homogenates was streaked onto two *Arcobacter* selective agar plate and further incubated as described above. Plates were checked for bacterial growth every 24 h for 4 days. All colonies obtained by direct isolation were counted and a maximum of 10 was picked and subcultured onto blood agar plates for further analysis. Analysis of the colonies on the selective agar plates obtained after enrichment was limited to four.

2.3. Characterization of the Arcobacter isolates

Identification of all isolates at species or genus level was performed by PCR assay as described above. In order to assess the diversity, *Arcobacter* isolates were characterized below species level by modified ERIC-PCR (Houf et al., 2002a). As shown in previous studies, DNA patterns that differed in one or more DNA fragments represented different types (Houf et al., 2002a; Van Driessche et al., 2003).

The Etest (AB Biodisk, Solna, Sweden) was performed for erythromycin, ciprofloxacin and tetracycline on Mueller–Hinton agar supplemented with 5% sheep blood according to the instructions provided by the manufacturer. Inocula were prepared by incubating the strains for 48 h at 37 °C under aerobic conditions in trypticase soy broth. After application of the Etest strips, plates were incubated in microaerophilic conditions at 37 °C for 48 h. The minimal inhibition concentrations (MIC) were read directly from the test strip at the point where the elliptical zone of inhibition intersected the MIC scale on the strip. Since the clinical and laboratory standards institute (CLSI) recommendations do not include specific breakpoints for testing *Campylobacteraceae* species, the MIC interpretative criteria used in this study for erythromycin and tetracycline were those for *Staphylococcus* species. For the other drugs, we used those for *Enterobacteriaceae* awaiting specific CLSI recommendations. The following MIC breakpoints for resistance were applied: ciprofloxacin MIC ≥ 4 mg/l, erythromycin MIC ≥ 8 mg/l, tetracycline MIC ≥ 16 mg/l (NCCLS, 2001).

For adhesion assays, bacterial cultures were prepared by inoculating Brain Heart Infusion medium with colonies grown on blood agar plates and incubating them for stationary subcultures aerobically at 37 °C without shaking. HEp-2 and Caco-2 cells were cultured in disposable flasks (Nunc) and routinely grown in Minimum Essential Medium (Gibco), supplemented with 10% heat inactivated fetal calf bovine serum (Omnilab), 1% nonessential amino acids (Gibco), 2% GlutaMAX-I Supplement (Gibco) and 0.4% gentamicin (Gibco). Cell lines were incubated at 37 °C in a 5% CO_2 atmosphere. For adhesion assays 10^5 cells/cm^2 were seeded onto circular glass cover slips (Sterilin, Barloworld Scientific, United Kingdom) placed at the bottom of the wells of 24-multiwell tissue culture plates (TPP) and incubated to confluency with cell culture medium without antibiotics for 48 h (Hep-2) and 72 h (Caco-2) at 37 °C in a 5% CO_2 atmosphere. Adhesion experiments were performed following a modified method of the Center for Vaccine Development (University of Maryland) (Cravioto et al., 1979). Confluent tissue culture on the glass cover slips in 24-well plates were washed once with PBS (Gibco), and a MOI (MOI; number of bacteria per number of mammalian cells) of 100:1 was added to the Hep-2 and Caco-2 monolayers in 1 ml cell line culture medium. The 24-well plates were centrifuged for 10 min at 1500 ×*g* at room temperature. Bacteria were allowed to adhere for 180 min at 37 °C in 5% CO_2. *A. butzleri* AHH 41 was included as positive adhering control. All experiments were performed in triplicates. After incubation, each well was rinsed five times with PBS, cell material was fixed by the addition of methanol for 10 min and stained with 10% Giemsa stain (Sigma-Aldrich). After staining of the biological material, cover slips were washed in ultra pure water and 1% acetic acid, contrasted with isopropanol, mounted on glass slides and examined under a light transmission microscope at a magnification of ×1000 (oil immersion).

3. Results

3.1. Validation of the isolation procedure

Arcobacter was not isolated from the five stool samples used in the validation tests. With the direct isolation method, the recovery of the different *Arcobacter* species at both spiking levels was concordant with the initial number added ($p<0.05$). The standard deviations calculated on the average of the ten

Table 2
Origin and characteristics of the Arcobacter isolates

Specimen N °	Sex	Region in Switzerland	Age	Species	Minimal inhibitory concentration (mg/l)			Adhesion experiments	
					Ciprofloxacin	Erythromycin	Tetracycline	Hep-2 cells	Caco cells
28	Male	Central part	41 years	*A. cryaerophilus*	0.064	0.500	0.500	+/−	−
36	Male	Northern part	44 years	*A. cryaerophilus*	0.023	0.064	0.125	+/−	−
55	Male	Western Part	34 years	*A. cryaerophilus*	0.032	0.750	0.190	−	−
57	Male	Northern part	49 years	*A. cryaerophilus*	0.004	0.094	0.016	+	+
96	Male	Northern part	36 years	*A. cryaerophilus*	0.006	0.064	0.047	−	−
162	Male	Northern part	38 years	*A. cryaerophilus*	0.064	0.500	0.380	+	+
456	Male	Western part	46 years	*A. cryaerophilus*	0.032	0.380	0.190	−	−

colony counts of each spiking level ranged from minimum 0.03 to a maximum of 0.13. There was no interspecies nor intraspecies difference in recovery, only a difference in the time required for outgrowth. For *A. butzleri*, a 24 h incubation was already sufficient to obtain visible colonies, whereas 24 and 72 h were necessary for *A. cryaerophilus* and *A. skirrowii,* respectively.

To determine the in-lab reproducibility, the spiked homogenates were stored for 5 days at 4 °C. Storage of these homogenates resulted in no significant decrease compared with the colony counts of the original spiked homogenates ($p<0.05$).

For the enrichment method, the detection limit was set on 10^0 cfu/g. At this concentration, *A. butzleri* could be isolated after 24 h of enrichment, followed by 24 h of plate incubation. *A. cryaerophilus* and *A. skirrowii* required an enrichment of 48 h followed by 48 h of plate incubation. An enrichment of 72 h was not required for any of the *Arcobacter* species tested.

For the specificity, it was noted that on some plates, yellow translucent colonies, resembling *Arcobacter* colony morphology appeared already after 24 h incubation. Biochemical examination of this accompanying flora identified them as belonging to the *Pseudomonas* genus. Examination of the selective agar plates with Henry transillumination revealed that all *Arcobacter* species (*A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. cibarius*) displayed a bluish bacterial growth in contrast to the accompanying flora, which facilitated the picking of the suspected colonies for further examination.

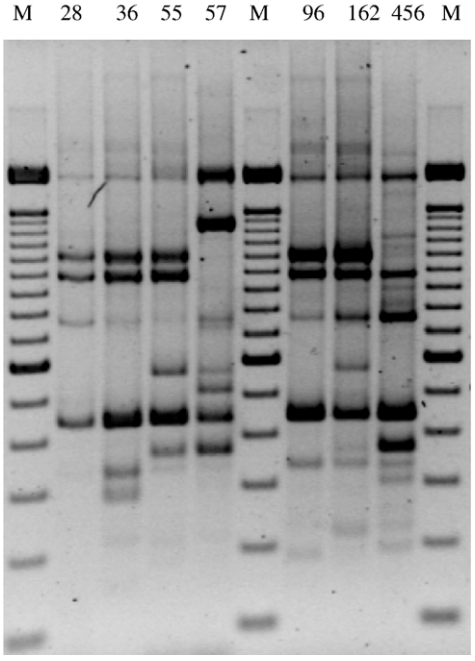

Fig. 1. ERIC fingerprints of the 7 *Arcobacter* isolates. The lane numbers correspond to the isolate numbers shown in Table 1. Lanes M contained a reference marker (100–1500 and 2072 bp).

3.2. Healthy human carriers

Prevalence and characteristics of the *Arcobacter* isolates are summarized in Table 2. None of the 500 samples was positive for the presence of arcobacters by direct isolation. Enrichment of the stool samples for 48 h followed by plating on selective agar resulted in 7 samples for which typical *Arcobacter* colonies were obtained. Of those carriers, one person worked in a slaughterhouse, three worked in meat processing plants, and three persons worked in logistics where they had no direct contact with animals or meat. The twenty-eight colonies picked for further examination were all identified as *A. cryaerophilus*. Characterization of the isolates revealed seven genotypes, with only small differences in the fingerprint patterns (Fig. 1). The four isolates per sample were all identical strains by which for all persons, only a single *A. cryaerophilus* strain was detected.

Within the tested antibiotics, a broad range of MIC values for the seven strains were obtained, especially for ciprofloxacin (0.006 to 0.064) but all strains were susceptible to the antibiotics tested (Table 2).

Adhesion experiments were performed on confluent monolayers of HEp-2 and Caco-2 under standardized conditions (stationary phase cultures, MOI 100:1, 180 min incubation time) and in triplicates for the two cell lines. According to these results, adhesion was considered positive (+), when adhesion was observed in all the replicates, ambivalent (+/−), when adhesion was observed in one or two of the replicates and negative (−), when no adhesion was observed in any of the experiments. Four isolates showed adhesion to HEp-2 and two isolates to Caco-2 cells (Table 2).

4. Discussion

Interest for arcobacters in veterinary and human public health enhanced since the first reports of the isolation of

arcobacters from food of animal origin ten years ago (de Boer et al., 1996). Since then, studies worldwide have reported the occurrence of arcobacters in raw meat samples and have highlighted the possible transmission to the human population. However, since the clarification of the taxonomic position of *Arcobacter*, only four studies have dealt with the clinical course of *Arcobacter* infection in humans (Engberg et al., 2000; Lastovica and Le Roux, 2000; Vandenberghe et al., 2004; Prouzet-Mauléon et al., 2006).

Our present study deals with two major aspects that actually hamper the risk assessment for these bacteria. First, besides the fact that clinical samples are not routinely tested for *Arcobacter* species as is done for *Salmonella* or *Campylobacter* species, most isolations are performed by methods designed for thermophilic *Campylobacter* species (Houf et al., 2001a). As for most studies mentioned above, the selective isolation is achieved by the incorporation of antimicrobial agents in the plating media. Although arcobacters have sporadically been isolated in this way, a study about the *Arcobacter* susceptibility to antimicrobials included in those media revealed that none of those selective supplements allowed the growth of all *Arcobacter* strains and at the same time sufficiently suppressed the accompanying flora in biological samples (Houf et al., 2001b). A second isolation strategy includes the filtration of a stool homogenate onto non-selective blood agar plates, as used by Lastovica and Le Roux (2000). Though theoretically this method allows the isolation of more sensitive species, it has not been validated for *Arcobacter* isolation. Furthermore, selective isolation based on filtration applied in food microbiology has shown to be less sensitive compared to selective isolation based on incorporation of antimicrobial agents (Gude et al., 2005; Houf et al., 2001a). Subsequently, isolates have to be identified which forms an additional problem in *Campylobacteraceae* research. In routine clinical laboratory, most of this identification is still performed by biochemical tests. Arcobacters, as campylobacters, display however low metabolic activity, often resulting in wrong identification (On, 1996). In the present study, the selective isolation method allowed the growth of all *Arcobacter* species presently associated with human illness and suppressed the accompanying flora in such way that isolation was not hindered. The performance of the direct isolation technique as well as the isolation after enrichment was concordant to those previously reported for animal feces (Van Driessche et al., 2003). The sensitivity, reproducibility, detection limits and optimal incubation periods were comparable (Van Driessche et al., 2003). Only on a minority of plates, accompanying flora was observed, which could easily be identified by the application of Henry transillumination.

Secondly, only little information was available about the prevalence of arcobacters in healthy people. Therefore the knowledge if arcobacters are part of the human commensal flora is lacking. Engberg et al. (2000) processed stool specimens from 107 healthy people by the filter method, but no arcobacters were isolated. In the present study, seven persons (1.4%) shed arcobacters in the feces at levels lower than 100 colony forming units per gram, and enrichment before isolation was therefore needed. All were identified as *A. cryaerophilus*. The absence of *A. butzleri* in the samples enhances the clinical relevance of this species in clinical samples. In the study of Vandenberghe et al. (2004) ages of patients with *A. butzleri* infection were 30 days–90 years with slightly more female than male patients. In the present study, no persons under 20 were included, so the prevalence of *Arcobacter* in children and adolescents has yet to be determined. In the present study all carriers were males but conclusions about a possible sex predisposition can not be drawn due to the low *Arcobacter* prevalence and the unequal number of males versus females among the test persons. Though *A. cryaerophilus* is the most heterogeneous *Arcobacter* species, characterization of the human isolates displayed only small genotypic differences and only one genotype per person was isolated. This contrasts with the findings in healthy farm animals from which multiple species and genotypes can be recovered (Houf et al., 2002a,b; Van Driessche et al., 2003). There was no relationship between the persons from whom arcobacters were isolated. They worked in different parts of Switzerland or in different companies, and six of them had no direct contact with animals or raw meat at the workplace.

To assess the pathogenicity of arcobacters for humans, evaluation of potential virulence factors is required. However, up to now, little is known about the mechanisms of pathogenicity. A necessary state in the successful colonization, establishment and ultimately production of disease by most microbial pathogens is the ability to adhere to host surfaces such as mucous membranes, gastric and intestinal epithelial or endothelial tissue (Boyle and Finlay, 2003). Up to now, no data in view of adhesion of *Arcobacter* species, and *A. cryaerophilus* in particular, are available in the literature, and if adhesion is even necessary in the pathogenicity is unknown. Specific adhesion to tissue cells is therefore considered an essential virulence factor for most bacterial pathogens (Klemm and Schembri, 2000). In this study, we investigated the potential of *A. cryaerophilus* strains to adhere to HEp-2 cells and to the human colon carcinoma cell line Caco-2, two well established and frequently used models for studying interactions of bacteria with human cells (Darfeuille-Michaud et al., 1990; Keller et al., 1998). Four strains out of 7 showed adhesion to at least one cell line. However, the significance of the *in vitro* adhesion capacity as well as other virulence factors of arcobacters have to be further determined, and more data is necessary about all human-related *Arcobacter* species to assess the ability of adhesion in pathogenicity.

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