International Journal of Food Microbiology 373 (2022) 109712

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Genetic characterization and biofilm formation of potentially pathogenic foodborne *Arcobacter* isolates

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ARTICLE INFO

Keywords:
Food
Prevalence
MLST
Virulence-associated genes
Adhesion to surfaces

ABSTRACT

Various species of the genus *Arcobacter* are regarded as emerging food pathogens and can be cause of human gastroenteric illness, among others. In order to gain knowledge on the risk associated with the presence of arcobacters in retail foods, this study aimed to determine their presence in a variety of products; to evaluate the genetic diversity and the occurrence of virulence and biofilm-associated genes in the isolated strains; and to assess their biofilm activity on polystyrene, borosilicate and stainless steel. *Arcobacters* were detected in the 22.3% of the analysed samples and the 83 recovered isolates were identified as *A. butzleri* ($n = 53$), *A. cryaerophilus* ($n = 24$), *A. skirrowii* ($n = 2$), *A. thereius* ($n = 3$) and *A. vitoriensis* ($n = 1$). They were isolated from virtually all tested food types, but mostly from squids and turkey meat (contamination levels of 60% and 40%, respectively). MLST differentiated 68 STs, most of which were novel (89.7%) and represented by a single strain (86.9%). Five novel STs were detected in various isolates derived from seafood, and the statistical analysis revealed their potential association with that type of food product ($p < 0,001$). All the isolates except one harboured virulence-associated genes and the highest incidence was noted for *A. butzleri*. Nineteen isolates (23.5%) were able to form biofilms on the different surfaces tested and, of note; glass enhanced the adhesion ability of the majority of them (84.2%). The results highlight the role that common food products can have in the transmission of *Arcobacter* spp., the pathogenic potential of the different species, and the survival and growth ability of several of them on different food contact surfaces. Therefore, the study provides interesting information regarding the risk arcobacters may pose to human health and the food industry.

1. Introduction

The genus *Arcobacter*, within the *Campylobacteraceae* family, was first described by Vandamme et al. in 1991. The taxonomy of this genus has been under debate during the last years (On et al., 2020, 2021; Pérez-Cataluña et al., 2018; Waite et al., 2017) but, at the time of writing and according to LPSN, the list of prokaryotic names with standing in nomenclature (Parte et al., 2020), the genus comprises 33 validly published species (https://lpsn.dsmz.de/genus/arcobacter; accessed on February 28th, 2022) that have been isolated from various different environments and sources. Certain species of the genus are associated with human disease. They mainly induce gastrointestinal symptoms (chronic watery diarrhoea and traveller's diarrhoea), but can also be the cause of bacteraemia, septicaemia, peritonitis and endocarditis (Collado and Figueras, 2011; Fanelli et al., 2019; Simaluiza et al., 2021). *A. butzleri* is the species most frequently associated with disease, followed by *A. cryaerophilus*, but infections due to *A. skirrowii*, *A. thereius* and *A. lanthieri* have also been reported (Kerkhof et al., 2021; Ramees et al., 2017; Ruíz de Alegría et al., 2021; Van den Abeele et al., 2014). Nevertheless, the mechanisms implied in the pathogenesis of these bacteria remain unclear. Various authors have demonstrated the in vitro cytotoxicity of *Arcobacter* species, along with their ability to adhere and

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https://doi.org/10.1016/j.ijfoodmicro.2022.109712
Received 7 August 2021; Received in revised form 1 May 2022; Accepted 5 May 2022
Available online 9 May 2022

invade different human cell lines (Buzzanca et al., 2021; Collado and Figueras, 2011; Karadas et al., 2013; Levican et al., 2013). On the other hand, the available *Arcobacter* genomes have shown the presence of various virulence-associated genes related to, among others, adaptation, cytotoxicity, adhesion, invasiveness and antibiotic resistance (Isidro et al., 2020; Miller et al., 2007; Müller et al., 2020a, 2020b). The correlation between the reported pathogenic capabilities and the presence of specific genes has not been established yet in *Arcobacter* spp. However, the virulence-associated gene content of the isolates can be indicative of the risk they may pose to human health.

The consumption of contaminated drinking water and/or undercooked or raw foods seems to be the main human transmission source of *Arcobacter* spp. They are commonly present in food products including vegetables, seafood, terrestrial animal food products and composite foods (Gónzalez and Ferrús, 2011; Kietsiri et al., 2021; Mottola et al., 2020; Nieva-Echevarria et al., 2013); but also in different waters including continental, coastal, sea, recreational, drinking and sewage (Sciortino et al., 2021). Moreover, arcobacters are often present in food processing environments such as slaughterhouses and dairy farms and/or plants (Ferreira et al., 2017; Giacometti et al., 2015b; Khodamoradi and Abiri, 2020) where, if the conditions are favourable, they may probably form biofilms. *Arcobacters* have the demonstrated ability to adhere to different surfaces and to form biofilms on them (Ferreira et al., 2013; Girbau et al., 2017; Šilha et al., 2021). When formed on food contact surfaces and/or materials, biofilms increase food safety risk. Reservoirs of food spoilage and/or pathogen bacteria in food industries are an important cause of product contamination that can lead not only to a reduced shelf life of foods, but also to health problems (Abebe, 2020; Adetunji et al., 2014). Therefore, the biofilm formation by food derived *Arcobacter* spp. can pose a risk for public health and a problem for the food industry. Tracking the infection source and the transmission routes of arcobacters is one of the necessary steps to assess the risk related to these pathogens. Among the molecular subtyping techniques available for the species, the Multilocus Sequence Typing (MLST) scheme proposed by Miller et al. (2009) is a reliable and reproducible technique that has been successfully utilized for characterization of *Arcobacter* isolates from different sources (Alonso et al., 2014; Caruso et al., 2020; Kietsiri et al., 2021; Niedermeyer et al., 2020). However, partly due to the limited available data in the *Arcobacter* MLST database (https://pubmlst.org/organisms/arcobacter-spp), partly due to the great genetic heterogeneity shown by *Arcobacter* isolates, no source-associated genetic marker has been reported so far for these species.

In order to increase the knowledge needed to assess the risk that arcobacters pose for human health; the purposes of this study were to confirm the presence of different *Arcobacter* species in retail food products; to evaluate, by MLST and virulence-associated gene detection, the genetic diversity of the isolates; and to investigate the biofilm production of all the recovered isolates. We complement, this way, our previous surveys on prevalence and characterization of arcobacters in foods (Alonso et al., 2014; Girbau et al., 2014, Girbau et al., 2017; Nieva-Echevarria et al., 2013) by the analysis of food products not previously surveyed.

2. Materials and methods

2.1. Sample collection and processing

Two hundred and twenty samples including cockle, squid, shrimp, quail meat, rabbit meat, turkey meat, fresh cheese, spinach, Swiss chard, lettuce and carrot, were purchased from different local retail shops and supermarkets in Vitoria-Gasteiz, Spain, from May to November 2015. All samples, 20 of each type of food, were kept in coolers, transported to the laboratory and processed within 2 h of purchase.

Ten grams of each sample were homogenized into 90 mL (1:10 wt/vol) of Arcobacter-CAT broth (Oxoid) as previously described (Nieva-Echevarria et al., 2013), and then incubated aerobically at 30 °C for 48 h. After enrichment, 0.2 mL of each broth were inoculated by passive filtration with 0.45-μm nitrocellulose membrane filters (Millipore) onto blood agar plates (Columbia agar supplemented with 5% sheep blood, Oxoid) and incubated under the aforementioned conditions for 48–72 h. After incubation, four to six suspect *Arcobacter* colonies (small, smooth and translucent to whitish) were picked from each plate and subcultured onto blood agar plates at least three times. Upon microscopic examination, those isolates presenting a curved to spiral shape and characteristic motility were subjected to PCR identification.

2.2. Arcobacter species identification

2.2.1. Genomic DNA isolation

DNA was isolated using PrepMan™ Ultra reagent (Applied Biosystems) according to the manufacturer's specifications. The concentration was determined spectrophotometrically (NanoDrop, Thermo Fisher Scientific), adjusted to 20 ng/μL and stored at −20 °C.

2.2.2. PCR and m-PCR

Suspicious colonies were identified by the genus-specific PCR described by Bastyns et al. (1995). To avoid the inclusion of clones in the collection, all *Arcobacter* isolates recovered from the same food sample were genotyped using the enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) protocol as previously described for *Arcobacter* (Houf et al., 2002). Patterns with at least one or more different bands were considered as different genotypes.

Species identification of the isolates was carried out by two previously described methods. The m-PCR proposed by Houf et al. (2000) that targets *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* was applied first, followed by the m-PCR proposed by Douidah et al. (2010) that simultaneously identifies *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. cibarius* and *A. thereius*. DNA from *A. butzleri* RM4018, *A. cryaerophilus* CCUG 17801T, *A. skirrowii* CCUG 30483, *A. cibarius* CECT 7203 and *A. thereius* CCUG 56002, together with deionized water, were used as positive and negative controls, respectively.

2.2.3. Phylogenetic and phylogenomic analysis

In order to determine the taxonomic position of one isolate which could not be identified to the species level by the aforementioned m-PCR methods, both, phylogenetic and phylogenomic analyses, were held (Alonso et al., 2020).

2.3. Genetic characterization

The 83 strains recovered in this study (53 *A. butzleri*, 24 *A. cryaerophilus*, three *A. thereius*, two *A. skirrowii* and one *A. vitoriensis*) were genetically characterized by Multilocus Sequence Typing (MLST) and virulence-associated gene detection.

2.3.1. MLST and minimum spanning trees

MLST was carried out according to the method of Miller et al. (2009) with minor modifications. The *glyA* gene from *A. cryaerophilus* was amplified with different annealing temperatures (55–59 °C) and the *glnA* gene from *A. thereius* was amplified by using the primers glnACR1 and glnATHF (5′-AAATGGAATGCCTTTTGATGGAG-3′). Allele numbers and sequence types (STs) were assigned using the PubMLST database (Jolley et al., 2018) at https://pubmlst.org/organisms/arcobacter-spp. New alleles and STs were submitted to the database curator to be assigned new allele or ST numbers. In order to detect possible recombination events, all the available concatenated MLST sequences were downloaded from the *Arcobacter* PubMLST database on January 2017 (n = 648) and subsequently analysed using five methods (RDP, Geneconv, MaxChi, Chimaera and 3Seq) implemented in the RDP3 software package (Martin et al., 2010) using default parameters. In order to visualize the relationships between the STs and their distribution among different food products, minimum spanning trees (MST) were created by

the goeBURST algorithm using the PHYLOViZ v2.0a software (Nascimento et al., 2017). MSTs were constructed based on the distances between the allelic profiles of all the *Arcobacter* isolates with an assigned ST available at the PubMLST database on January 2021 ($n = 997$), including those identified in the present study.

2.3.2. Detection of virulence genes

The presence of ten putative virulence genes (*cadF*, *Cj1349*, *ciaB*, *mviN*, *pldA*, *tlyA*, *irgA*, *hecA*, *hecB* and *iroE*) was determined using the primer pairs designed by Douidah et al. (2012) and Karadas et al. (2013), following the protocol described by Girbau et al. (2015). DNA from *A. butzleri* RM4018 was used as positive control and deionized water as negative one. Additionally, DNAs from *A. cryaerophilus* CCUG 17801 and Ac-L7 (Girbau et al., 2015), *A. skirrowii* CCUG 30483 and *A. thereius* CCUG 56902 were also included as controls whenever any of these species were subjected to the aforementioned PCRs.

2.4. Biofilm production

The ability to form biofilms of the strains recovered in this survey was investigated by biofilm-associated gene detection and in vitro phenotypic assays.

2.4.1. Biofilm-associated gene detection

Genes to be detected (*flaA*, *flaB*, *fliS*, *luxS*, *pta*, *waaF* and *spoT*) were selected based on their association with adherence to abiotic surfaces in other campylobacteria. Based on comprehensive analyses and alignments of the published genome sequences of *A. butzleri* RM4018^T, *A. cryaerophilus* ATCC 43158^T, *A. skirrowii* CCUG 10374^T, *A. thereius* LMG 24486^T and *A. vitoriensis* F199^T (GenBank, accession numbers GCA_000014025, NZ_CP032823, NZ_VZOH00000000, NZ_CP035926.1 and PDKB00000000, respectively), 35 PCR primers were designed using Clone Manager 9 Professional Edition software (Sci Ed Software LLC). Once designed, the primers were tested *in silico* by blasting them against completed *Arcobacter* genome sequences available in the GenBank database. The primer sequences and the expected amplicon sizes depending on the species are listed in Table S1.

All PCRs were carried out in final volumes of 50 μL containing 100 ng of DNA as template, 1.25 U of DreamTaq DNA Polymerase (Thermo Scientific), 0.2 mM of each dNTP, 1× buffer and 0.5 μM of each primer set. An initial denaturation step at 94 °C for 3 min was followed by 30 cycles of denaturation at 94 °C for 45 s; primer annealing at different temperatures ranging from 50 °C to 56 °C for 45 s; and elongation at 72 °C for 1 min. A final elongation step at 72 °C for 3 min was performed.

2.4.2. Motility assay

In order to test the motility of those strains for which flagellin gene detection failed, individual colonies were spotted onto 0.4% thioglycolate plates (Scharlau). The plates were incubated at 30 °C, and the growth and expansion of colonies was examined after 16–24 h.

2.4.3. Static biofilm assays

The biofilm formation ability was first assessed at 30 °C under aerobic conditions using polystyrene microtiter plates, as described previously (Girbau et al., 2017). Borosilicate glass tubes and stainless steel coupons were also used for further testing of the ability of those strains showing adherence to polystyrene. For each assay, every isolate was examined in three replicates and the experiments were performed at least on three separate occasions.

Those biofilms formed on polystyrene and borosilicate were expressed by the biofilm formation index (BFI) according to Niu and Gilbert (2004), and subsequently categorized as strong, moderate, weak or none biofilm formation according to Naves et al. (2008). The biofilms formed on stainless steel were evaluated by plate count method on Mueller-Hinton agar (Oxoid) after gently washing the coupons with sterile distilled water (Girbau et al., 2017).

2.5. Statistical analysis

Data were analysed with the SPSS 26 statistical package program (SPSS Inc., Chicago, IL, USA). The Chi-square and Fisher's exact tests were performed in order to compare the distribution of the isolates and species among samples and to assess possible associations between variables. The isolates derived from spinach ($n = 1$), lettuce ($n = 1$), cheese ($n = 1$) and rabbit ($n = 3$), along with those identified as *A. thereius* ($n = 3$), *A. skirrowii* ($n = 2$) and *A. vitoriensis* ($n = 1$), were excluded from the analyses based on their low representation.

Once tested the normality of the numerical variable "biofilm formation" using the Kolmogorov-Smirnov method, the Kruskal-Wallis test was used to compare the values obtained for biofilm formation ability on each surface among the isolates. Student *t*-test was used to compare the values obtained for the formed biofilms on polystyrene versus borosilicate for each strain. Results were considered significant at *p* values of <0.05.

3. Results

3.1. Occurrence of Arcobacter spp. in food samples

The occurrence of *Arcobacter* spp. in the 220 food samples analysed in this study is summarized in Table 1. Overall, they were detected in all the tested types of products except chard, with a gross occurrence of 22.3% (49 out of 220 samples). The arcobacters were mostly detected in seafood products, which showed a statistically significant ($p < 0.001$) contamination level of 43.3%. Foods of terrestrial animal and vegetable origin showed lower contamination levels, 21.3% and 7.5%, respectively. Specifically, those products from which *Arcobacter* spp. were mainly recovered were squid (60.0%) and turkey meat (40.0%). The recovery from squid was statistically significant ($p < 0.001$).

Among the 266 isolates identified as *Arcobacter* spp. by the genus-specific PCR (Bastyns et al., 1995), 83 were selected for further identification to the species level based on the ERIC-PCR results (Houf et al., 2002). Out of them, 53 isolates were identified as *A. butzleri*, 24 as *A. cryaerophilus* and two as *A. skirrowii* by both m-PCR (Douidah et al., 2010; Houf et al., 2000); and three as *A. thereius* by one of them (Douidah et al., 2010). The identification of the remaining *Arcobacter* isolate required phylogenetic and phylogenomic analyses, which identified it as *Aliarcobacter* (now *Arcobacter*) *vitoriensis* (Alonso et al., 2020). The distribution of the recovered species according to the type of food differed significantly ($p = 0.009$) and it was as follows: *A. butzleri* was isolated from all type of products except chard; *A. cryaerophilus* from seafood and meat products; and *A. skirrowii* and *A. thereius* only from seafood. *A. vitoriensis* was recovered from a carrot sample that was simultaneously contaminated with *A. butzleri*. Three cockle samples were doubly contaminated with *A. butzleri* and *A. cryaerophilus*; a shrimp sample with *A. butzleri* and *A. thereius*; and a squid sample with *A. cryaerophilus* and *A. thereius*. *A. butzleri* was the most commonly isolated species from all of the food products except cockle and shrimp, where *A. cryaerophilus* prevailed. Approximately the half (50.9%) of the *A. butzleri* isolates were recovered from terrestrial animal products and the majority of the *A. cryaerophilus* from seafood (87.5%). Indeed, Fisher exact test established an association between seafood products and *A. cryaerophilus* ($p < 0.001$).

3.2. Genotyping by MLST

Eighty two isolates out of the 83 analysed were successfully typed by MLST and numerous alleles and STs were identified (Table S2). The allele sequences of the *A. vitoriensis* isolate could not be determined. A total of 351 alleles were identified across all seven loci, ranging from 42 alleles at *gltA* to 62 at *glyA*. Overall, 172 out of the 351 (49%) alleles were previously unreported, ranging their frequency from 33.3% (*gltA*) to 64.5% (*glyA*). Sixty eight STs (41 of *A. butzleri*, 22 of *A. cryaerophilus*,

Table 1
Arcobacter species recovered from the 220 food samples purchased at the retail level in Vitoria-Gasteiz between May and November 2015.

			No. (%) of samples positive for:					No. of genotypes identified by ERIC-PCR for:				
Type of sample	No.	*Arcobacter* spp.	*A. butzleri*	*A. cryaerophilus*	*A. skirrowii*	*A. thereius*	*A. vitoriensis*	*A. butzleri*	*A. cryaerophilus*	*A. skirrowii*	*A. thereius*	*A. vitoriensis*
Seafood												
Cockle	20	7 (35.0)[a]	4 (57.1)	6 (85.7)	–	–	–	6	9	–	–	–
Squid	20	12 (60.0)[b,*]	7 (58.3)	3 (25)	1 (8.3)	2 (16.7)	–	9	4	1	2	–
Shrimp	20	7 (35.0)[c]	3 (42.9)	3 (42.9)	1 (14.3)	1 (14.3)	–	3	8	1	1	–
Subtotal	60	26 (43.3)[*]	14 (53,8)	12 (46.2)	2 (7.7)	1 (3.8)	–	18	21[*]	2	3	
Terrestrial animal products												
Turkey meat	20	8 (40.0)	7 (87.5)	1 (12.5)	–	–	–	15	1	–	–	–
Rabbit meat	20	3 (15.0)	2 (66.7)	1 (33.3)	–	–	–	2	1	–	–	–
Quail meat	20	5 (25.0)[d]	5 (100)	1 (20)	–	–	–	9	1	–	–	–
Fresh cheese	20	1 (5.0)	1 (100)	–	–	–	–	1	–	–	–	–
Subtotal	80	17 (21.3)	15 (88,2)[*]	3 (17,6)	–	–	–	27	3	–	–	–
Vegetables												
Carrot	20	4 (20.0)[e]	4 (100)	–	–	–	1 (20)	6	–	–	–	1
Spinach	20	1 (5.0)	1 (100)	–	–	–	–	1	–	–	–	–
Lettuce	20	1 (5.0)	1 (100)	–	–	–	–	1	–	–	–	–
Chard	20	–	–	–	–	–	–	–	–	–	–	–
Subtotal	80	6 (7.5)	6 (100)[*]	–	–	–	1 (16.7)	8	–	–	–	1
Total	220	49 (22.3)	35 (71.4)	15 (30,6)	2 (4.1)	1 (2.0)	1 (2.0)		53	24	2	3 1

[a] *A. butzleri* and *A. cryaerophilus* were simultaneously detected in three cockle samples.
[b] *A. cryaerophilus* and *A. thereius* were simultaneously detected in a squid sample.
[c] *A. butzleri* and *A. thereius* were simultaneously detected in a shrimp sample.
[d] *A. butzleri* and *A. cryaerophilus* were simultaneously detected in a quail meat sample.
[e] *A. butzleri* and *A. vitoriensis* were simultaneously detected in a carrot sample.
[*] Statistically significant differences ($p < 0.05$) between results, based on Fisher's exact test.

two of *A. skirrowii* and three of *A. thereius*) were identified among the 82 genotyped isolates and their occurrence in seafood, foods of terrestrial animal origin and vegetables was 33, 28 and 7, respectively (Table 2). Most of the STs (89.7%) were previously unreported and resulted from new allele's sequences ($n = 50$) or new combinations of known alleles ($n = 11$). Fifty three out of the 61 novel STs (86.9%) were represented by a single strain; five STs (three of *A. butzleri* and two of *A. cryaerophilus*) by two; ST-512 by three (*A. butzleri*); ST-513 by four (*A. butzleri*) and ST-517 by five (*A. butzleri*). The analysis of the relatedness and distribution of the identified STs and the other *Arcobacter* spp. STs available in de PubMLST database (Fig. 1) revealed that, overall, the STs clustered by species. The *A. butzleri* genotypes identified in this study distributed among all the species-specific clades except one mainly populated by isolates of human origin, and they mostly grouped in a cluster principally composed by isolates derived from food products. The *A. cryaerophilus* genotypes identified were more closely related to each other and they all except two grouped in the main species-specific clade. The three *A. thereius* and two *A. skirrowii* identified genotypes also clustered close to each other. No apparent host-associated STs were identified. Nevertheless, three novel STs from *A. butzleri* (ST-512, ST-513 and ST-517, represented by two, three and five isolates, respectively) and two novel STs from *A. cryaerophilus* (ST-521 and ST-596, represented by two isolates each) only included isolates from seafood products (Table 2 and Fig. 1). Moreover, the Fisher exact test assessed an association ($p < 0{,}001$) between seafood derived isolates of these study and the above mentioned STs. Among the previously identified STs, all except ST-16 were shared with isolates derived from different food products (Fig. 1). The recombinant analysis (RDP3) held with the 648 available concatenated MLST sequences (3341 positions) detected a potential single recombination event on ST-599, at the region between nucleotide positions 1968 and 2081, within the sequence of *glyA* allele. ST-609 (*glyA*-613, *A. cryaerophilus*) and ST-250 (*glyA*-263, *A. skirrowii*) were identified as the potential parents (major and minor, respectively). This event was statistically supported by all the implemented methods: RDP (1.686×10^{-06}), Geneconv (1.233×10^{-10}), MaxChi (1.602×10^{-04}), Chimaera (2.141×10^{-04}), and 3Seq (5.106×10^{-11}).

3.2.1. Putative virulence genes

The presence and distribution of the ten putative virulence genes investigated is shown in Table 3. The profiles of virulence genes identified in the different species are shown in Table S2. Overall, all the genes were detected among the isolates analysed and, based on the virulence gene content of each isolate, 28 different profiles were identified. None of the isolates harboured all ten virulence genes, and only one isolate with apparently no virulence gene content was identified. Among the arcobacters isolated *ciaB* (97.6%) and *mviN* (94%) were the

Table 2
Distribution of the STs identified among *Arcobacter* species.

Source of isolation	*A. butzleri*			*A. cryaerophilus*			*A. skirrowii*			*A. thereius*			*Arcobacter* spp.	
	No. of strains	No. of ST	Identified ST	No. of strains	No. of ST	Identified ST	No. of strains	No. of ST	Identified ST	No. of strains	No. of ST	Identified ST	No. of strains	No. of ST
Seafood	18	9		21	19		2	2		3	3		44	33
Cockle	6	5	475, **513**, **517**[a] ($n = 2$), 519, 530,	9	9	415, 534, 535, **596**[a], 597, 598, 599, 606, 607								
Squid	9	6	18, 172, **512**[a] (n = 2), **513**[a] (n = 2), **517**[a] (n = 2), 518	4	4	**521**[a], 605, 608, 609	1	1	532	2	2	623, 624		
Shrimp	3	3	**512**[a], **513**[a], **517**[a]	8	8	**521**[a], 522, **596**[a], 601, 602, 603, 610, 611	1	1	622	1	1	625		
Terrestrial animal products	27	25		3	3								30	28
Turkey meat	15	14	16, 452, 514, 515, 516, **520** (n = 2), 523, 524, 525, 527, 528, 536, 594, 595	1	1	604								
Rabbit meat	2	2	406, 586	1	1	626								
Quail meat	9	8	3, 506, 507, 508, 509, **510** (n = 2), 511, 587	1	1	600								
Fresh cheese	1	1	533											
Vegetables	8	7											8	7
Carrot	6	5	**526** (n = 2), 531, 588, 589, 590											
Spinach	1	1	593											
Lettuce	1	1	529											
Total food products	53	41		24	22		2	2		3	3		82	68

Boldface entries represent STs detected in two or more isolates.
[a] Fisher's exact test based statistically significant association ($p < 0.001$) between STs and seafood derived *A. butzleri* and *A. cryaerophilus*.

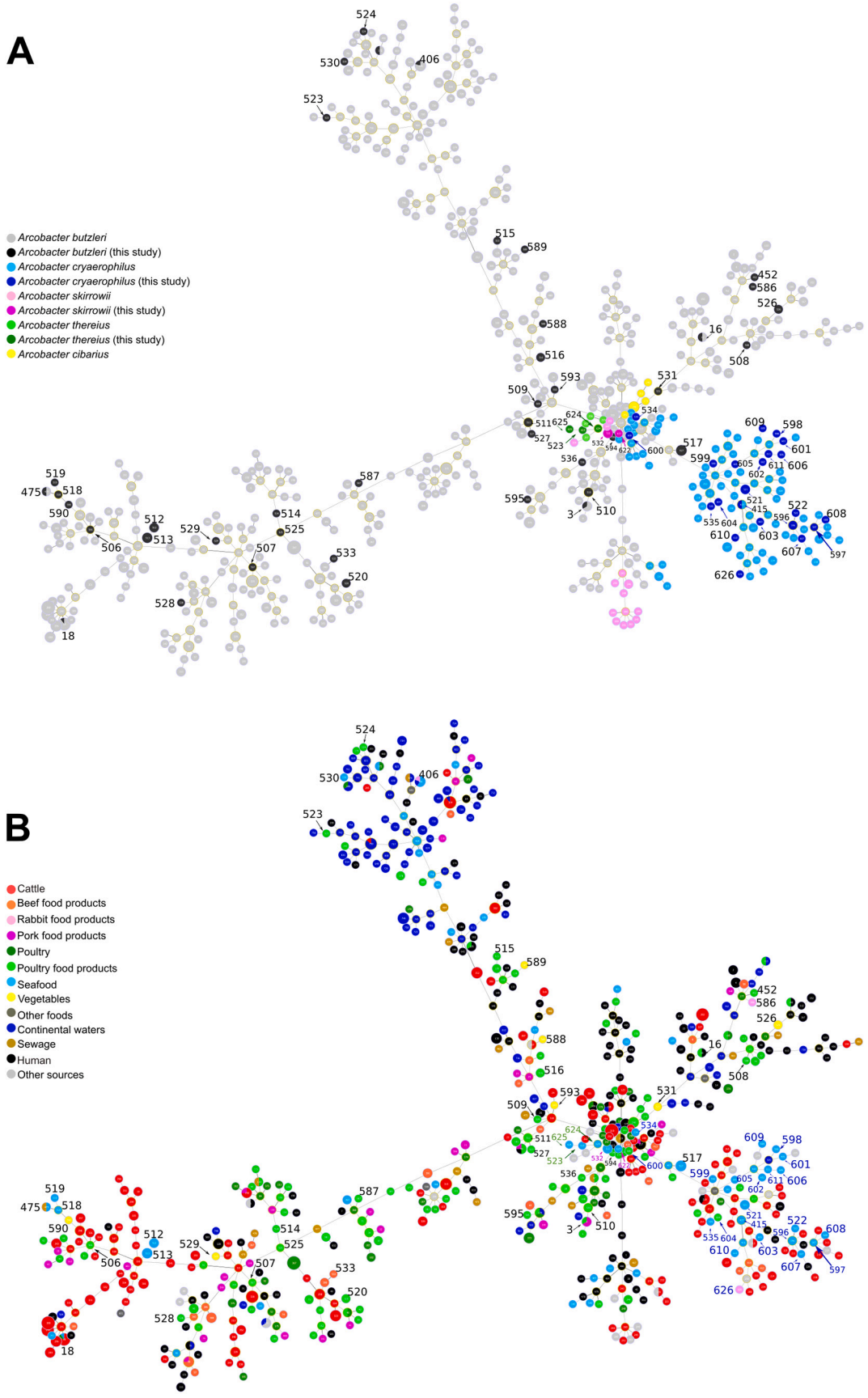

Fig. 1. Minimum spanning trees based on the MLST profiles of the isolates genotyped in this study and all other *Arcobacter* isolates from diverse sources available in the PubMLST, showing the relatedness and distribution of the STs among species (A) and diverse sources (B). Each circle represents an ST type and the size of the circle correlates to the number of isolates. The number next to the nodes indicates STs in the present study.

Table 3
Presence and distribution of putative virulence genes in *Arcobacter* spp. analysed in this study.

Species/source	No. of strains	No. (%) of strains generating specific gene amplicon									
		cadF	*ciaB*	*Cj1349*	*hecA*	*hecB*	*irgA*	*mviN*	*pldA*	*tlyA*	*iroE*
By species											
A. butzleri	53	53 (100)*	53 (100)	53 (100)*	9 (17)	16 (30.2)*	6 (11.3)	52 (98.1)	53 (100)*	53 (100)*	12 (22.6)
A. cryaerophilus	24	8 (33.3)	22 (91.7)	0	6 (25)	0	1 (4.2)	22 (91.7)	0	3 (12.5)	3 (12.5)
A. skirrowii	2	0	2 (100)	0	0	0	0	2 (100)	0	1 (50)	0
A. thereius	3	2 (66.7)	3 (100)	0	0	0	0	1 (33.3)	0	0	0
A. vitoriensis	1	0	1 (100)	0	0	0	0	1 (100)	0	0	0
Arcobacter spp.	83	63 (75.9)	81 (97.6)	53 (63.9)	15 (18.1)	16 (19.3)	7 (8.4)	78 (94)	53 (63.9)	57 (68.7)	15 (18.1)
By source of isolation											
Seafood											
Cockle	15	11 (73.3)	13 (86.7)	6 (40.0)	3 (20.0)	3 (20.0)	0	14 (93.3)	6 (40.0)	7 (46.7)	2 (13.3)
Squid	16	10 (62.5)	16 (100)	9 (56.3)*	5 (31.3)	5 (31.3)	1 (6.3)	14 (87.5)	9 (56.3)*	10 (62.5)	3 (18.8)
Shrimp	13	4 (56.8)	13 (100)	3 (23.1)	3 (23.1)	1 (7.7)	0	12 (92.3)	3 (23.1)	4 (30.8)	0
Subtotal	44	25 (56.8)	42 (95.5)	18 (40.9)	11 (25)	9 (20.5)	1 (2.3)	39 (88.6)	18 (40.9)	21 (47.7)	5 (11.4)
Terrestrial animal products											
Rabbit meat	3	3 (100)	3 (100)	2 (66.7)	2 (66.7)	1 (33.3)	1 (33.3)	3 (100)	2 (66.7)	2 (66.7)	2 (66.7)
Quail meat	10	10 (100)*	10 (100)	9 (90.0)*	1 (10.0)	0	3 (30.0)	10 (100)	9 (90.0)*	9 (90.0)*	1 (10.0)
Turkey meat	16	16 (100)*	16 (100)	15 (93.8)*	1 (6.3)	4 (25.0)	2 (12.5)	16 (100)	15 (93.8)*	16 (100)*	3 (18.8)
Fresh cheese	1	1 (100)	1 (100)	1 (100)	0	1 (100)	0	1 (100)	1 (100)	1 (100)	1 (100)
Subtotal	30	30 (100)*	30 (100)	27 (90.0)*	4 (13.3)	6 (20)	6 (20.0)	30 (100)	27 (90)*	28 (93.3)*	7 (23.3)
Vegetables											
Spinach	1	1 (100)	1 (100)	1 (100)	0	0	0	1 (100)	1 (100)	1 (100)	0
Lettuce	1	1 (100)	1 (100)	1 (100)	0	0	0	1 (100)	1 (100)	1 (100)	1 (100)
Carrot	7	6 (85.7)*	7 (100)	6 (85.7)*	0	1 (14.3)	0	6 (85.7)	6 (85.7)*	6 (85.7)*	2 (28.6)
Subtotal	9	8 (88.9)*	9 (100)	8 (88.9)*	0	1 (11.1)	0	8 (88.9)	8 (88.9)*	8 (88.9)*	3 (33.3)
Total all food products	83	63 (75.9)	81 (97.6)	53 (63.9)	15 (18.1)	16 (19.3)	7 (8.4)	78 (94)	53 (63.9)	57 (68.7)	15 (18.1)

* Statistically significant differences ($p < 0.05$) between results, based on Fisher's exact test.

most prevalent genes, and *irgA* (8.4%) the least one. The prevalence of the other genes varied from 75.9% for *cadF* to 18.1% for *iroE* and *hecA*. The statistical analyses revealed that *cadF*, *Cj1349*, *pldA* and *tlyA* genes were significantly ($p < 0.001$) more prevalent among the strains derived from terrestrial animal products (100%, 90%, 90% and 93.3%, respectively) and vegetables (88.9% each gene) than in those derived from seafood (56.8%, 40.9%, 40.9% and 47.7%, respectively). Specifically, *cadF* was common to all quail and turkey derived isolates and significantly more common in carrot derived ones (85.7%); and *Cj1349*, *pldA* and *tlyA* were significantly more common in quail (90.0% each), turkey (93.8% *Cj1349*; 93.8% *pldA*; 100% *tlyA*) and carrot (90.0% *Cj1349* and *pldA*; 93.3% *tlyA*) derived isolates. The genes *Cj1349* and *pldA* were also significantly prevalent in those isolates obtained from squids (56.3% each).

Significant differences ($p < 0.05$) of the gene distribution were also observed when the species were considered. All the 53 *A. butzleri* isolates

Table 4
Presence and distribution of the biofilm-associated genes in *Arcobacter* spp. analysed in this study.

Species/source	No. of strains	No. (%) of strains generating specific gene amplicon						
		fliS	*luxS*	*pta*	*waaf*	*Spot*	*flaA*	*flaB*
By species								
A. butzleri	53	53 (100)	53 (100)	53 (100)	53 (100)	53 (100)	36 (67.9)	36 (67.9)
A. cryaerophilus	24	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)	24 (100)	17 (58,3)
A. skirrowii	2	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)	2 (100)
A. thereius	3	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	0
A. vitoriensis	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Arcobacter spp.	83	83 (100)	83 (100)	83 (100)	83 (100)	83 (100)	66 (79.5)	56 (67.5)
By source of isolation								
Seafood								
Cockle	15	15 (100)	15 (100)	15 (100)	15 (100)	15 (100)	13 (86.7)	13 (86.7)
Squid	16	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	14 (87.5)	9 (56.3)
Shrimp	13	13 (100)	13 (100)	13 (100)	13 (100)	13 (100)	12 (92.3)	9 (69.2)
Subtotal	44	44 (100)	44 (100)	44 (100)	44 (100)	44 (100)	39 (88.6)	31 (70.5)
Terrestrial animal products								
Rabbit meat	3	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	2 (66.7)
Quail meat	10	10 (100)	10 (100)	10 (100)	10 (100)	10 (100)	7 (70)	6 (60)
Turkey meat	16	16 (100)	16 (100)	16 (100)	16 (100)	16 (100)	9 (56.3)	10 (62.5)
Fresh cheese	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	0
Subtotal	30	30 (100)	30 (100)	30 (100)	30 (100)	30 (100)	19 (63.3)	18 (60)
Vegetables								
Carrot	7	7 (100)	7 (100)	7 (100)	7 (100)	7 (100)	6 (85.7)	6 (85.7)
Spinach	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	0	0
Lettuce	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)
Subtotal	9	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	7 (7.8)	7 (7.8)
Total all food products	83	83 (100)	83 (100)	83 (100)	83 (100)	83 (100)	66 (79.5)	56 (67.5)

were positive for *cadF*, *ciaB*, *Cj1349*, *pldA* and *tlyA*, while the detection rate of the other genes ranged from 11.3% for *irgA* to 98.1% for *mviN*. The most frequent combination of genes in *A. butzleri* was *cadF*, *ciaB*, *Cj1349*, *mviN*, *pldA* and *tlyA*, which was detected in 47.2% of the isolates. The gene content of the *A. cryaerophilus* isolates was notably lower: only five out of the 24 tested strains showed four genes or more. The most prevalent genes in this species were *ciaB* and *mviN* (91.7% each), whose combination was noted in 37.5% of the isolates. *Cj1349*, *hecB* and *pldA* were not detected and the prevalence of the remaining genes was variable, ranging from 4.2% (*irgA*) to 33.3% (*cadF*). Both *A. skirrowii* isolates were positive for *ciaB* and *mviN*, being one of them also positive for *tlyA*. All the three *A. thereius* isolates possessed *ciaB*, two of them *cadF* and the third one also *mviN*. *Arcobacter vitoriensis* was positive just for *ciaB* and *mviN*.

3.3. Biofilm production

All the *Arcobacter* isolates were positive for *fliS*, *luxS*, *pta*, *waaF* and *spoT* genes, but 17 out of the 83 (20.5%) isolates resulted negative for *flaA* and *flaB* and ten (12%) for *flaB* (Table 4). Among these, all were motile upon examination on thioglycolate soft agar plates (data not shown).

The biofilm activity could only be tested with 81 of the 83 strains included in the study (we were unable to recover two *A. cryaerophilus* isolates, Ac-BER3 and Ac-CH1, from the strain collection). The initially measured adherence by microtitter assay is shown in Table S3. Table 5 summarizes the distribution and categorization of the tested isolates among food products and surfaces. Overall, 19 isolates (23.5%) were able to form biofilms on polystyrene surfaces under the experimental conditions. Among them, eight (42.1%) were categorized as weakly adherent, another eight (42.1%) as moderate, and three (15.8%) as strongly adherent. Nevertheless, based on Kruskal-Wallis, no significantly higher adhesion ability was identified among these adherent isolates. The proportion of adherent isolates differed significantly ($p = 0.037$) among species: 32.1% (17 isolates) in *A. butzleri*, 9.1% (2 isolates) in *A. cryaerophilus* and 0% in *A. skirrowii*, *A. thereius* and

Table 5
Distribution and categorization of the isolates based on their adhesion ability among food sources and surfaces.

	Polystyrene									Borosilicate			
No. () of tested strains	No. (%) of adherent strains on:						No. (%) of strains categorized as:			No. (%) of adherent strains on polystyrene categorized as:			
							WA[b]	MA[c]	SA[d]	NA[a]	WA[b]	MA[c]	SA[d]
By species													
A. butzleri (53)	17 (32.1)*						8 (47.1)	6 (35.3)	3 (17.6)	1 (5.9)	2 (11.8)	–	14 (82.3)
A. cryaerophilus (22)	2 (9.1)						–	2 (100)	–	–	–	1 (50)	1 (50)
A. skirrowii (2)	–						–	–	–	–	–	–	–
A. thereius (3)	–						–	–	–	–	–	–	–
A. vitoriensis (1)	–						–	–	–	–	–	–	–
Arcobacter spp. (81)	19 (23.5)						8 (42.1)	8 (42.1)	3 (15.8)	1 (5.3)	2 (10.5)	1 (5.3)	15 (78.9)
By source of isolation	*Arcobacter*	Ab[e]	Ac[f]	As[g]	At[h]	Av[i]							
Seafood (42)													
Cockle (6 Ab, 8 Ac)	4 (9.5)	4 (66.7)*	–	–	–	–	1 Ab (25)	2 Ab (50)	1 Ab (25)	–	1 Ab (25)	–	3 Ab (75)
Squid (9 Ab, 3 Ac, 1 As, 2 At)	5 (11.9)	5 (55.6)*	–	–	–	–	3 Ab (60)	2 Ab (40)	–	1 Ab (20)	–	–	4 Ab (80)
Shrimp (3 Ab, 8 Ac, 1 As, 1 At)	3 (7.1)	1 (33.3)	2 (25)	–	–	–	1 Ab (100)	2 Ac (100)	–	–	–	1 Ac (50)	1 Ab (100), 1 Ac (50)
Subtotal (18 Ab, 19 Ac, 2 As, 3 At)	12 (28,6)	10 (55.6)*	2 (10.5)	–	–	–	5 (41.7)	6 (50)	1 (8.3)	1 (8.3)	1 (8.3)	1 (8.3)	9 (75)
Terrestrial animal products (30)													
Rabbit meat (2 Ab, 1 Ac)	1 (3.3)	1 (50)	–	–	–	–	1 Ab (100)	–	–	–	–	–	1 Ab (100)
Quail meat (9 Ab, 1 Ac)	3 (10)	3 (33.3)	–	–	–	–	2 Ab (66.7)	–	1 Ab (33.3)	–	–	–	3 Ab (100)
Turkey meat (15 Ab, 1 Ac)	1 (3.3)	1 (6.7)	–	–	–	–	–	–	1 Ab (100)	–	1 Ab (100)	–	–
Fresh cheese (1 Ab)	–	–	–	–	–	–	–	–	–	–	–	–	–
Subtotal	5 (16,7)	5 (18.5)	–	–	–	–	3 (60)	–	2 (40)	–	1 (20)	–	4 (80)
Vegetables (8)													
Spinach (1)	1 (12.5)	1 (100)	–	–	–	–	–	1 Ab (100)	–	–	–	–	1 Ab (100)
Lettuce (1)	–	–	–	–	–	–	–	–	–	–	–	–	
Carrot (6 Ab, 1 Av)	1 (12.5)	1 (16.7)	–	–	–	–	–	1 Ab (100)		–	–	–	1 Ab (100)
Subtotal	2 (25)	2 (25)	–	–	–	–	–	2 (100)	–	–	–	–	2 (100)
Total all food products (81)	19 (23.5)	17 (32.1)*	2 (9.1)	–	–	–	8 (42.1)	8 (42.1)	3 (15.8)	1 (5.3)	2 (10.5)	1 (5.3)	15 (78.9)

[a] NA, no adherent.
[b] WA, weakly adherent.
[c] MA, moderately adherent.
[d] SA, strongly adherent.
[e] Ab, *A. butzleri*.
[f] Ac, *A. cryaerophilus*.
[g] As, *A. skirrowii*.
[h] At, *A. thereius*.
[i] Av, *A. vitoriensis*.
* Statistically significant differences ($p < 0.05$) between results, based on Fisher's exact test.

A. vitoriensis. Regarding the source of isolation, adherent isolates were detected in all type of food products except lettuce and fresh cheese. The distribution of the *A. butzleri* adherent isolates varied significantly ($p = 0.03$) among sources, and was as follows: ten adherent isolates derived from seafood (58.8%), five from foods of terrestrial animal origin (29.4%) and two from vegetables (11.8%). Specifically, the number of adherent strains among cockle and squid-derived isolates was significantly higher ($p = 0.034$). Both *A. cryaerophilus* adherent isolates derived from seafood.

The influence of the material on the adhesion ability of the 19 adherent strains was also tested, and the results are shown in Table 6. Eighteen isolates (94.7%) were able to adhere to borosilicate surfaces, and all of them to stainless steel. The adhesion ability of the majority of the strains (84.2%) was higher on borosilicate than on polystyrene, ranging the increase in BFI values from approximately double to more than 14 times higher. In fact, six weakly and seven moderately adherent isolates were categorized as strongly adherent when tested on borosilicate. The increased biofilm formation capability on borosilicate surface was significant for Ab-CH8 ($p = 0.02$), Ab-CH11 ($p = 0.037$) and Ac-G2 ($p = 0.044$) according to the Student *t*-test. Based on Kruskal-Wallis, no strain was significantly more adherent than other on borosilicate. Regarding stainless steel, the number of viable cells that adhered to the coupons ranged from 0.69 ± 0.43 to 3.66 ± 0.26 log CFU per cm^2, and based on Kruskal-Wallis, the adhesion ability of Ab-CH11 was significantly higher ($p = 0.002$).

4. Discussion

Various members of the genus *Arcobacter* are regarded as emerging food and waterborne pathogens (Collado and Figueras, 2011; Ramees et al., 2017); and their distribution in foods has been widely studied worldwide (Cruzado-Bravo et al., 2020; Fernández et al., 2015; Gónzalez et al., 2017; Hsu and Lee, 2015; Kietsiri et al., 2021; Laishram et al., 2016; Mottola et al., 2016a, 2016b, 2020, 2021; Uljanovas et al., 2021; Zhang et al., 2019). However, these studies are not so abundant in Spain, being even less common those combining various products in the same survey. In this study, different types of seafood, meat, vegetables and fresh cheese purchased in local markets in the city of Vitoria-Gasteiz were examined for *Arcobacter* spp., and the recovered isolates were genetically characterized by MLST and virulotyping. Additionally, the biofilm activity of the isolates was studied.

Arcobacter spp. was isolated from virtually all the tested food products with an overall prevalence of 22.3%. The most frequently isolated species among the five identified was *A. butzleri* (71.4%), but the enrichment broth employed for the procedure may have favoured this result, as it is known to benefit the recovery of this species over others (Levican et al., 2016).

In line with a previous study carried out in the same geographical area (Nieva-Echevarria et al., 2013), the most highly contaminated products ($p < 0.001$) were those coming from the sea (43.3%), especially the squids (60%). This result confirms the cephalopods to be an important reservoir of *Arcobacter* spp. (Rathlavath et al., 2017; Zhang et al., 2019) and highlights the importance of squids as a potential source of human infection if consumed raw or poorly cooked. It is known that shellfish such as bivalves constitute natural reservoirs of various marine *Arcobacter* species (Collado and Figueras, 2011). The seafood derived isolates of this study were identified as *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. thereius*. The isolation of three *A. thereius* strains from squid and shrimp samples was an interesting finding that, in accordance with previous observations (Levican et al., 2014; Zhang et al., 2019), confirms that *A. thereius* can also be isolated from other sources apart from animal faeces and abortions. Not in line with other reports (Levican et al., 2014; Morejón et al., 2017; Mottola et al., 2016b; Nieva-Echevarria et al., 2013; Rathlavath et al., 2017; Zhang et al., 2019), *A. cryaerophilus* prevailed above *A. butzleri* among seafood derived isolates.

In contrast, *A. butzleri* was the most common species isolated from meat (90%) and vegetable (88.9%) products, especially from poultry and carrots. The predominance of these species in meat products has been frequently reported (Collado and Figueras, 2011; Khodamoradi and Abiri, 2020; Kim et al., 2019; Nieva-Echevarria et al., 2013; Ohnishi and Hara-Kudo, 2021). In addition, the observed prevalence of arcobacters in turkey (40%) and rabbit (15%) meats is consistent with that reported by Collado et al. (2009) for the same products in the same country (33.3% and 10%, respectively); regardless of the differences noted in comparisons between our results and those obtained

Table 6
Biofilm formation ability of the adherent *A. butzleri* and *A. cryaerophilus* isolates on different abiotic surfaces.

Isolate	Polystyrene		Borosilicate		Stainless steel
	BFI[a]	Classification[b]	BFI[a]	Classification[b]	log CFU/cm^2
Ab-BER1	0.57 ± 0.12	Weak	0.69 ± 0.42	Weak	0.69 ± 0.43
Ab-BER4	1.05 ± 0.15	Moderate	1.86 ± 1.06	Strong	1.92 ± 0.16
Ab-BER6	0.96 ± 0.05	Moderate	3.29 ± 2.21	Strong	2.12 ± 0.3
Ab-BER7	2.48 ± 1.16	Strong	10.56 ± 8.75	Strong	1.92 ± 0.23
Ab-CH8	0.46 ± 0.04	Weak	3.67 ± 0.17*	Strong	1.79 ± 0.24
Ab-CH9	0.74 ± 0.22	Moderate	1.94 ± 0.25	Strong	1.59 ± 0.03
Ab-CH10	0.41 ± 0.34	Weak	1.95 ± 1.1	Strong	3.28 ± 0.23
Ab-CH11	0.76 ± 0.13	Moderate	2.55 ± 0.48*	Strong	3.66 ± 0.26•
Ab-CH12	0.41 ± 0.22	Weak	0.04 ± 0.503	None	2.29 ± 0.2
Ab-CZ3	0.53 ± 0.26	Weak	2.74 ± 3.04	Strong	1.11 ± 0.03
Ab-CZ5	0.62 ± 0.62	Weak	1.96 ± 1.46	Strong	1.31 ± 0.34
Ab-CZ6	3.00 ± 2.9	Strong	1.90 ± 0.77	Strong	2.99 ± 0.66
Ab-PV7	1.50 ± 1.01	Strong	0.40 ± 0.44	Weak	1.83 ± 0.15
Ab-CN1	0.65 ± 0.64	Weak	3.78 ± 1.76	Strong	1.07 ± 0.26
Ab-E1	0.74 ± 0.39	Moderate	1.82 ± 0.83	Strong	2.84 ± 0.42
Ab-G1	0.65 ± 0.44	Weak	2.96 ± 2.45	Strong	1.99 ± 0.48
Ab-Z7	0.78 ± 0.13	Moderate	11.18 ± 11.45	Strong	2.06 ± 0.18
Ac-G2	0.73 ± 0.13	Moderate	5.11 ± 1.35*	Strong	3.15 ± 2.66
Ac-G4	0.81 ± 0.23	Moderate	0.83 ± 0.9	Moderate	3 ± 2.68

[a] BFI, biofilm formation index. Values are expressed as means ± standard errors.
[b] Biofilm formation pattern according to Naves et al. (2008).
• Kruskal-Wallis based statistically significant ($p = 0.002$) differences obtained when comparing the values obtained for biofilm formation ability on stainless steel for each isolate.
* Student *t*–based statistically significant ($p < 0.05$) differences obtained when comparing biofilm formation on polystyrene versus borosilicate. The higher BFI value, representing the most suitable surface for biofilm formation, is indicated.

somewhere else (Khodamoradi and Abiri, 2020; Rahimi, 2014). Differences in farming and/or food processing practices between countries could be the underlying reasons for this disparity, but also the utilization of different food matrices, sample sizes and/or isolation approaches. On the other hand, 5% of the spinach and lettuce samples tested were positive for *Arcobacter* spp. Curiously, these samples were purchased washed and packed. Probably, being pre-prepared vegetables, they had been contaminated at any point of the production chain due to various possible causes (Mottola et al., 2021). Green vegetables have been previously found to be positive to *Arcobacter* spp. with variable prevalence (Gónzalez and Ferrús, 2011; Gónzalez et al., 2017; Mottola et al., 2016a, 2021), but to our knowledge this is the first survey held with fresh carrots. Indeed, these resulted to be the main contaminated vegetables (20%) and, of note, the unique representative of *A. vitoriensis*, a minority but potentially pathogenic species, was isolated from them (Alonso et al., 2020).

As far as we know this is also the first Spanish study reporting the prevalence of *Arcobacter* spp. in fresh cheese at the retail. One (5%) out of the total feta (3), mozzarella (4), Burgos (10) and goat (3) cheese samples analysed resulted positive for *Arcobacter* (Burgos cheese is a Spanish fresh cheese made with either raw or pasteurized cow and sheep's milk applying soft heat treatment (28–30 °C) while enzymatic curdling). Various other researches (Cruzado-Bravo et al., 2020; Mudadu et al., 2021; Scarano et al., 2014; Yesilmen et al., 2014) have also reported the presence of *Arcobacter* in fresh cheese, with contamination levels varying from 10.2% in Brazilian Minas cheese (Cruzado-Bravo et al., 2020) to 56% in village cheese in Turkey (Yesilmen et al., 2014). In those cases, factors as using raw milk for cheese production (Yesilmen et al., 2014), an inadequate pasteurization of the milk (Cruzado-Bravo et al., 2020) or processing and post processing contamination (Mudadu et al., 2021; Scarano et al., 2014) were assumed as the source of contamination for the cheeses. We did not investigate the source of the products' contamination, but noticed that the only positive sample for *Arcobacter* was a Burgos cheese portion cut by the shopkeeper of the poulterer's where it was purchased. If arcobacters were able to survive and grow on fresh Burgos cheese the way they do on fresh ricotta (Giacometti et al., 2015a), this ready to eat product could be considered a potential source of *Arcobacter* infection for humans. Therefore, tracking the origin of *Arcobacter* contamination of Burgos cheese and studying the survival of the species of the genus in it would be of interest for future studies.

Foodborne arcobacters have shown great genetic diversity (Alonso et al., 2014; Caruso et al., 2020; De Cesare et al., 2015). In our study, the genetic heterogeneity of the recovered isolates was confirmed by MLST, especially that of those belonging to the species *A. butzleri* and *A. cryaerophilus*. Apparently, and in agreement with previous observations (Miller et al., 2009), sequence diversity among the analysed *A. cryaerophilus* isolates is higher than among *A. butzleri* (22 STs identified among 24 isolates versus 41 STs among 53 isolates, respectively). Regardless of the species, genetic variation also seems to be higher among the isolates derived from terrestrial animal food products (28 STs among 30 isolates) than from seafood (33 STs among 44 isolates). Up to date, no study has confirmed the hypothesis of the association between certain STs and specific food products for *Arcobacter* spp. In fact, the result of the phylogenetic analysis of the STs held in this study goes in line with previous ones, where no STs clustered by host (Alonso et al., 2014; Kietsiri et al., 2021; Miller et al., 2009). Despite the observed significant association ($p < 0.001$) between seafood-derived *A. butzleri* isolates from this study and ST-517, ST-513 and ST-512, the information on the *A. butzleri* genotypes on seafood is not so abundant and therefore, further MLST studies are necessary to elucidate whether ST-517, ST-513 and ST-512 are dominant genetic markers in *A. butzleri* strains derived from sea products. On the other hand, based on the potential recombination event identified in ST-599 we can confirm that recombination events due to horizontal gene transfer take place between *Arcobacter* species (Alonso et al., 2014; Miller et al., 2009).

Despite being recognised as a risk for human health (ICMSF, 2002), the mechanisms underlying the pathogenesis potential of arcobacters remain unclear. However, recent comparative genome analyses revealed that the virulome of *Arcobacter* spp. is composed of a great range of virulence-associated genes (Buzzanca et al., 2021; Isidro et al., 2020; Müller et al., 2020a, 2020b). The pathogenic potential of the isolates recovered in this study was estimated by single PCR detection of ten genes typically employed as virulence markers (Douidah et al., 2012; Girbau et al., 2015; Kietsiri et al., 2021; Šilha et al., 2019; Mottola et al., 2021; Uljanovas et al., 2021): *cadF* and *Cj1349*, coding for fibronectin binding proteins that promote the binding of bacteria to intestinal cells; *ciaB* (*Campylobacter* invasive antigen E), that contributes to host cell invasion through a secretion system; *hecA*, involved in attachment, aggregation and epidermal cell killing; *hecB* and *tlyA*, haemolysin activation protein and haemolysin encoding genes, respectively; the phospholipase encoding gene *pldA*, also associated with erythrocyte lysis; *mviN*, coding for a protein essential for peptidoglycan biosynthesis; and *irgA* and *iroE*, that code for functional components for iron acquisition and are required for establishing and maintaining infection. The vast majority of the isolates (98.8%) harboured at least one of the studied genes. Overall, and consistent with previous data (Girbau et al., 2015; Rathlavath et al., 2017; Šilha et al., 2019; Uljanovas et al., 2021; Zacharow et al., 2015), *ciaB*, *Cj1349*, *mviN*, *pldA*, *tlyA* and *cadF*, that showed different distribution ($p < 0.001$) depending on the origin of the isolates, were the most detected genes among the *Arcobacter* isolates. It is not unusual to notice significant differences on the distribution of the detected virulence markers when the source of the isolates is considered (Girbau et al., 2015; Kietsiri et al. 2021; Rathlavath et al., 2017; Šilha et al., 2019; Uljanovas et al., 2021; Zacharow et al., 2015). The type of matrix probably influences not only the species, but also the strains present on them and, consequently, the genetic determinants of the isolates obtained. In accordance with other studies (Girbau et al., 2015; Šilha et al., 2019; Uljanovas et al., 2021; Zacharow et al., 2015), the detection rates and profiles of the virulence-associated genes were higher in *A. butzleri* than in the rest of species. This variability between species might derive from the limitations of the PCR procedure employed (Douidah et al., 2012), which are becoming more evident as pangenome analyses highlight the heterogeneity and polymorphisms of the selected genes among and within *Arcobacter* species (Buzzanca et al., 2021; Isidro et al., 2020; Müller et al., 2020a, 2020b). As commonly reported (Buzzanca et al., 2021; Girbau et al., 2015; Kietsiri et al., 2021; Piva et al., 2017; Rathlavath et al., 2017), *cadF*, *ciaB*, *Cj1349*, *mviN*, *pldA* and *tlyA* were detected in virtually all *A. butzleri* isolates. In *A. cryaerophilus*, *ciaB* and *mviN* were the most detected genes, as reported elsewhere (Girbau et al., 2015; Uljanovas et al., 2021; Zacharow et al., 2015); and only two out of the 24 isolates were positive for more than four genes. Irrespective of the aforementioned procedure limitation, a recent genomic analysis of *A. cryaerophilus* strains (Müller et al., 2020b) also indicates that, despite the wide range of virulence-associated genes described for this species, it is not frequent to find strains with many of them. All the *A. skirrowii*, *A. thereius* and *A. vitoriensis* isolates were also positive for *ciaB*, confirming the stability of this gene among *Arcobacter* species (Zhang et al., 2019). The gene profiles of these minority species resulted from the combination of *ciaB* with at least one other gene, mainly *mviN*. Regardless of the species, all the isolates studied here but one harboured genes related at least to host cell adhesion and invasion, which highlights the pathogenic potential of the different *Arcobacter* species from food and the role that food products can play in the transmission of potentially pathogenic arcobacters.

Biofilms play a key role in the pathogenesis of many bacteria (Parsek and Singh, 2003). Many foodborne pathogens are able to form them (Galié et al., 2018), and *Arcobacter* species are not an exception (Girbau et al., 2017; Hrušková et al., 2013; Šilha et al., 2021). Considering the risk for human health these structures can pose if present on food contact surfaces or factory equipment, the biofilm forming ability of the isolates recovered in this survey was also investigated. First, and based

on their association with the biofilm formation in campylobacteria (Joshua et al., 2006; McLennan et al., 2008; Naito et al., 2010; Reeser et al., 2007), the presence of the flagellar *flaA*, *flaB* and *fliS* genes; *luxS*, responsible of the AI-2 autoinducer involved in *quorum sensing*; *cj0688* (*pta*), coding for a phosphate acetyltransferase; *spoT*, involved in the regulation of the stringent response; and *waaF*, participant in the synthesis of lipooligosaccharide; was assessed by PCR. All the genes but *flaA* and *flaB* were detected in all the isolates studied. The lower detection rate of these two genes (79.5 and 67.5%, respectively) is probably a consequence of the designed primer-pairs, which may not be as specific as initially expected, and/or the detection protocol. The tests carried out demonstrated the motility of those isolates negative for *flaB* or both flagellar genes and, consequently, the presence of a functional flagellum in all of them. However, as far as we know, this is the first survey where the presence of these biofilm-associated genes is tested in a set of *Arcobacter* isolates, so further studies would assess their prevalence among isolates. The biofilm formation was monitored at 30 °C under aerobic conditions, and three different surfaces were employed. The low number of studies held with no poultry or milk derived foodborne isolates, as well as with species other than *A. butzleri*, difficult the comparison of results. Nevertheless, a recent study held with *A. butzleri* and *A. cryaerophilus* isolates from the Czech Republic (Šilha et al., 2021) reported biofilm formation capability for virtually all the tested *A. butzleri* (96.7%) and most of the *A. cryaerophilus* (69%) isolates on polystyrene surfaces under experimental conditions similar to ours. Our results indicate, however, that the ability to adhere can differ significantly among species ($p = 0.037$). In line with previous reports (Girbau et al., 2017; Šilha et al., 2021), our results do not point to arcobacters as strong biofilm producers. Nevertheless, factors such as temperature, nutrient availability, surface material and atmosphere influence their biofilm activity (Girbau et al., 2017; Šilha et al., 2021). Therefore, it would not be surprising if any of the studied strains would show higher biofilm activity under any of the environmental conditions they may encounter in any of the very different food processing environments. Indeed, our results also indicate that this capability is variable among surfaces. Overall, all the adherent isolates on polystyrene were also able to form biofilms on borosilicate glass and stainless steel coupons, but borosilicate enhanced the adhesion ability of the vast majority of the isolates (84.2%). Indeed, the increased adherence observed for three seafood derived isolates (two *A. butzleri* and *one A. cryaerophilus*) was statistically significant ($p < 0.05$). On the other hand, we identified an association ($p = 0.034$) between adherent *A. butzleri* isolates and cockle and squid samples, which made us think of seafood as a potential relevant source of adherent arcobacters. In fact, adherent isolates derived from mussels and clams had been previously reported (Girbau et al., 2017). Further investigations would elucidate whether other species of *Arcobacter* present higher numbers of adherent strains derived from seafood than from other food products.

5. Conclusions

This study shows that a variety of seafood, vegetables and terrestrial animal food products harbour potentially human pathogenic *Arcobacter* species and therefore, highlights the important role that common food products obtained at the retail could have in their transmission. Additionally, the results confirm the biofilm formation by foodborne arcobacters on different food contact surfaces (polystyrene, borosilicate glass and stainless steel) and point towards an enhanced biofilm activity on borosilicate, which let us hypothesize that glass surfaces favour the survival and cross-contamination of *Arcobacter* spp. Of note, we report for the first time the presence of *Arcobacter* species in fresh Burgos cheese and carrots, and point out seafood, especially squids, as an important source of adherent arcobacters. These findings should be taken into consideration, since Burgos cheese is a ready-to-eat product and carrots and seafood are frequently consumed undercooked or raw. Future studies on the survival and growth ability of *Arcobacter* on these products, especially on ready to eat ones, may be of interest in order to assess the implications of these findings for food safety. Finally, the novel STs of *A. butzleri* ST-517, ST-513 and ST-512 were detected in multiple seafood derived isolates and, in our sample, a statistically significant association between these STs and the origin of the isolates was identified. Further MLST studies would elucidate if these STs can be proposed as host-associated genetic markers.

Funding

This work was supported by the Spanish Ministry of Economy and Competitiveness [project number AGL2014-56179-P, co-financed with FEDER funds]; the University of the Basque Country [grant number PPG17/27]; and by the Basque Government [Project number PA20/03].

Declaration of competing interest

None.

Acknowledgments

The authors would like to acknowledge the University of Basque Country UPV/EHU, that kindly provided the Open Access funding. They would also like to thank Sandra Ubeira, for the help she provided with language.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2022.109712.

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