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Prevalence, virulence gene distribution and genetic diversity of *Arcobacter* in food samples in Germany

Zur Prävalenz, der Verteilung von Virulenzgenen und der genetischen Diversität von Arcobacter aus Lebensmitteln in Deutschland

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Summary

This study was carried out to determine the prevalence of *Arcobacter* spp. in food samples in Germany. In addition, the presence of putative virulence genes and the genetic diversity was tested for *Arcobacter* (*A.*) *butzleri* strains isolated during this study. The prevalence of *Arcobacter* spp. was 34% in fish meat, 26.8% in poultry meat and 2% in minced meat (beef and pork). All investigated *A. butzleri* isolates carried the genes *cadF*, *ciaB*, *cj1349*, *mviN* and *pldA*. The gene *tlyA* was detectable in 97.5% of the strains. Lower detection rates were observed for *hecA* (47.5%), *hecB* (45%), *iroE* (40%) and *irgA* (35%). Genotyping by ERIC-PCR demonstrated a high genetic diversity of *A. butzleri* strains from different foods. In conclusion, this study shows that about one third of fish meat and poultry meat samples contained *Arcobacter* spp. These data highlight the need to strengthen our effort to elucidate the importance of *Arcobacter* on veterinary public health.

Keywords: *Arcobacter*, food, prevalence, genotype, virulence genes

Zusammenfassung

Ziel der Studie war, die Prävalenz von *Arcobacter* spp. in Lebensmittelproben in Deutschland zu bestimmen. Zusätzlich wurde die Anwesenheit putativer Virulenzgene und die genetische Diversität der *Arcobacter* (*A.*) *butzleri*-Stämme untersucht. Die Prävalenz von *Arcobacter* spp. betrug 34 % in Fisch, 26,8 % in Geflügelfleisch und 2 % in Hackfleisch (Rindfleisch und Schweinefleisch). In allen untersuchten *A. butzleri*-Isolaten waren die Gene *cadF*, *ciaB, cj1349*, *mviN* und *pldA* nachweisbar. Das Gen *tlyA* war in 97,5 % der Stämme zu finden. Für h*ecA* (47.5 %), *hecB* (45 %), *iroE* (40 %) und *irgA* (35 %) wurden niedrigere Nachweisraten beobachtet. Die Genotypisierung der *A. butzleri*-Stämme aus verschiedenen Lebensmitteln mittels ERIC-PCR zeigte eine hohe genetische Vielfalt dieser Stämme. Zusammenfassend belegt diese Studie, dass etwa ein Drittel der Fisch- und Geflügelfleischproben *Arcobacter* spp. enthielten. Diese Daten unterstreichen die Notwendigkeit, die Bedeutung von *Arcobacter* für den gesundheitlichen Verbraucherschutz weitergehend zu betrachten.

Schlüsselwörter: *Arcobacter*, Lebensmittel, Prävalenz, Genotyp, Virulenzgene

Introduction

Arcobacter spp. are gram-negative, motile and spiral-shaped bacteria belonging to the family of *Campylobacteraceae*. They have been specified by the International Commission on Microbiological Specifications for Foods as serious hazards for human health (ICMSF, 2002). Among *Arcobacter* spp., *A. butzleri* is the most important species associated with human disease, resulting in abdominal pain with acute diarrhoea or prolonged watery diarrhoea for up to two months (Vandenberg et al., 2004). As *Arcobacter* spp. is not detected in routine diagnostics, the prevalence of *Arcobacter* associated human diseases is not known so far. However, outbreaks and single case reports have been published (Vandamme et al., 1992; Rice et al., 1999; Lappi et al., 2013; Figueras et al., 2014). Nonetheless, *Arcobacter* spp. are mostly reported as commensals in the gastrointestinal tract of animals (Ho et al., 2006).

Arcobacter spp. were already isolated from a wide range of food of animal origin. This might be explained by contaminations during the slaughtering process. Highest prevalences in food are reported for poultry meat, followed by pork and beef (Houf, 2009; Shah et al., 2011). Some studies described the presence of *Arcobacter* spp. in marine waters (esp. sediments), possibly representing a reservoir for shellfish contamination (Collado et al., 2009; Collado et al., 2014; Levican et al., 2014). Little is known about the presence of *Arcobacter* in fish or fish meat.

Different methods have been applied to genotype *Arcobacter* spp., with focus on *A. butzleri*. Among these, ERIC (enterobacterial repetitive intergenic consensus)-PCR is one of the most used typing method applied for outbreak investigations and typing of isolates originating from different foods (Collado and Figueras, 2011; Nieva-Echevarria et al., 2013).

Limited information is available about the pathogenic mechanisms and putative virulence genes of *A. butzleri*. So far, it is not known under what conditions *Arcobacter* spp. are pathogenic and whether they are pathogenic by themselves or by interactions with other bacteria. Bücker et al. (2009) demonstrated that *A. butzleri* is able to induce barrier dysfunctions in HT-29/B6 cells in vitro, thereby facilitating translocation of bacteria and probably inducing diarrhoea. The genomic sequence of *A. butzleri* RM 4018 demonstrated the presence of ten putative virulence genes: *cadF, mviN, pldA, tlyA, cj1349, hecB, irgA, hecA, ciaB* and *iroE* (Miller et al., 2007), but it is still unknown whether these putative virulence factors have similar functions as described for their homologs in other microbial species. CadF, HecA, and Cj1349 promote adherence of bacteria to host cells, while CiaB contributes to host cell invasion (Konkel et al., 1999; Rojas et al., 2002; Flanagan et al., 2009). HecB, TlyA and PldA might be involved in lysis of erythrocytes (Grant et al., 1997; Wren et al., 1998; Miller et al., 2007). IrgA and IroE are functional components for the iron acquisition and therefore required for establishing and maintaining infections (Mey et al., 2002; Zhu et al., 2005; Rashid et al., 2006). MviN is an essential protein required for peptidoglycan biosynthesis in *E. coli* but the contribution to virulence is not established yet (Ruiz, 2008). However, no correlation between virulence gene pattern of *A. butzleri* isolates and adhesive and invasive phenotypes could be shown by in vitro assays (Karadas et al., 2013; Levican et al., 2013).

The aim of this study was to detect the prevalence of *Arcobacter* spp. in different foods, to genotype *A. butzleri* strains isolated from these sources using ERIC-PCR and to examine the distribution of putative virulence genes.

Material and Methods

Samples

Samples of fresh fish meat (n = 50, originated from aquacultures and wild-caught fish), mixed minced meat (pork and beef) (n = 51) and poultry meat (n = 56) were purchased from supermarkets and retail shops through-

TABLE 1: *Primers used in this study*

Primer	sequence	Amplicon	study
ARCO R	CGTATTCACCGTAGCATAGC		Houf et al. (2000)
BUTZ F	CCTGGACTTGACATAGTAAGAATGA	401	
SKIR F	GGCGATTTACTGGAACACA	641	
CRY1	TGCTGGAGCGGATAGAAGTA	257	
CRY2	AACAACCTACGTCCTTCGAC		
16SrRNAF1	AGAGTTTGATCCTGGCTGAG	approx. 1500	Coenye et al. (1999)
16SrRNAF1	AAGGAGGTGATCCAGCCGCA		
16F358	CTCCTACGGGAGGCAGCAGT	for sequencing	
16R1093	GTTGCGCTCGTTGCGGGACT	for sequencing	
cadF F	TTACTCCTACACCGTAGT	283	Douidah et al. (2012)
cadF R	AAACTATGCTAACGCTGGTT		
pldA F	TTGACGAGACAATAAGTGCAGC	293	
pldA R	CGTCTTTATCTTTGCTTTCAGGGA		
irgA F	TGCAGAGGATACTTGGAGCGTAACT	437	
irgA R	GTATAACCCCATTGATGAGGAGCA		
hecA F	GTGGAAGTACAACGATAGCAGGCTC	537	
hecA R	GTCTGTTTTAGTTGCTCTGCACTC		
hecB F	CTAAACTCTACAAATCGTGC	528	
hecB R	CTTTTGAGTGTTGACCTC		
Cj1349 F	GAATTGTAAAAGTAGGGCATAA	556	Karadas et al. (2013)
Cj1349 R	TTTGTTTGATTTCGCTCTTT		
ciaB F	GGAATAAATAAAGAGTTGGTTGC	498	
ciaB R	ATTTACTCTATGGTCATTTGTGC		
mviN F	TTTTGCTGTTAGTAATTGTTGTTC	409	
mviN R	TGATACTTGGTTAGCTTCTCTTTT		
tlyA F	AAATAAAGTTAAATGTGATGGTG	529	
tlyA R	GTTGTCTCTTGCTTTTTGTATTG		
iroE F	AATGGCTATGATGTTGTTTAC	415	
iroE R	TTGCTGCTATGAAGTTTTG		
ERIC 1R	ATGTAAGCTCCTGGGGATTCAC		Houf et al. (2002)
ERIC 2	AAGTAAGTGACTGGGGTGAGCG		

out Berlin, Germany. To ensure sample diversity, packs were chosen with a wide range of European Community identifiers and best-before dates. The time between purchase and isolation varied between one and 60 h. During this period, samples were stored at 4–6°C.

Isolation and identification of *Arcobacter*

Arcobacter spp. were isolated by a modified version of a protocol described by Atabay et al. (2003). Briefly, 1 g of each sample was aseptically removed, placed in 10 ml of *Arcobacter* Broth (Oxoid Deutschland, Wesel, Germany) supplemented with cefoperazone-amphotericin-teicoplanin selective supplement (Oxoid) and mixed thoroughly. The homogenate was incubated microaerobically at 30°C. After two days, the enriched culture was 10-fold diluted in *Brucella* Broth (BD Biosciences, Heidelberg, Germany) and 300 µl were spread onto a 0.6 µm pore size membrane filter (GE Healthcare Europe, Freiburg, Germany) laid on the surface of a Mueller Hinton agar plate (Oxoid) with 5% sheep blood (MHB). The plates were incubated aerobically for 1 h at 30°C before the filter was removed. For better colony separation, another 100 µl of sterile *Brucella* Broth was dropped on the plates before streaking, and incubation continued under the same conditions for up to seven days or until colonies were visible. Presumptive *Arcobacter* colonies were picked, streaked onto MHB agar plates and incubated microaerobically at 30°C for 48 h.

From each isolate, DNA was extracted using the chelex method described by Karadas et al. (2013). Identification

of the isolates was performed using a multiplex PCR assay according to Houf et al. (2000). Briefly, 2 µl of DNA were added to 23 µl of the reaction mixture containing 1x PCR buffer (Qiagen, Hilden, Germany), 1.3 mM MgCl$_2$ (Qiagen), 0.2 mM of each dNTP (Thermo Fisher, St. Leon-Rot, Germany), 0.75 U of *Taq* DNA polymerase (Qiagen), 1 µM of each primer ARCO, BUTZ, CRY1 and CRY2 and 0.5 µM of the primer SKIR (Tab. 1). PCR involved 32 cycles of denaturation (94°C for 45 s), primer annealing (61°C for 45 s), and chain extension (72°C for 30 s). Prior to cycling, samples were heated at 94°C for 2 min. Amplified products were detected by electrophoresis in 3% agarose gels.

Isolates found positive were further characterized by sequencing a fragment of the 16S rRNA gene as described by Coenye et al. (1999). PCR was performed in 50 µl reaction mixture composed of 4 µl DNA, 45 µl ReddyMix PCR Master Mix (Thermo Fisher) and 1 µM of each primer 16SrRNAF1 and 16SrRNAR1 (Tab. 1). Amplicons were purified using a GeneJE PCR Purification Kit (Thermo Fisher) and sequencing was performed at GATC Biotech (Konstanz, Germany) using the primers 16F358 and 16R1093 according to Coenye et al. (1999).

Detection of virulence genes in *A. butzleri*
Detection of putative virulence genes in *A. butzleri* strains originating from fish meat and poultry meat was performed by PCR. Primers are listed in Table 1 and PCR protocols for partial amplification of *cadF*, *pldA*, *irgA*, *hecA* and *hecB* were used according to Douidah et al. (2012). Briefly, PCRs were carried out in a total volume of 25 µl containing 1x PCR-buffer (Qiagen), 0.2 mM of each dNTP (Thermo Fisher), 1 µM of each primer, 0.5 U of *Taq* DNA polymerase (Qiagen) and 2 µl DNA. An initial denaturation step at 95°C for 4 min was followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 56°C (*hecA* and *irgA*) or 55°C (*cadF*, *hecB* and *pldA*) for 45 s and elongation at 72°C for 45 s, and a final elongation step for 5 min at 72°C. For partial amplification of *cj1349*, *ciaB*, *mviN*, *tlyA* and *iroE* primers (Tab. 1) and protocols were used according to Karadas et al. (2013). PCRs were carried out in a total volume of 25 µl containing 1x PCR-buffer (Qiagen), 0.2 mM of each dNTP (Thermo Fisher), 0.5 µM of each primer, 0.5 U of *Taq* DNA polymerase (Qiagen) and 2 µl DNA. An initial denaturation step at 95°C for 4 min was followed by 30 cycles of denaturation

at 94°C for 30 s, annealing at 50°C for 30 s and elongation at 72°C for 30 s, and a final elongation step for 6 min at 72°C.

Genotyping of *A. butzleri*
Isolates belonging to the genus *A. butzleri* were further characterized by ERIC-PCR according to Houf et al. (2002). Briefly, 0.5 µl of DNA was added to 24.5 µl of the reaction mixture containing 1x PCR buffer (Qiagen), 4 mM MgCl$_2$ (Qiagen), 0.2 mM of each dNTP (Thermo Fisher), 2.5 U of *Taq* DNA polymerase (Qiagen) and 0.5 µM of the primers ERIC 1R and 2 (Tab. 1). PCR involved 40 cycles of denaturation (94°C for 60 s), primer annealing (25°C for 60 s), and chain extension (72°C for 120 s). Prior to cycling, samples were heated at 94°C for 5 min. Amplified products were detected by electrophoresis in 3% agarose gels and visualized with UV transillumination. Band pattern were analysed using BioNumerics version 6.1 (Applied Maths, Sint-Martens-Latem, Belgium). After normalisation, the similarities between profiles, based on peak position, were calculated using Pearson correlation coefficient. For cluster analysis, the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was used with a cluster cut-off of 90%.

Results and Discussion

Arcobacter prevalence
Arcobacter spp. were isolated from fish meat, poultry meat and minced meat (beef and pork) (Tab. 2). Species were identified by mPCR and further confirmed by 16SrRNA sequencing. For fish meat, 34% (17/50) of the samples contained *Arcobacter* spp., with *A. butzleri* as the dominant species detectable in 32% (16/50) of the samples. In a single sample *A. cryaerophilus* was detectable. To our knowledge, no comparable data on *Arcobacter* in fish meat are available yet. Nonetheless, comparable prevalences were detectable in shellfish (73.3%) and mussels (41.1%) in northern Spain (Collado et al., 2009; Nieva-Echevarria et al., 2013). These data suggest that seafood represents an important reservoir for *Arcobacter* spp.

In 26.8% of the poultry meat samples *A. butzleri* was detectable. That corresponds well with published data, demonstrating prevalence in poultry meat of between 15% and 73% (Rivas et al., 2004; Keller et al., 2006).

Of the minced meat samples, 2% were *A. butzleri* positive. When investigating minced pork, Van Driessche and Houf (2007) detected a prevalence of 19.2%. For minced beef, prevalences ranged from 4–10% (Rohder et al., 2007; Nieva-Echevarria et al., 2013). These data imply that minced pork and beef meat is of minor importance as source for *Arcobacter* spp. but as raw minced meat is consumed in Germany it should still be considered as a reservoir for human infections.

TABLE 2: *Prevalence of Arcobacter spp. in food samples*

Samples	n	No. (%) of positive samples			
		Arcobacter spp.	*A. butzleri*	*A. cryaerophilus*	*A. skirrowii*
Fish meat	50	17 (34%)	16 (32%)	1 (2%)	–
Poultry meat	56	15 (26.8%)	15 (26.8%)	–	–
Minced meat	51	1 (2.0%)	1 (2.0%)	–	–
Total	157	33 (21.0%)	32 (20.4%)	1 (0.6%)	–

- Not detectable

TABLE 3: *Presence of putative virulence genes in A. butzleri strains isolated from different foods*

Source	n	No. (%) of strains demonstrating specific gene amplicons									
		cadF	*ciaB*	*cj1349*	*irgA*	*hecA*	*hecB*	*mviN*	*pldA*	*tlyA*	*iroE*
Fish meat	21	21 (100%)	21 (100%)	21 (100%)	8 (38.1%)	14 (66.7%)	12 (57.1%)	21 (100%)	21 (100%)	21 (100%)	9 (42.9%)
Poultry meat	19	19 (100%)	19 (100%)	19 (100%)	6 (31.6%)	5 (26.3%)	6 (31.6%)	19 (100%)	19 (100%)	18 (94.7%)	7 (36.8%)
Total	40	40 (100%)	40 (100%)	40 (100%)	14 (35%)	19 (47.5%)	18 (45%)	40 (100%)	40 (100%)	39 (97.5%)	16 (40%)

Gonzalez et al. (2010) detected even higher prevalences of *Arcobacter* spp. in chicken and waste water samples when applying molecular methods (i. e. RT-PCR) compared to culture dependent detection methods, suggesting that also prevalences in fish meat might be higher than observed in our study.

Virulence gene distribution in *A. butzleri*

The distribution of putative virulence genes among the isolated *A. butzleri* strains is shown in Tab. 3. All investigated *A. butzleri* isolates carried the genes *cadF, ciaB, cj1349, mviN* and *pldA*. The gene *tlyA* was detectable in 97.5% (39/40) of the strains. Lower detection rates were observed for *hecA* (47.5%), *hecB* (45%), *iroE* (40%) and *irgA* (35%). This is in general agreement with previous studies carried out by Douidah et al. (2012) and Karadas et al. (2013) who detected the presence of *cadF, ciaB, cj1349, mviN, pldA* and *tlyA* in all strains and a lower presence of *hecA, hecB, iroE* (only Karadas et al., 2013) and *irgA*. Interestingly, a study by Collado et al. (2014) carried out on *Arcobacter* isolates from mollusc samples showed a lower presence of the investigated virulence genes in *A. butzleri* (ranging from 19.4% for *hecA* to 79% for *mviN*). Of all strains tested in our study, 22.5% (9/40) possessed all ten genes (Fig. 1). No differences in the distribution of putative virulence genes and the source of strains (fish meat vs. poultry meat) were detectable. Therefore, it could be suggested that isolates derived from fish meat have the same pathogenic potential as strains derived from poultry meat.

Genotyping of *A. butzleri*

To investigate the genetic diversity of *A. butzleri* strains isolated from different foods, ERIC-PCR assays were carried out (Fig. 1). These assays demonstrated a high genetic diversity of the *A. butzleri* strains, even though some strains with similar ERIC-PCR pattern were isolated from different fish and chicken samples (i. e. isolate 96, 98 and 120, 121, 123). This high heterogeneity in *A. butzleri* strains originating from different foods was already described by using ERIC-PCR and MLST (Houf et al., 2002; Alonso et al. 2014). A comparable genetic diversity has also been described for *Campylobacter* spp. (Meinersmann et al., 2002). In general, no correlation between genotype distribution and source of strains (i. e. type of food) was obtained. This is in agreement with a study by Alonso et al. (2014) who applied MLST analysis and demonstrated that no association of alleles or sequence types with food source was detectable. Further, no correlation between ERIC-PCR and virulence gene pattern has been observed as strains encoding all virulence genes are distributed over the complete ERIC-PCR dendrogram (Fig. 1). These data confirm that genetic diversity of *A. butzleri* strains is also common among isolates originated from fish products.

In conclusion, further efforts are needed to elucidate the role of *A. butzleri* in veterinary public health as fish meat has been detected to be a further food matrix for *A. butzleri*. This study shows that about one third of fish meat and poultry meat samples contained *A. butzleri*. A
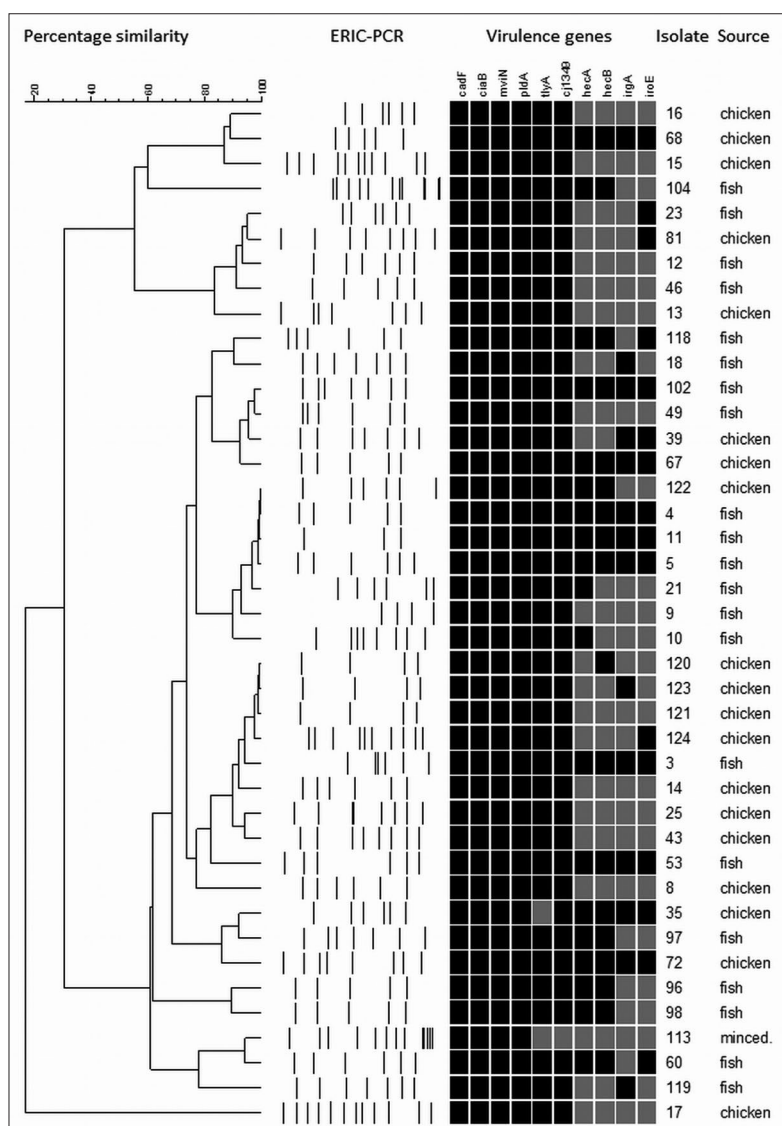

FIGURE 1: *Dendrogram based on ERIC-PCR of A. butzleri strains isolated from different foods with the respective virulence gene pattern. Virulence genes pattern: black = gene present, grey = gene absent*

lower prevalence was detected for minced meat. Several of the putative virulence genes were found in these isolates, implying their pathogenic potential. Also high genetic diversity of this species was observed with no correlation between genotype and source of isolates. Further insights into patho-mechanisms might help to elucidate which *A. butzleri* isolates have an impact on human infection.

Conflict of interest

The authors declare that they have no conflict of interest.

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