Introduction

Arcobacter spp. are gram-negative, motile and spiral-shaped bacteria belonging to the family of Campylobacteracea. 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 (Vandenberge 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 (Vandename 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).

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

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
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 diarrhea. The genomic sequence of A. butzleri is 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 throughout 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 chexel method described by Karadas et al. (2013). Identification

### Table 1: Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>sequence</th>
<th>Amplicon</th>
<th>study</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARCO R</td>
<td>CGATTCCAGCTTAGATGCC</td>
<td>Houf et al. (2000)</td>
<td></td>
</tr>
<tr>
<td>BUTZ F</td>
<td>CCTGGACTGAGCACATGATAGGC</td>
<td>401</td>
<td></td>
</tr>
<tr>
<td>SKIR R</td>
<td>GCAGATTACCTGGGAGAACAC</td>
<td>641</td>
<td></td>
</tr>
<tr>
<td>CR1 Y</td>
<td>TCTGGAGCGCATGAGTAATGA</td>
<td>257</td>
<td></td>
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<tr>
<td>CR2 Y</td>
<td>AACAACTACGCCTCTTCCAGC</td>
<td>approx. 1500</td>
<td></td>
</tr>
<tr>
<td>16S rDNA F</td>
<td>AGAGTTTGATCCTGGCAGG</td>
<td>Coenye et al. (1999)</td>
<td></td>
</tr>
<tr>
<td>16S rDNA F</td>
<td>AGAGTTTGATCCTGGCAGG</td>
<td>approx. 1500</td>
<td></td>
</tr>
<tr>
<td>Cry1 F</td>
<td>AGGAGTACCTGGACGACG</td>
<td>for sequencing</td>
<td></td>
</tr>
<tr>
<td>Cry2 F</td>
<td>AACAACCTACGTCCTTCGAC</td>
<td>257</td>
<td></td>
</tr>
<tr>
<td>16SrRNAF1</td>
<td>AAGGAGGTGATCCAGCCGCA</td>
<td>537</td>
<td></td>
</tr>
<tr>
<td>16SrRNAF1</td>
<td>AAGGAGGTGATCCAGCCGCA</td>
<td>537</td>
<td></td>
</tr>
<tr>
<td>CadF R</td>
<td>CCTGGACTTGACATAGTAAGAATGA</td>
<td>401</td>
<td></td>
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<tr>
<td>CadF R</td>
<td>CCTGGACTTGACATAGTAAGAATGA</td>
<td>401</td>
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<tr>
<td>PldA F</td>
<td>TTGAGCAGACAAATAATGGGCA</td>
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<tr>
<td>PldA R</td>
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<tr>
<td>IrgA F</td>
<td>TGCAAGAATCTTTGAGGGTAGCTC</td>
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<td>IrgA R</td>
<td>GTATAACCCCCATTGATAGGACGCA</td>
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<tr>
<td>HeCA F</td>
<td>GTGAAGTCAACAGATACAGCGGCTC</td>
<td>537</td>
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<tr>
<td>HeCA R</td>
<td>GTCTTTTAGTTGCTCGTCTGACTC</td>
<td>537</td>
<td></td>
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<tr>
<td>HeCB F</td>
<td>CTTAAAACCTACACATGCTCGC</td>
<td>528</td>
<td></td>
</tr>
<tr>
<td>HeCB R</td>
<td>CTTTTGGAGTTGGAGC</td>
<td>528</td>
<td></td>
</tr>
<tr>
<td>Cj1349 F</td>
<td>GAATTGAAAGATGGGATATAAA</td>
<td>556</td>
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<tr>
<td>Cj1349 R</td>
<td>TTTGTGGATTTGCCCTT</td>
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<tr>
<td>CiaB F</td>
<td>GGAAATATAAAGAATTGTTGTC</td>
<td>498</td>
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<td>CiaB R</td>
<td>ATTTACCTCTAGGTATTTTAGTGC</td>
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<tr>
<td>MviN F</td>
<td>TTTCGGCTTGATTAATGGTTTC</td>
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<tr>
<td>MviN R</td>
<td>TGATACCTGTTAGCCTCCTCTT</td>
<td>409</td>
<td></td>
</tr>
<tr>
<td>TlyA F</td>
<td>AACAAAGATTAAATGATGCTG</td>
<td>529</td>
<td></td>
</tr>
<tr>
<td>TlyA R</td>
<td>GTGTTCACTCCTGCTTGGTATG</td>
<td>529</td>
<td></td>
</tr>
<tr>
<td>IroE F</td>
<td>AATGAGTTTGAGGGATTTC</td>
<td>415</td>
<td></td>
</tr>
<tr>
<td>IroE R</td>
<td>GCCGGCTAGAATTTTG</td>
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<td></td>
</tr>
<tr>
<td>ERIC 1 R</td>
<td>ATGAAGTCTCTGGGAGATCAC</td>
<td>Houf et al. (2002)</td>
<td></td>
</tr>
<tr>
<td>ERIC 2</td>
<td>CAAAGGTGACTGGGAGATCCG</td>
<td>555</td>
<td></td>
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</table>

### Notes

**TABLE 1: Primers used in this study**

- **Primers** used in this study:
  - **ARCO R**
  - **BUTZ F**
  - **CR1 Y**
  - **CR2 Y**
  - **16S rDNA F**
  - **Cry1 F**
  - **Cry2 F**
  - **16SrRNAF1**
  - **CadF R**
  - **PldA F**
  - **IrgA F**
  - **HeCA F**
  - **HeCB F**
  - **Cj1349 F**
  - **Cj1349 R**
  - **CiaB F**
  - **MviN F**
  - **TlyA F**
  - **IroE F**
  - **ERIC 1 R**
  - **ERIC 2**

- **Sequence** of the primers:
  - **ARCO R**
  - **BUTZ F**
  - **CR1 Y**
  - **CR2 Y**
  - **16S rDNA F**
  - **Cry1 F**
  - **Cry2 F**
  - **16SrRNAF1**
  - **CadF R**
  - **PldA F**
  - **IrgA F**
  - **HeCA F**
  - **HeCB F**
  - **Cj1349 F**
  - **Cj1349 R**
  - **CiaB F**
  - **MviN F**
  - **TlyA F**
  - **IroE F**
  - **ERIC 1 R**
  - **ERIC 2**

- **Amplicon** size:
  - **ARCO R**
  - **BUTZ F**
  - **CR1 Y**
  - **CR2 Y**
  - **16S rDNA F**
  - **Cry1 F**
  - **Cry2 F**
  - **16SrRNAF1**
  - **CadF R**
  - **PldA F**
  - **IrgA F**
  - **HeCA F**
  - **HeCB F**
  - **Cj1349 F**
  - **Cj1349 R**
  - **CiaB F**
  - **MviN F**
  - **TlyA F**
  - **IroE F**
  - **ERIC 1 R**
  - **ERIC 2**

- **Studies**:
  - Houf et al. (2000)
  - Coenye et al. (1999)
  - Karadas et al. (2013)
  - Houf et al. (2002)
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 MgCl₂ (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 16S rRNA F1 and 16S rRNA R1 (Tab. 1). Amplicons were purified using a GeneJet 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.

### 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% (Röhder 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.

#### 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 MgCl₂ (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%.

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>n</th>
<th>Arcobacter spp</th>
<th>A. butzleri</th>
<th>A. cryaerophilus</th>
<th>A. skirrowii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meat</td>
<td>50</td>
<td>17 (34%)</td>
<td>16 (32%)</td>
<td>1 (2%)</td>
<td>–</td>
</tr>
<tr>
<td>Poultry meat</td>
<td>56</td>
<td>15 (26.8%)</td>
<td>15 (26.8%)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Minced meat</td>
<td>51</td>
<td>1 (2.0%)</td>
<td>1 (2.0%)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>157</td>
<td>33 (21.0%)</td>
<td>32 (20.4%)</td>
<td>1 (0.6%)</td>
<td>–</td>
</tr>
</tbody>
</table>

*Not detectable*
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 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.

**References**


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