

RESEARCH ARTICLE

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Multilocus sequence typing and biocide tolerance of *Arcobacter butzleri* from Danish broiler carcasses

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Abstract

Background: *Arcobacter* spp. have in recent years received increasing interest as potential emerging enteropathogens and zoonotic agents. They are associated with various animals including poultry and can be isolated from meat products. The possibilities of persistence and cross-contamination in slaughterhouses during meat processing are not well established. We have evaluated the occurrence and persistence of *Arcobacter* spp. in a Danish slaughterhouse and determined the sensitivity of isolates to sodium hypochlorite, a commonly used biocide.

Results: *Arcobacter* contamination was examined in a broiler slaughterhouse by selective enrichment of 235 swabs from the processing line during two production days and after sanitizing in between. In total 13.6% of samples were positive for *A. butzleri* with the majority (29 of 32 isolates) originating from the evisceration machine. No *Arcobacter* spp. was isolated after cleaning. *A. butzleri* isolates confirmed by PCR were typed by multilocus sequence typing (MLST) resulting in 10 new sequence types (STs). Two sequence types were isolated on both processing days. Minimum inhibitory concentration (MIC) to sodium hypochlorite was determined to 0.5% hypochlorite biocide (500 ppm chlorine) for most isolates, which allows growth of *A. butzleri* within the working concentration of the biocide (0.2 - 0.5%).

Conclusions: *A. butzleri* was readily isolated from a Danish broiler slaughterhouse, primarily in the evisceration machine. Typing by MLST showed high strain variability but the recurrence of two STs indicate that some persistence or cross-contamination takes place. Importantly, the isolates tolerated sodium hypochlorite, a biocide commonly employed in slaughterhouse sanitizing, at levels close to the disinfection concentration, and thus, *A. butzleri* may survive the disinfection process although this was not observed in our study.

Keywords: *Arcobacter butzleri*, MLST, Chicken slaughterhouse, MIC, Sodium hypochlorite

Background

Arcobacter is member of the family *Campylobacteraceae* [1] and was first isolated in 1977 from aborted bovine and porcine fetuses [2,3]. The genus includes several species of which *A. butzleri*, *A. cryaerophilus* and to some extent *A. skirrowii* have been considered as animal pathogens implicated in abortions, mastitis and gastrointestinal disorders [4] and as human pathogens causing gastroenteritis and occasionally bacteraemia [5-8]. The genome sequence of *A. butzleri* revealed the presence of homologues of several virulence genes known to contribute to the pathogenesis of *Campylobacter jejuni* but also close resemblance to species of the *Helicobacteraceae* [9]. *Arcobacter* spp. have also

been isolated from healthy humans and animals [8,10-12] but in general the prevalence of this emerging pathogen in human disease is likely to be underestimated due to lack of a standard detection procedure [13,14]. Methods for detection and isolation of *Arcobacter* spp. have been suggested and are continuously developing [15-18].

Arcobacter spp. have been isolated from a variety of meat products such as beef, pork, poultry and lamb, with notably high prevalence in poultry meat [19-22]. Consistently, poultry seem to be a major reservoir with flock prevalence between 4.3% and 100%, determined with various isolation techniques [23-28]. Some controversies have occurred about the location of *A. butzleri* in broilers, but the intestines and the cloacae seem to be colonized [18,23,29,30]. In poultry slaughterhouses, *Arcobacter* spp. have been isolated from the processing line, process water and carcasses and an increasing prevalence through a slaughter week has been indicated [27,28]. In a Danish study, all of 30 chicken

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carcasses sampled at a processing plant were positive for *A. butzleri* and six of these were concurrently positive for *A. cryaerophilus* [25]. *A. butzleri* has the ability to attach to surfaces of stainless steel and plastic and is capable of growing at 10°C and forming biofilm [31,32]. These findings suggest that *A. butzleri* may be able to persist in the food processing environment for extended periods of time and contaminate processing equipment despite of sanitizing [28]. In light of this information, we have addressed if *A. butzleri* chicken slaughterhouse isolates tolerate the commonly used biocide sodium hypochlorite in disinfection concentration causing the risk of isolates persisting in this environment. Further, multilocus sequence typing (MLST) of *A. butzleri* isolates was used to evaluate the heterogeneity of the isolates obtained on two consecutive production days and after the sanitizing in between.

Methods

Bacterial strains

Reference strains used in this study are listed in Table 1. Bacteria were routinely grown in Brain-Heart-Infusion broth (BHI; OXOID, Greve, Denmark) or *Arcobacter* broth (OXOID) and on *Arcobacter* agar (OXOID; Agar No. 3 incl. *Arcobacter* broth and 50 mL L⁻¹ defibrinated calf blood) at 28°C in microaerobic atmosphere generated using Campy Gen (OXOID) in sealed jars. For dilution and washing of bacteria peptone-saline water was used (NaCl 9.0 g L⁻¹ and peptone 1.0 g L⁻¹).

Specifications of the broiler slaughterhouse

A Danish slaughterhouse was selected for an investigation of the occurrence of *Arcobacter* during two processing days. The slaughter capacity was 180,000 chickens per day and the products ranged from whole chickens, breast fillets, wings and chicken legs. After slaughter, scalding, defeathering, evisceration and washing, chickens were conveyed through a 3-hour air chilling step before cutting and packaging. In general, the temperature in the processing areas was 10–12°C. The temperature of the scalding water was 53 ± 2°C. The cleaning following directly after each processing day was performed with flusher and foam

followed by disinfection. A biocide containing sodium hypochlorite was used for disinfection six days of the week and once per week a quaternary ammonium compound was used. The disinfection biocide containing sodium hypochlorite (Divosan Hypo Plus, JohnsonDiversey, Nivaa, Denmark) was used in a 0.2 – 0.5% working solution, corresponding to 200–500 ppm active chlorine and with a contact time of ten minutes as determined by the manufacturer.

Sample collection

Samples were collected three times at four sampling points during two consecutive production days (see Table 2 for details). Sterile cotton swabs moistened with *Arcobacter* broth sampled surfaces directly or indirectly in contact with parts of the carcasses. A total of 235 swabs were taken with 55 samples collected from the scalding tank (scalding tank water and overflow), 60 samples from the evisceration machine, 60 samples from the area “cutting of breast fillet” and 60 samples from the area “boning of chicken legs”. *A. butzleri* LMG 10828, *A. cryaerophilus* LMG 7536 and *A. skirrowii* LMG 6621 on agar were used as positive controls and sterile broth samples were used as negative controls. After sampling, swabs were stored individually in 5 mL *Arcobacter* broth in transport tubes under cooled conditions and processed within 30 h.

Selective isolation

For selective enrichment favoring *Arcobacter* spp. the samples were added 50 mL L⁻¹ defibrinated calf blood and selective supplement comprising of 5-flourouacil (100 mg L⁻¹), amphotericin B (10 mg L⁻¹), cefoperazone (16 mg L⁻¹), novobiocin (32 mg L⁻¹) and trimethoprim (64 mg L⁻¹) [33] and incubated in 48 h in microaerobic atmosphere at 28°C. The samples were then stored in 15% glycerol at – 80°C until further processing. The samples were thawed in refrigerator at 5°C and aliquots of 250 µL were subcultured individually in 7 mL *Arcobacter* broth, 5% defibrinated calf blood and the selective supplement before incubation for 48 h in microaerobic atmosphere at 28°C. Subsequent 100 µL was inoculated on *Arcobacter* agar with selective supplement and incubated in 48 to 96 h in microaerobic atmosphere at 28°C. Furthermore, the frozen enrichments were subjected to direct PCR identification of *Arcobacter* spp. on crude DNA lysates. After thawing, 300 mL enrichment culture was centrifuged (8.000 × g for 5 min), the supernatant was removed and the pellet was added 300 mL miliQ water. Bacteria were lysed by heating to 99°C for 15 min, cooled down and centrifuged (10.000 × g for 2 min). The supernatants were used as DNA templates in a genus specific PCR amplifying a 181-bp DNA fragment of the 16S rRNA gene from *Arcobacter* spp. [34].

Table 1 *Arcobacter* strains used in this study

Species	ID	Obtained from
<i>A. butzleri</i>	LMG 10828	BCCM/LMG (Ghent, Belgium)
<i>A. cryaerophilus</i>	LMG 7536	CCUG (Göteborg, Sweden)
	LMG 10829	BCCM/LMG (Ghent, Belgium)
<i>A. skirrowii</i>	LMG 6621	CCUG (Göteborg, Sweden)
<i>A. trophiarum</i>	LMG 25534	BCCM/LMG (Ghent, Belgium)
<i>A. cibarius</i>	LMG 21996	BCCM/LMG (Ghent, Belgium)
<i>A. thereius</i>	LMG 24486	BCCM/LMG (Ghent, Belgium)

Table 2 Prevalence of *Arcobacter* spp. in the processing line of a Danish broiler slaughterhouse determined by cultural enrichment and isolation or by direct PCR on enrichment cultures

Place	Processing day 1		After cleaning		Processing day 2	
	Enrichment	PCR	Enrichment	PCR	Enrichment	PCR
Scalding tank	0/15	0/15	0/20	0/20	3/20	2/20
Evisceration machine	10/20	13/20	0/20	2/20	19/20	17/20
Area "cutting of breast fillet"	0/20	0/20	0/20	0/20	0/20	0/20
Area "boning of chicken leg"	0/20	2/20	0/20	0/20	0/20	0/20
Prevalence	10/75	15/75	0/80	2/80	22/80	19/80
In percent	13.3%	20%	0%	2.5%	27.5%	23.8%

Species identification by multiplex-PCR

Presumptive *Arcobacter* colonies, determined by colony morphology (<1.5 mm greyish/white colonies) and cell morphology (motile, small, curved rods by microscopy), were subcultured on *Arcobacter* agar and incubated in microaerobic atmosphere at 28°C for 48 h. BHI cultures, grown for 20 h at 28°C, were centrifuged at 11,000 × g for 3 min. Pellets were suspended in 0.5 mL peptone-saline water and centrifuged for 3 min at 14,000 × g. DNA was extracted using QIAGEN DNeasy® Blood & Tissue kit following the manufacturer's instructions with pretreatment for Gram-negative bacteria. All PCR assays were carried out using DreamTaq™ Green PCR Master Mix (2x) (Fermentas Life Sciences) and primers were obtained from TAG Copenhagen (Copenhagen, Denmark). Multiplex-PCR (mPCR) was used for confirmation and identification at species level [35] using a range of *Arcobacter* species as controls (Table 1). Isolates not positive in the mPCR were furthermore evaluated by PCR using primers specific for *A. trophiarum* [36].

Multilocus sequence typing (MLST)

MLST, a typing method based on partial sequence information of seven housekeeping loci (*aspA*, *atpA*, *glnA*, *gltA*, *glyA*, *pgm* and *tkl*), was used for characterization of *A. butzleri* isolates below species level [37]. The MLST gene products were sequenced by Macrogen Inc. (Seoul, Korea) and the sequencing results were processed with CLC Main Work Bench version 5.7.1. To compare with known *A. butzleri* MLST profiles the profiles from the isolates were subjected to the *Arcobacter* specific MLST scheme: [http://pubmlst.org/arcobacter/] [38].

Phylogenetic analysis

Concatenated *A. butzleri* alleles were aligned using ClustalX [39]. Phylogenetic analyses were conducted using MEGA (Version 5.1; [40]). Un-rooted neighbor-joining phylogenetic trees were constructed with bootstrap analysis (1000 replicates).

Determination of minimal inhibitory concentration (MIC)

The tolerance towards a slaughterhouse biocide containing sodium hypochlorite (NaClO; Divosan Hypo Plus) was assessed using a standard dilution assay for determination of MIC. The MICs were determined by preparing dilutions starting from the maximal recommended working solution (0.5% and two-fold dilutions from 0.4 to 0.05%) of the biocide in 96-well microtiterplates [41,42]. BHI cultures of the slaughterhouse isolates and *A. butzleri* LMG 10828, grown for 20 h at 28°C, were diluted in BHI broth to give absorbance at 600 nm (A_{600}) of 0.01 and were inoculated to biocide broth dilutions to a total volume of 200 µL per well. Positive (no NaClO) and negative (no inoculum) growth controls were included and the experiment was performed with quadruple determinations. The lowest NaClO concentration to inhibit bacterial growth after 20 h of incubation at 28°C in microaerobic atmosphere was considered as the MIC. To verify that inhibition from the slaughterhouse biocide was caused by the active ingredient, NaClO, the experiment was repeated with NaClO from Sigma-Aldrich (Broendby, Denmark; active chlorine; 4.00 - 4.99%).

Results and discussion

Occurrence of *Arcobacter* in a Danish slaughterhouse

A Danish chicken slaughterhouse, with a capacity of 180,000 chickens per day, was subjected to swab sampling on selected sites over two consecutive processing days during production, and after the sanitizing in between, to evaluate the *Arcobacter* contamination. In total, 32 of 235 (13.6%) samples were confirmed positive for *A. butzleri* by multiplex PCR after selective enrichment [33,43] but none of the samples taken on clean surfaces after sanitizing were positive. The majority of the isolates (29 of 32) were recovered from the evisceration machine; hence 48.3% of the samples from this site were positive. The distribution in the processing line is shown in Table 2. Using a direct PCR-technique, 36 of 235 (15.3%) samples were found positive for *Arcobacter* spp. and by this method, two samples from the meat processing area (cutting and

boning) were positive as well as two samples from the evisceration machine after cleaning, which were not found in the selective enrichment. Otherwise, *Arcobacter* positive samples were identical using the two methods, except for three additional positives in the evisceration machine (Day 1) and two 'false negative' samples from the evisceration machine (Day 2) that were disqualified due to un-specific bands on agarose gels. The high prevalence of *A. butzleri* in the evisceration area supports earlier reports of *Arcobacter* positive chicken gut samples [23,26,28] and suggests that contamination is likely to originate from the chicken gut.

A. butzleri was the only species isolated. This may be due to the enrichment procedure, as controls of *A. cryaerophilus* and *A. skirrowii* failed to grow under the enrichment conditions. Enrichment culturing has previously been shown to favor outgrowth of *A. butzleri* [33], but variations in the enrichments culturing used in this study (prolonged cooled storage before enrichment and inclusion of a freeze storage stage of enriched samples) have likely had a negative effect on the survival of *Arcobacter* isolates, especially non-*A. butzleri* species [29]. This is supported by the difference in prevalence of *Arcobacter* on the first and second sampling day, as the prevalence was twice as high in samples from the second day, which had a shorter storage time. The *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* controls were all detected using direct PCR. Interestingly, we detected *Arcobacter* in two out of 20 swabs from the evisceration machine after cleaning, but these were not isolated using selective plating. *Arcobacter* spp. have previously been recovered from cleaned processing machines in poultry slaughterhouses, even after a three-day break in the production, as well as from slaughtered broilers before evisceration [27]. Furthermore, *A. butzleri* is able to multiply at the temperature present in slaughterhouses (10°C) and to form biofilm on stainless

steel surfaces [31], which suggest that *A. butzleri* may be able to establish itself in the slaughterhouse environment. In consequence, we investigated the heterogeneity of *A. butzleri* isolated from chicken carcasses and slaughterhouse equipment by MLST and the ability of the obtained isolates to tolerate disinfection with the routinely used biocide (sodium hypochlorite).

Characterization of *A. butzleri* isolates by multilocus sequence typing (MLST) and phylogeny

Typing of *Arcobacter* by MLST was demonstrated to be useful for discrimination below species level of isolates of *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. cibarius* and a database for *Arcobacter* MLST profiles are available online [<http://pubmlst.org/arcobacter/>] [37,38]. We performed MLST analysis of 32 *A. butzleri* isolates obtained in the chicken slaughterhouse, resulting in identification of 10 new sequence types (STs) comprising both known alleles and 13 new alleles (Table 3). No known STs were identified. The relatively high numbers of new alleles and STs show the heterogeneity of the *A. butzleri* isolates, but also reflects the limited number of profiles in the *Arcobacter* MLST database. With three isolates on the first sampling day and 12 on the second sampling day, ST 367 was found to be the most prevailing (47% of isolates, see Table 4). Furthermore, ST 367 (15 isolates) and ST 373 (five isolates) were isolated during both production days in the evisceration machine. The two STs 367 and 373 have no identical alleles and it seems more likely that the reoccurrence of these STs are due to cross-contamination rather than identical sequence types being present at different broiler flock farms.

Isolation of *A. butzleri* from the scalding water during production on the second sampling day suggests a risk of cross-contamination within a production period. However, *Arcobacter* spp. are unlikely to survive more than few

Table 3 Overview of MLST results of *A. butzleri* LMG 10828 and slaughterhouse isolates with allelic profiles according to the *Arcobacter* MLST database [37]

Isolation area	Allelic profile							ST
	<i>aspA</i>	<i>atpA</i>	<i>glnA</i>	<i>gltA</i>	<i>glyA</i>	<i>pgm</i>	<i>tkt</i>	
Evisceration machine	153	4	11	11	177	102	11	367
Evisceration machine	5	12	122*	15	36	86	2	368
Evisceration machine	30	34	9	30	144	35	175*	369
Scalding tank	206*	143*	2	37	166	53	162	370
Evisceration machine	14	49	26	55	449*	102	176*	371
Evisceration machine	23	44	24	14	450*	101	20	372
Evisceration machine	205*	144*	123*	55	447*	233*	177*	373
Evisceration machine	5	5	5	19	120	11	65	374
Evisceration machine	20	25	7	2	354	32	2	375
Evisceration machine	175	25	41	19	88	101	14	376

* New allele.

Table 4 Occurrence and isolation site of 32 *Arcobacter butzleri* isolates assigned ten different MLST sequence types (ST)

ST	Day 1		Day 2	
	No. of isolates	Area	No. of isolates	Area
367	3	Evisceration	12	Evisceration
368	1	Evisceration	-	-
369	1	Evisceration	-	-
370	-	-	3	Scalding tank
371	-	-	2	Evisceration
372	-	-	1	Evisceration
373	4	Evisceration	1	Evisceration
374	1	Evisceration	-	-
375	-	-	1	Evisceration
376	-	-	2	Evisceration

minutes in the scalding water ($53 \pm 2^\circ\text{C}$) [44] and the ST found in scalding water was not found elsewhere in the production. Phylogenetic analysis of the concatenated *A. butzleri* alleles was conducted to evaluate possible clustering within the isolates. As established with the MLST, the strain diversity is high and in conjunction with the relatively low number of isolates this results in a phylogenetic tree (Figure 1) that is not very robust (low bootstrap values) and shows no clustering of isolates. *Arcobacter* have previously been characterized by molecular typing using ERIC-PCR and RAPD-PCR [45,46], by MLST [9] and by AFLP and PFGE profiling [47]. In general, these molecular fingerprinting techniques show a high discrimination of strains due to considerable heterogeneity of *Arcobacter* [15]. A great advantage of MLST genotyping is the online available database of *Arcobacter* spp. isolates collected worldwide [38] and thereby the possibility to

compare results. Yet, so far it has not been possible to relate specific STs with hosts or geographical areas [37].

Biocide tolerance

In the slaughterhouse, cleaning is performed on a daily basis by foaming all surfaces with a detergent and high pressure cleaning with hot water. Following cleaning, disinfection is performed with a biocide containing sodium hypochlorite (NaClO/Chlorine) at concentrations of 5–15%. The working solution of the biocide is 0.2 – 0.5% corresponding to 200–500 ppm active chlorine and the contact time is approximately 10 min. To address the susceptibility of *A. butzleri* to sodium hypochlorite we determined the minimum inhibitory concentration (MIC) by a standard broth dilution method used in similar studies [41,42]. We found that the majority of *Arcobacter* were only inhibited by the sodium hypochlorite containing biocide in the maximum recommended working solution; 0.5% corresponding to 500 ppm active chlorine. *A. butzleri* LMG 10828 had a MIC of 0.5% sodium hypochlorite, as did 31 of the 32 slaughterhouse isolates (97%) (data not shown). The last slaughterhouse isolate and the remaining *Arcobacter* type strains (Table 1) had a MIC of 0.2% corresponding to 200 ppm active chlorine. This tolerance was confirmed using reagent grade sodium hypochlorite (Sigma-Aldrich). During regular cleaning operations the biocides are allowed a contact time of 10 min when applied for disinfection. Here we determine that the lower working concentration (0.2%) of sodium hypochlorite does not have a lethal effect on *A. butzleri* even after 20 h of exposure. Despite the biocide tolerance of *Arcobacter* isolates we did not obtain any isolates in the slaughterhouse after cleaning and disinfection with chlorine containing detergent, although two of a total of 80 swabs taken after sanitizing were *Arcobacter* positive by direct PCR. This

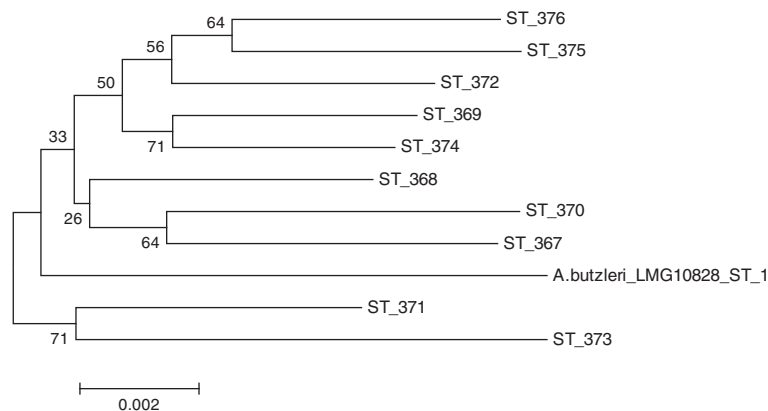


Figure 1 Neighbour-joining phylogenetic tree of concatenated *A. butzleri* alleles from ST 367 to ST 376 (and including *A. butzleri* LMG 10828 ST 1) isolated at a Danish chicken slaughterhouse. Bootstrap analysis with 1000 replications was performed, based on 3,341 bp, and bootstrap values are shown at the nodes.

suggests that the combined cleaning and disinfection procedures are generally working satisfactorily, but in highly contaminated areas it might not be sufficient.

We isolated *A. butzleri* almost exclusively from the evisceration area in the chicken slaughterhouse. Previous studies report findings of arcobacters in the intestines of chickens [23,48] and increased prevalence of *A. butzleri* on neck skin after evisceration with identical genotypes in the intestines and on the carcasses within a flock [28]. These findings suggest that arcobacter contamination occurs at the evisceration step and that contamination from the intestinal content to the carcass surface takes place. We determined a high biocide tolerance of the *A. butzleri* isolates obtained from the slaughterhouse, illustrating that *A. butzleri* can grow at concentrations close to the working solution of NaClO and we confirmed the presence of *Arcobacter* in two swab samples taken after sanitizing. Considering the disinfection contact time of 10 min, it is likely that *A. butzleri* may survive sanitizing. This assumption is supported by the isolation of two cases of identical STs of *A. butzleri*, determined by MLST, reoccurring on two consecutive production days, suggesting either persistence or continuous re-contamination of *A. butzleri*.

Conclusions

The MLST genotyping of *A. butzleri*, isolated on two consecutive production days in a chicken slaughterhouse, showed high heterogeneity of the isolates but nonetheless reoccurrence of two sequence types on both production days. We further show that *A. butzleri* can survive and multiply in prolonged exposure (20 h) to working concentrations of a commonly used disinfecting biocide, sodium hypochlorite. Thus, *A. butzleri* may be able to persist in the slaughterhouse after disinfection.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LHR conceived the research idea and performed the laboratory work, analyzed the data and wrote the manuscript. JK helped in sample collection, analyzing and interpreting data and preparation of the manuscript. JPC and HI helped in designing the study and supervision of the work and revised the manuscript. All authors read and approved the final manuscript.

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