Viability and biofilm formation of *Arcobacter* spp. at various processing temperatures

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Summary
The genus *Arcobacter* has become increasingly important in recent years because some species have been considered emerging enteropathogens and potential zoonotic agents. *Arcobacter* spp. can be isolated from food of animal origin and from various kind of water. This study assessed the survival and biofilm activity of *Arcobacter* strains (including the less described *A. defluvii*) at processing temperatures applied in the food industry. Broth cultures of five strains were stored at various temperatures (–18, 5, 15, 25, 37 °C) to determine their survival. Tested *Arcobacter* strains survived freezing (for 1–7 days) and cooling (for 3–21 days) as well as long term storage (for more than 6 months) at 15 °C and 25 °C. The lethal temperature was determined for arcobacters in nutritionally distinct media (distilled water, physiological saline, brain heart infusion, semi-skimmed milk). The lethal temperatures (10 min exposure) of 50.5–56.0 °C, 45.5–55.0 °C, or 48.0–52.0 °C were determined for *A. butzleri*, *A. cryaerophilus* and *A. defluvii*, respectively. Further, the biofilm formation of arcobacters at various processing temperatures was evaluated. Intermediate or high biofilm-forming activity was observed at all tested temperatures.

Keywords
Arcobacter spp.; temperature condition; survival; lethal temperature; inactivation; biofilm formation

Bacteria from the *Arcobacter* genus are gram-negative, slightly curved (helical or S-shaped), thin, non-sporulating rods, 0.5–3.0 μm long and 0.2–0.9 μm wide. The size of the bacterial cells may vary. Arcobacters can form almost coccoid forms in old cultures and sporadically free spiral fibres up to 20 μm long [1, 2]. Arcobacters are taxonomically included in the family *Campylobacteraceae* together with the *Campylobacter* genus. Differentiation of *Arcobacter* spp. is usually based of their ability to grow at temperatures of 15–30 °C [3]. However, with the increasing number of arcobacters being described and ranked, it becomes evident that this differentiation is inadequate [4].

In recent years, various laboratories have focused on this bacterial genus. In 2002, *A. butzleri* was included in the list of microorganisms harmful to human health [5]. Four species are usually listed as pathogenic, namely, *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. thereius*, which were shown to be linked to human disease [6]. However, more than 25 species of arcobacters are currently described [7]. Sensitivity of arcobacters to various external influences was described [8, 9], but they are abundantly isolated from a wide range of sample types. For example, they were detected in food of animal origin (poultry, pork, beef, lamb, milk and seafood), in water samples (drinking, waste, river and brackish waters), in marine sediments and in oil fields. The presence of arcobacters was also demonstrated in human biological material [10, 11].

A temperature of 30 °C is indicated as an optimal culture temperature for *Arcobacter* spp. [12], but rare survival of an *A. butzleri* clinical isolate was also reported at 42 °C [13]. The resistance of arcobacters to various temperatures was partly described, in the literature, for some *A. butzleri* strains, mainly collection strains [14–17]. From the food industry point of view, it is important to monitor the influence of freezer and refrigerator temperatures, as well as other technological temperature ranges. The ability of arcobacters to survive at low temperatures (< 15 °C) was previously demonstrated in several studies [14, 16–18].

Although the presence of *Arcobacter* spp. is

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known in the food industry and processing plants, there is not enough general information in the literature on the behaviour of arcobacters at various industrial temperatures. This study provides an overview of the behaviour of selected *Arcobacter* representatives at various process temperatures. Moreover, the comparison is also made regarding temperature-related biofilm formation, which is often a major source of contamination in various food industries and environments.

MATERIALS AND METHODS

Bacterial strains

The microorganisms used in this study were: *A. butzleri* CCUG 30484, *A. butzleri* UPa KK (strain isolated from chicken neck), *A. cryaerophilus* CCM 7050, *A. cryaerophilus* CCM 3933 and *A. defluvii* LMG 25694, obtained from the Czech Collection of Microorganisms (CCM, Brno, Czech Republic), Culture Collection University of Göteborg (CCUG, Göteborg, Sweden), Belgian Co-ordinated Collections of Microorganisms (LMG, Ghent, Belgium) or isolated at the University of Pardubice (UPa, Pardubice, Czech Republic). Cultures were grown on tryptone soya agar (TSA; HiMedia, Mumbai, India) for 48 h at 30 °C before testing. Cells were harvested and suspended in physiological saline to a value of 0.5 of McFarland scale ($3–9 \times 10^8$ CFU·ml^{-1}). The suspension of cells was then diluted to an appropriate density before testing. To verify the exact number of cells in the prepared suspension, a presumptive density of 10^3 CFU·ml^{-1} was counted on TSA.

Growth and viability of arcobacters in broth at processing temperatures

Brain heart infusion (BHI, HiMedia, Mumbai, India) culture at a density of 10^6 CFU·ml^{-1} of the test strain was stored in Eppendorf microtubes (250 μl) at freezer and refrigerator temperatures, i.e. –18 °C and 5 °C, respectively. Similarly, the BHI culture was maintained in a thermostat at 15, 25 and 37 °C. The growth and viability of arcobacters were evaluated after exposure for 6, 24, 72 h, then after 7 days and subsequently every week, by spreading 100 μl of the cell suspension on TSA in duplicates to detect the survival of *Arcobacter* strains at the selected temperatures. In parallel, it was always verified whether the cells were not in the so-called VBNC (viable but non-culturable) state by inoculating fresh BHI broth and culturing at 30 °C for 72 h. The experiments were repeated three times.

Determination of lethal temperature and *D*-value

BHI culture with a cell density of 10^6 CFU·ml^{-1} of the test strain was heated in Eppendorf microtubes (250 μl) for 10 min in a thermoblock (block heater Stuart SBH130DC; Bibby Scientific, Stone, United Kingdom). After warming up (temperature range 40–60 °C), a viability assay of the *Arcobacter* cells was immediately performed by spreading on TSA. A temperature without a positive growth of *Arcobacter* spp. (including verification of a possible VBNC state, as mentioned above) was considered a lethal temperature (T_{leth}). The experiments were repeated three times. T_{leth} was expressed as arithmetic mean ± standard deviation.

The dependence of cell counts of BHI culture with the initial *Arcobacter* density of 10^6 CFU·ml^{-1} on exposure time (1–10 min) was determined for individual lethal temperatures. After each minute of exposure of cell suspension to a given temperature (1–10 min), the number of surviving *Arcobacter* cells was determined by spreading on TSA (data not shown). The experiments were repeated three times. Decimal reduction time (*D*-value) for experimentally determined T_{leth} was expressed from graphs. The experiments were repeated three times and results are expressed as arithmetic mean ± standard deviation.

Determination of biofilm formation at processing temperatures

Biofilm formation was monitored in 96-well polystyrene flat-bottomed microtitre plates (SPL Life Sciences, Pocheon-si, South Korea) as previously described by Christensen et al. [19] with modifications. Briefly, the strain cultured in BHI broth was prepared at a final cell concentration of 10^7 CFU·ml^{-1}. After incubation at experimental temperatures (T = 5, 15, 25, 30 °C) for 24 h under aerobic conditions, the microtitre plate was repeatedly washed with sterile distilled water and dried. Fixation of the biofilm was performed with 2% sodium acetate (during 15 min), and attached cells were stained with 100 μl of 1% crystal violet (Sigma-Aldrich, St. Louis, Missouri, USA). After 15 min of staining, the plate was repeatedly washed and dried. Then, the biofilm-associated crystal violet was solubilized with 96% ethanol and the absorbance of the solution was measured in a new plate at 595 nm (Infinite M200, Tecan, Männedorf, Switzerland). The results were interpreted as weak biofilm-forming activity ($0.112 < A \leq 0.120$), intermediate biofilm-forming activity ($0.120 < A \leq 0.150$) and high biofilm-forming activity ($A > 0.150$). There were 8 replicate wells in each experiment, experiments were independently repeated three times. The obtained

values were statistically evaluated using Excel 2010 (Microsoft, Redmond, Washington, USA) and Statistica 12 (StatSoft, Tulsa, Oklahoma, USA). Extreme values were tested by the Dean-Dixon test, and any outlier values were excluded with 95% probability. The median and standard deviation were determined from the remaining values. A possible source of mistakes, which is the effect of insufficient dye washing resulting in an increase in absorbance, was also considered and absorbance values that were too high compared to other measured values were excluded.

RESULTS AND DISCUSSION

Survival of *Arcobacter*-strains at processing temperatures

The survival of arcobacters was studied under various temperature conditions (Tab. 1). From the obtained data it is clear that arcobacters are sensitive, especially to freezing temperature (–18 °C). The strains *A. cryaerophilus* CCM 3933 and *A. cryaerophilus* CCM 7050 survived the longest of all the tested arcobacters at this temperature, i.e. for 14 days. After 2 weeks of exposure, their viability was tested by resuscitation in BHI broth to exclude VBNC form. They were found to be in VBNC state after 14 days, however, they were devitalized after 21 days. The strains *A. butzleri* CCUG 30484 and *A. butzleri* UPa KK were culturable after exposure to the freezing temperature for 72 h. They were in the VBNC state after 7 days but were devitalized after 14 days. *A. defluvii* LMG 25694 was the least resistant in this respect, with complete inhibition already after 24 h. An earlier study reported a decrease in cell viability by 2 logs after 24 h of storage at a freezing temperature of –20 °C followed by almost constant viability over a period of 21 days [16]. Another study reported the survival of one isolated *A. butzleri* strain for up to 6 months at –20 °C, but in a special nutrient medium [18].

The arcobacters were considerably more resistant at a refrigeration temperature of 5 °C, at which they did not significantly multiply and were gradually losing viability. The strains *A. cryaerophilus* CCM 7050 and *A. butzleri* CCUG 30484 were the most resistant at this temperature, being still culturable after 21 and 14 days, respectively. These results are consistent with an earlier study of only one particular strain (*A. butzleri* NCTC 12481) [16], although different experimental conditions were used in this study. On the other hand, *A. cryaerophilus* CCM 3933 and *A. butzleri* UPa KK isolate survived only 7 days, while *A. defluvii* LMG 25694 was again the least resistant strain (lethal time shorter than 7 days). A previous study described the survival of *A. butzleri* ATCC 49616 in BHI broth and in chicken meat juice at 5 °C for over 70 days [14]. It was also previously determined that *A. butzleri* survives refrigeration temperatures better than freezing conditions [16], which is in line with our results.

Temperatures from 15 °C to 25 °C were determined to support the survival of arcobacters. Even though the temperature of 15 °C is sometimes considered a minimum temperature for occurrence of arcobacters [14], very long-term survival was recorded at it. In strains *A. butzleri* CCUG 30484 and *A. cryaerophilus* CCM 7050, the survival was almost the same throughout the experiment, for more than 6 months. An earlier study also suggested the ability of arcobacters to survive in BHI broth at temperatures lower than 15 °C [14]. The temperature of 37 °C is, on the contrary, considered to be the maximum temperature compatible with the growth and survival of arcobacters, except for some previously published exceptions, such as a human *A. butzleri* strain [13]. At that temperature, considerable variations in survivability of arcobacters was observed. Long-term survival at physiological temperatures was reported for *A. cryaerophilus* CCM 7050 (up to 98 days) and *A. butzleri* CCUG 30484 (35 days). However,

Tab. 1. Survival period of *Arcobacter* cultures in brain heart infusion broth at processing temperatures.

Strain	–18 °C	5 °C	15 °C	25 °C	37 °C
	Survival time [d]				
A. butzleri CCUG 30484	7	14	189*	189*	35
A. butzleri UPa KK	7	7	77	77	7
A. cryaerophilus CCM 3933	14	7	119	142	1
A. cryaerophilus CCM 7050	14	21	189*	189*	98
A. defluvii LMG 25694	1	3	129	129	1

* – last time point examined.

Tab. 2. Lethal temperatures and calculated *D*-values for *Arcobacter* strains in various media.

Strain	Distilled water		Physiological saline		BHI broth		Semi-skimmed milk	
	T_{leth} [°C]	*D*-value [min]	T_{leth} [°C]	*D*-value [min]	T_{leth} [°C]	*D*-value [min]	T_{leth} [°C]	*D*-value [min]
A. butzleri CCUG 30484	50.5 ± 1.2	8.3 ± 0.2	52.0 ± 0.8	9.5 ± 0.2	53.5 ± 0.8	9.8 ± 0.7	55.0 ± 0.8	9.8 ± 0.2
A. butzleri UPa KK	52.0 ± 0.4	7.9 ± 0.1	53.0 ± 0.8	8.1 ± 0.2	55.0 ± 0.0	8.9 ± 0.3	56.0 ± 0.8	9.1 ± 0.2
A. cryaerophilus CCM 3933	45.5 ± 0.4	8.1 ± 0.3	48.0 ± 0.8	8.1 ± 0.2	50.0 ± 0.8	8.0 ± 0.2	50.5 ± 0.7	8.5 ± 0.3
A. cryaerophilus CCM 7050	50.0 ± 0.8	7.9 ± 0.4	51.0 ± 0.0	8.3 ± 0.4	54.0 ± 0.8	8.1 ± 0.3	55.0 ± 0.8	8.9 ± 0.3
A. defluvii LMG 25694	48.0 ± 0.8	8.1 ± 0.3	51.0 ± 0.8	8.9 ± 0.4	51.5 ± 0.4	8.9 ± 0.2	52.0 ± 0.8	9.2 ± 0.3

The results are expressed as mean ± standard deviation (n = 3).
T_{leth} – lethal temperature, *D*-value – decimal reduction time at lethal temperature, BHI – brain hearth infusion.

for *A. cryaerophilus* CCM 3933, *A. defluvii* LMG 25694 and *A. butzleri* UPa KK it was only 1 day or 7 days. No data are available in literature on survivability at various process temperatures, in particular regarding *A. defluvii*.

T_{leth} is the lowest temperature at which microorganisms are killed after a certain period of time and under defined conditions. It is known that the determination of T_{leth} is dependent on many experimental conditions. Because the environment may affect T_{leth}, the assay was performed for several nutritionally distinct matrices (distilled water, physiological saline, BHI broth, semi-skimmed milk) in order to obtain more informative results. The determined T_{leth} values (Tab. 2) differed slightly between individual strains and differences were also observed at different experimental conditions. T_{leth} determined for an exposure of 10 min ranged from 45.5 °C to 56.0 °C. The determined T_{leth} values depended on the medium used, increasing in the order distilled water < physiological saline < BHI broth < semi-skimmed milk, confirming the protective effect of nutritionally richer culture media on the survival of arcobacters. A similar protective effect on the survival of arcobacters was previously observed when adding organic material to drinking water [17]. The lowest T_{leth} values (45.5–50.5 °C) were determined for *A. cryaerophilus* CCM 3933, while *A. butzleri* UPa KK (with T_{leth} 52.0–56.0 °C) was rated as the most resistant to increased temperature. *A. butzleri* species was rated as the most resistant to increased temperature.

Furthermore, *D*-values were expressed for experimentally determined T_{leth} (experiment time 10 min). *D*-values ranged from 7.9 min to 9.8 min (Tab. 2), depending on the conditions of the experiment and the given strain. The lowest *D*-values were recorded for T_{leth} determined in distilled water (7.9 min, 52.0 °C) for *A. butzleri* UPa KK and 7.9 min (50.0 °C) for *A. cryaerophilus* CCM 7050. Overall, the highest *D*-values in all media were observed in *A. butzleri* CCUG 30484. It can be concluded that *A. butzleri* CCUG 30484 survived for a long time at T_{leth} with a relatively high cell density (gradual decline of surviving cells). The literature provides *D*-values for *A. butzleri* NCTC 12481 in exponential, resp. stationary phase (1.7 min, resp. 2.1 min at 50 °C; 1.1 min, resp. 0.4 min at 55 °C). [16], however, the assay was performed for only one collection strain in the environment of a phosphate buffer, thus under different experimental conditions. Higher *D*-values (5.41 min at 50 °C and 0.57 min at 55 °C) for arcobacters were described elsewhere [18]. In the literature, *D*-values are usually determined for temperatures of 50 °C and 55 °C, not for specific T_{leth} of the given strains, so those data cannot be directly compared to our results.

Biofilm formation at processing temperatures

The biofilm-forming activity of arcobacters was monitored at various temperatures (Fig. 1) that are significant for the food industry or due to temperature demands of these bacteria. All the tested strains formed biofilm (A > 0.112). Biofilm-forming activity varied depending on the experimental temperature. In most cases, the lowest biofilm formation was recorded at 25 °C, which can be explained by the fact that that is an optimal culture temperature and the cells "do not need" to form biofilm to such an extent. However, *A. defluvii* LMG 25694 did not follow this rule as its biofilm-forming activity grew with increasing experimental temperature. Overall, high biofilm formation (A = 0.200) was observed for this strain. A previous study gave similar results, as the tested *Arcobacter* strains exhibited greater biofilm-forming activity with the increasing culture temperature [14]. However, the *A. butzleri* UPa KK isolate formed the most biofilm at 15 °C (A = 0.129), while at other experimental

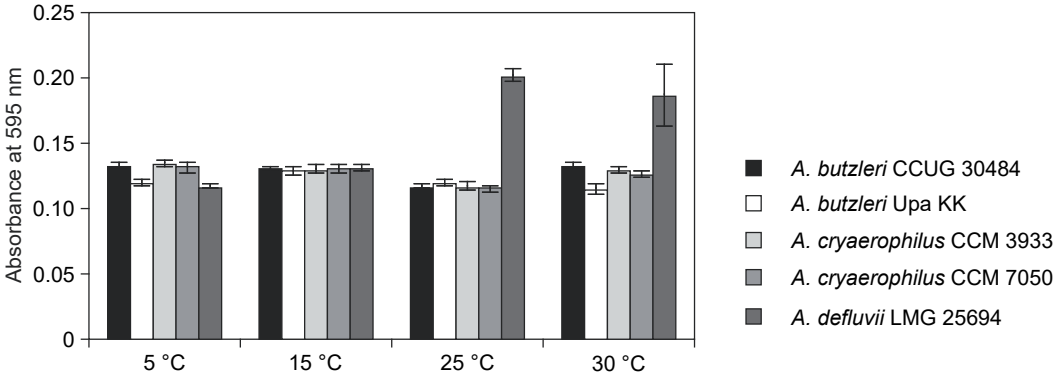

Fig. 1. Biofilm formation of *Arcobacter* strains at various temperatures.
The results are expressed as median ± standard deviation (n = 8).

temperatures, the biofilm-forming activity was weak ($0.112 < A \leq 0.120$). *A. cryaerophilus* strains included in this study exhibited similar biofilm activity under all experimental conditions.

CONCLUSION

Arcobacter spp. have been associated with cases of human illness and are widely distributed in water, animals and foods of animal origin. This study provides an overview of the behaviour of selected *Arcobacter* strains (traditionally known and recently described strains) at various process temperatures. The results of the study show that arcobacters are able to survive at various technological temperatures during a significantly long time. Lethal temperatures varied significantly, depending on the conditions of the experiment. The feared property of bacteria is their ability to produce biofilm. The results of this study showed significant biofilm formation at all experimental temperatures.

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