Effect of pH and Water Activity on the Growth of *Arcobacter* sp. in Culture

Libor ČERVENKA[1], Iveta ZACHOVÁ[1], Pavlína MINAŘÍKOVÁ[1] and Jarmila VYTŘASOVÁ[2]

[1]Department of Analytical Chemistry and [2]Department of Biological and Biochemical Sciences, Faculty of Chemical Technology, University of Pardubice, Pardubice, Czech Republic

Abstract

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The effect was studied of pH value and water activity (a_w) on the growth of *Arcobacter butzleri* and *Arcobacter cryaerophilus* in culture media at 30°C. Various weak organic acids were used to achieve target pH, and different humectants were used to control a_w. Generally, the growth of arcobacters was inhibited at medium pH. Compared to propionic, lactic, malic and ascorbic acids (pH 5.5–5.0), formic, citric and tartaric acids in the pH range of 6.0–5.5 were more inhibitory to both arcobacter species. Both arcobacter strains were extremely sensitive to broth environment with a_w values of < 0.980 using NaCl, glycerol and sucrose as humectants. This sensitivity to a_w and pH may well be an important constraint for the distribution and survival of *Arcobacter* sp. in the environment, particularly in foods and food products.

Keywords: *Arcobacter* sp.; weak organic acids; water activity

The genus *Arcobacter* is a member of the family *Campylobacteriaceae* and was classified by Vandamme *et al*. (1991) as "aerotolerant campylobacter" which was differentiated from *Campylobacter* sp. by the ability to grow at 25°C in the presence of oxygen. There exist four species: *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. nitrofigilis*. The first two species were found to be associated with human illness (Hsueh *et al*. 1997; Yan *et al*. 2000). Although *Arcobacter* sp. do not seem to play a major role in human foodborne diseases presently, they can be ranked to the group of "emerging foodborne pathogen". As more sophisticated detection, isolation, and identification techniques have been developed, more items of information are gathered on their epidemiology and their sources in the environment and in food (Phillips 2001). Therefore, *Arcobacter butzleri* and *A. cryaerophilus* have been recognised as widespread species around the world. *Arcobacter* sp. was recently isolated from cattle and swine faeces as well as from cloacal chicken swabs in Japan (Kabeya *et al*. 2003), from beef and dairy cattle in Texas (Golla *et al*. 2002), from raw ground pork collected from slaughterhouse facilities across the United States (Ohlendorf & Murano 2002), from broiler flocks slaughtered in poultry slaughterhouses in Belgium (Houf *et al*. 2003), and also from chicken carcasses sold in retail markets in the Czech Republic (Vytřasová *et al*. 2003) and in Turkey (Atabay *et al*. 2003).

Although there are many already existing as well as newly designed treatments for successful elimination of *Campylobacter* sp., these may not be equally effective for the inactivation or removal of *Arcobacter* sp., even though these microorganisms are closely related. In foods liable to contamination by *Arcobacter* sp. such as pork and beef, decontamination with organic acids was demonstrated to be effective in reducing contamination by other pathogens (Smudlers 1995). However, although

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closely related to *Campylobacter*, *Arcobacter* does not necessarily respond to the potential control treatments in the same way as *Campylobacter* sp. For example, *A. butzleri* is more tolerant to irradiation treatment than *C. jejuni* (Collins *et al*. 1996). Thus, each treatment regime should be evaluated for *Arcobacter* sp. rather than assumptions being made from *C. jejuni* studies. It was known that continuous exposure to sodium lactate in pure culture was not effective against *A. butzleri* while citric and lactic acids eliminated its growth (Phillips 1999). Short-term treatments with both trisodium phosphate and EDTA (alone and in combination with nisin) were effective in reducing the viable count of *A. butzleri* in pure culture (Phillips & Duggan 2001). Decreasing temperature appeared to have an effect in reducing the growth of *A. butzleri* but it was still able to survive in culture (Phillips & Duggan 2002). Further, *A. butzleri* was also recovered from frozen chicken carcasses (Atabay *et al.* 2003) and a negative effect of meat fat level on the prevalence of *Arcobacter* sp. was determined (Ohlendorf & Murano 2002). Arcobacter strains grow at pH values between 5.5–9.5, the majority of strains growing between pH 6.8–8.0 (Hilton *et al.* 2001). However, the growth or survival of microorganisms depend not only on pH values but also on the acidulat used, particularly on the undissociated acid concentration (Östling & Lindgren 1993; Conner & Kotrola 1995; El-Ziney *et al.* 1997). The minimal water activity for the growth of *Arcobacter* sp. was not determined so far but the ability to grow on media containing various amounts of sodium chloride was reported (Atabay *et al.* 1998; Maugeri *et al.* 2000).

In this paper, the effect of pH values adjusted by the addition of weak organic acids, and that of water activity adjusted by the addition of humectants on the survival of *Arcobacter butzleri* and *Arcobacter cryaerophilus* were studied.

MATERIALS AND METHODS

Bacterial strains. *Arcobacter butzleri* CCUG 30484 (Culture collection, University of Göteborg, Sweden) and *Arcobacter cryaerophilus* CCM 3934 (Czech Collection of Microorganisms, Masaryk University, Czech Republic) were used in this study. The stock culture was maintained on CASO agar (Merck, Darmstadt, Germany) slopes at 4°C and re-cultured every 14 days at 30°C. To prepare inocula for the test media, the cultures were activated by transfer into brain heart infusion broth (BHI, HiMedia Lab. Bombay, India) at 30°C for 48 h.

pH experiment. An appropriate amount of formic, citric, tartaric (Lachema. Ltd., Brno), propionic (Aldrich, Steinheim, Switzerland), DL-lactic (Fluka, Steinheim, Switzerland), DL-malic (Merck, Darmstadt, Germany), or ascorbic acid (Farmacon, Olomouc, Czech Republic) was added into test tubes containing 5 ml of BHI broth to reach target pH of 6.5, 6.0, 5.5, 5.0, and 4.5. BHI broth without any acid addition (pH 7.4) served as the control. pH values were measured by pH meter (Cyberscan 510, Singapore) before autoclaving. Dilution of 24-h culture of *Arcobacter butzleri* CCUG 30484 or *Arcobacter cryaerophilus* CCM 3934 with physiological solution was carried out using 0.5 McFarland turbidimetric standard (1×10^8 CFU/ml), then 100 µl was added to reach approximately 2×10^6 CFU/ml in each test tube. The sets of acidified BHI broth were incubated at 30°C for 48 h after which the turbidity of the broth was measured by visual observation. Obvious turbidity of broths occurring within 2-day period was recorded as positive growth. After 48 h, all remaining test tubes with no sign of turbidity were examined for populations of viable cells by surface streaking of 10 µl using calibrated bacteriological loop (Decon Laboratories, Inc., USA) on CASO agar in duplicates. Tubes were recorded as negative if no viable cell population was detected after incubation at 30°C for 48 h.

The undissociated concentrations of weak acids were calculated from the following expression:

For a monobasic acid:

$$[AH] = \frac{Ca\,[H^+]}{[H^+] + K_1} \quad (1)$$

For a dibasic acid:

$$[AH_2] = \frac{Ca\,[H^+]^2}{[H^+]^2 + [H^+] + K_1 + K_1K_2} \quad (2)$$

For a tribasic acid:

$$[AH_3] = \frac{Ca\,[H^+]^3}{[H^+]^3 + [H^+]^2K_1 + [H^+]K_1K_2 + K_1K_2K_3} \quad (3)$$

where $[AH]$, K_1; $[AH_2]$, K_2; $[AH_3]$, K_3 are the undissociated concentrations and equilibrium constants of monobasic, dibasic, and tribasic acids, and Ca is the total acid concentration.

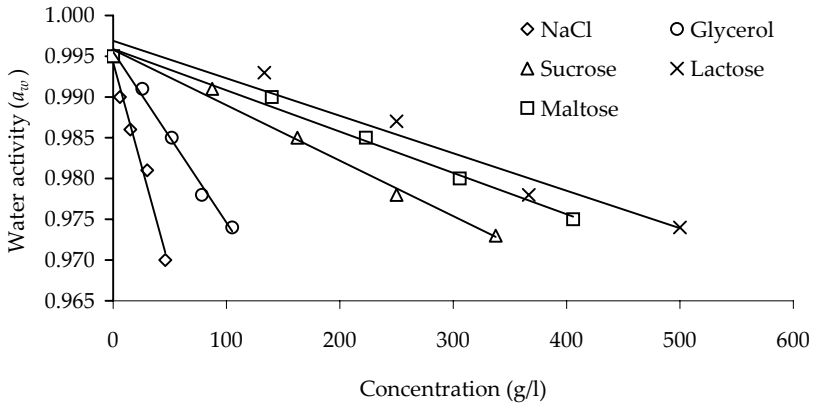

Figure 1. Influence of humectants on water activity of BHI agar at 25°C before incubation

Water activity experiment. BHI agar (HiMedia Lab. Bombay, India) was adjusted to water activity values ranging from 0.995 to 0.970 using various concentrations of NaCl, glycerol, sucrose, lactose, or maltose, respectively. The amounts of humectants required to obtain the desired a_w values are shown in Figure 1. Water activity value of 0.995 represented BHI agar in its basic formulation (5 g/l NaCl). The water activity of BHI agar was determined at 25°C using Thermoconstanter A_w Sprint Novasina TH 500 (Axair Ltd., Pfäffikon, Switzerland) before incubation. The mean values were determined from six analyses of each test medium. Loopfulls of *Arcobacter butzleri* CCUG 30484 or *Arcobacter cryaerophilus* CCM 3934 fresh culture were inoculated onto the surface of BHI agar (in duplicates) adjusted to different water activity values after which the colony formation was observed after 48 h incubation at 30°C. The plates were recorded as negative if no colony formation occurred. Experiments were repeated in two or three separate trials.

RESULTS AND DISCUSSION

The results of *Arcobacter* sp. growth tests under various pH conditions are summarised in Table 1. *Arcobacter butzleri* grows at pH 5.5 but no viable cells were detected in BHI broth acidified to pH 5.0 with ascorbic, malic, lactic, or propionic acid after 48 incubation at 30°C. Formic, citric and tartaric acids were more inhibitory towards *A. butzleri* with no growth observed at pH < 5.5 whereas at pH 6.0 the growth was evident. *Arcobacter cryaerophilus* was

Table 1. Growth of *Arcobacter* sp. in BHI broth adjusted to selected pH using weak organic acids after 48 h incubation at 30°C

Strain	pH	Growth in BHI broth with given acid						
		formic	propionic	lactic	ascorbic	malic	tartaric	citric
A. butzleri CCUG 30484	4.5	–	–	–	–	–	–	–
	5.0	–	–	–	–	–	–	–
	5.5	–	+	+	+	+	–	–
	6.0	+	+	+	+	+	+	+
	6.5	+	+	+	+	+	+	+
A. cryaerophilus CCM 3934	4.5	–	–	–	–	–	–	–
	5.0	–	–	–	–	–	–	–
	5.5	–	–	–	–	–	–	–
	6.0	–	+	+	+	+	–	–
	6.5	+	+	+	+	+	+	+

(+) growth was interpreted as development of turbidity after 48 h incubation at 30°C

more susceptible to all acids tested in comparison with the growth of *A. butzleri*. Formic, citric, and tartaric acids ihibited the growth at higher pH (6.0) while other acidulants allowed the growth of *A. cryaerophilus* in BHI broth at this pH. Acetic acid, a commonly used acidulant, is not included in this study, however, it was found to depress the growth of *A. butzleri* at pH 5.9 (data not shown). It is evident, in general, from the results of the present study that the strains of arcobacters tested were mildly acid-tolerant microorganisms capable to grow at pH > 5.5, as concluded by Hilton *et al.* (2001) using inorganic acid. A similar sensitivity of *Campylobacter jejuni* to a moderately acidic environment was determined in pasteurised milk whose pH had been adjusted with lactic or propionic acids to pH value in the range of 4.2–5.3 (Čuk *et al.* 1987). Citric acid inhibited the growth of *Arcobacter butzleri* in Arcobacter selective broth acidified to pH 4.5 with no viable counts detected after 6 h incubation at 30°C while viable cells were found at low pH values using lactic acid as preservative (Phillips 1999). This finding was supported by our results where pH adjusted by the addition of citric acid was found to be more inhibitory for both arcobacters than the same pH adjusted by lactic acid. Because of differences in the dissociation properties of the acids tested, different amounts of acids were required to achieve the target pH values at which the growth was inhibited. Thus, the concentrations at the respective pH values were calculated and found to differ between the acids tested (Table 2). On this basis, the tested concentrations of citric (7.9–10.2 mmol/l), tartaric (11.8–20.8 mmol/l), and malic (22.0–29.7 mmol/l) acids were the lowest observed, while the concentrations of formic (54.6–73.3 mmol/l) and ascorbic (43.7–53.4 mmol/l) acids were the highest. Weak organic acids have optimal inhibitory activity at low pH values because these favour the undissociated state of the molecule which is mainly responsible for the acid antimicrobial activity (Stradford 2000). At the limited pH values obtained in this study, the concentrations of undissociated acids were calculated using equations (1)–(3). In the pH range of 6.0–5.5, citric acid (4.5×10^{-4}–6.4×10^{-4} mmol/l) and tartaric acid (2.6×10^{-4}–4.4×10^{-4}) should be considered as the most effective due to the lowest concentration of undissociated acid used to achieve the target pH in comparsion to formic acid. Among acids which inhibited the growth of arcobacters in a lower pH range (5.5–5.0), the concentration of undissociated malic acid of 7.3×10^{-2}–0.58 mmol/l appeared to be the most effective. At concentrations below 1 mmol/l, ascorbic acid stimulated the growth of *Campylobacter jejuni* in nutrient broth whereas at 5 mmol/l the culture of *C. jejuni* (10^4 cells/ml) was destroyed (Juven & Kanner 1986). Similar results were obtained in our work in which undissociated ascorbic acid in concentrations of > 4.9 mmol/l (pH < 5.0) inhibited the growth of *A. butzleri* and *A. cryaerophilus* after 48 h incubation at 30°C. *Arcobacter cryaerophilus* was found in the current study to be more susceptible to the decrease of pH values, regardless of the acidulant used. This may partly explain its lower occurrence in meat in which pH can drop from the initial value of around 7.0 to 5.4–5.5 (Adams & Moss 1995), as compared to a higher prevalence of *A. butzleri* (Kabeya *et al.* 2003; Houf *et al.* 2003). Overall, *A. butzleri* and *A. cryaerophilus* appear to be medium acid-tolerant, similarly to campylobacters.

Table 2. Inhibited pH ranges and concentrations of acids tested in BHI broth for *Arcobacter* sp. growth

Acid		Inhibition pH range	Total acid concentration (mmol/l)	Undissociated acid concentration (mmol/l)
Formic	$pK_1 = 3.75$	6.0–5.5	54.6–73.3	0.3–1.3
Tartaric	$pK_1 = 2.98$, $pK_2 = 4.37$	6.0–5.5	11.8–20.8	2.6×10^{-4}–4.4×10^{-4}
Citric	$pK_1 = 3.14$, $pK_2 = 4.77$, $pK_3 = 6.39$	6.0–5.5	7.9–10.2	4.5×10^{-4}–6.4×10^{-4}
Propionic	$pK_1 = 4.88$	5.5–5.0	27.4–41.8	5.3–18.0
Lactic	$pK_1 = 3.86$	5.5–5.0	29.5–43.9	0.7–3.0
Ascorbic	$pK_1 = 4.0$, $pK_2 = 11.79$	5.5–5.0	43.7–53.4	1.3–4.9
Malic	$pK_1 = 3.46$, $pK_2 = 5.21$	5.5–5.0	22.0–29.7	7.3×10^{-2}–0.58

Table 3. Water activity requirements for the growth of *Arcobacter* sp. on BHI agar

Solute	a_w	*A. butzleri* CCUG 30484	*A. cryaerophilus* CCM 3934
NaCl	0.995	+	+
	0.990	+	+
	0.986	+	–
	0.981	–	–
	0.970	–	–
Glycerol	0.995	+	+
	0.991	+	+
	0.985	+	+
	0.979	–	–
	0.974	–	–
Sucrose	0.995	+	+
	0.991	+	+
	0.985	+	+
	0.978	–	–
	0.973	–	–

(+) growth was interpreted as colony forming units observed on BHI agar after 48 h incubation at 30°C

It was reported by Atabay *et al.* (1998) that in media containing 2%, 3.5% and 4.0% NaCl, 100%, 70% and 15% of *A. butzleri* isolates of different sources couli grow, while the growth of *A. cryaerophilus* isolates was observed only in media containing 2% NaCl. On the contrary, none of 49 *A. butzleri* chicken isolates was able to grow in the presence of 4% NaCl (Atabay *et al.* 2003). Additionally, 2.0%, 3.5%, and 4.0% NaCl solutions corresponded with water activity values of 0.989, 0.980 and 0.977, respectively (Resnik & Chirife 1988). In this study, it was confirmed that both *A. butzleri* and *A. cryaerophilus* were sensitive to the decrease of the water activity with no colony formation detected on agar plates at a_w < 0.980 adjusted with NaCl as well as with glycerol or sucrose (Table 3). The solute used to control a_w can influence the growth pattern of the microorganisms. NaCl and sucrose generally behave similarly, while glycerol, because it freely enters the bacterial cells, does not appear to osmotically stress the cell as most other solutes do (Chirife & del Pilar Buera 1996). Therefore, bacteria can grow at lower water activity values in the presence of glycerol in comparison to other solutes which was also confirmed in our recent study (Červenka *et al.* 2002). A similar a_w requirement for *C. jejuni* growth in BHI broth was determined (0.970–0.980 a_w) using sodium chloride as humectant, whereas in the presence of glycerol the growth was observed at 0.967 a_w (Uradzinski 1988). In the present work, glycerol, sodium chloride, and sucrose had the same effect on arcobacter growth except for *A. cryaerophilus* which appeared to be more sensitive to low water activity controlled by NaCl than by glycerol. This suggestion is supported by the work of Atabay *et al.* (1998) who found *A. cryaerophilus* more sensitive to sodium chloride content in culture medium. This finding is also in agreement with Marshall *et al.* (1971) who found sodium chloride, at similar levels of a_w, more inhibitory than glycerol to salt sensitive species. In addition, two non-ionic humectants, lactose and maltose, did not allow the growth of both *A. butzleri* and *A. cryaerophilus* on the surface of BHI agar plates adjusted to a_w < 0.990, i.e. the growth was observed only in BHI agar without any addition of these sugars (data not shown). The reason why arcobacter did not grow on BHI agar adjusted to low a_w with lactose and maltose contrary to the adjustment to the same water activity values with sucrose, remaines unclear.

In conclusion, it appears that both *A. butzleri* and *A. cryaerophilus* strains are extremely sensitive to the environment with a_w values of < 0.980 and with pH values higher than 5.0–5.5. It is also well established for many bacteria, including *Escherichia coli*, *Salmonella typhimurium*, *Listeria monocytogenes* and *Lactobacillus* sp., that the survival at low pH is enhanced by prior exposure to mildly acidic pH (Stradford 2000). Foodborne bacteria also possess specific ability to respond to either to the loss or to the gain of water which enhances their survival, particularly in food with low a_w (O'Byrne & Booth 2002). It was described by Murphy *et al.* (2003) that *C. jejuni* induced adaptive tolerance response enhancing its survival under acidic and aerobic stress. However, comparative *Arcobacter* sp. studies have not been yet published. The sensitivity to a_w and pH may well be an important constraint for the distribution and survival of *Arcobacter* sp. in the environment, particularly in food and food products.

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Souhrn

Červenka L., Zachová I., Minaříková P., Vytřasová J. (2003): **Vliv pH a aktivity vody na růst** *Arcobacter* sp. **v laboratorním médiu**. Czech J. Food Sci., **21**: 203–209.

Stanovovali jsme vliv pH a aktivity vody na růst *Arcobacter butzzeri* a *Arcobacter cryaerophilus* v laboratorním médiu při 30 °C. pH média bylo upraveno přídavky různých slabých organických kyselin a a_w byla snížena přídavky různých osmotolerantních látek. Růst arkobakterů byl inhibován v slabě kyselém prostředí. *A. cryaerophilus* byl citlivější ke snížené hodnotě pH ve srovnání s *A. butzleri*, a to bez ohledu na použitou kyselinu. Přídavky kyseliny mravenčí, citronové a vinné inhibovaly růst arkobakterů již při pH 6,0–5,5, zatímco kyselina mléčná, propionová, jablečná a askorbová při nižším pH (5,5–5,0). Oba testované druhy arkobakterů byly schopny růst v prostředí s hodnotou a_w < 0,980, která byla ovlivněna přídavky NaCl, glycerolu a sacharosy. Citlivost k nízkým hodnotám a_w a pH představuje významný faktor k omezení výskytu a růstu patogenní bakterie *Arcobacter* sp. v prostředí, zejména v mase a masných výrobcích.

Klíčová slova: *Arcobacter* sp.; slabé organické kyseliny; aktivita vody

Corresponding author:

Ing. Libor Červenka, Ph.D., Univerzita Pardubice, Fakulta chemicko-technologická, katedra analytické chemie, Štrossova 239, 530 03 Pardubice, Česká republika
tel.: + 420 466 037 111, fax: + 420 466 037 068, e-mail: libor.cervenka@upce.cz