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ORIGINAL ARTICLE

Adaptation of *Campylobacter* field isolates to propionic acid and sorbic acid is associated with fitness costs

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Abstract

Aims: To reduce the burden of *Campylobacter* at different stages of the food chain, recent studies have shown the effectiveness of organic acids as a risk mitigation strategy. However, very little is known about possible adaptation responses of *Campylobacter* that lead to reduced susceptibility to organic acids. Here we investigated the adaptive responses of *Campylobacter* field isolates to organic acids and estimated the fitness costs.

Methods and Results: Exposure of two *Campylobacter jejuni* and one *Campylobacter coli* isolate to subinhibitory concentrations of propionic acid or sorbic acid resulted in twofold to fourfold increased minimal inhibitory concentration values for the adapted variants. With one exception, the decreased susceptibility was stable in at least 10 successive subcultures without selection pressure. Growth competition experiments revealed a reduced fitness of adapted variants compared to the wild-type isolates. A linear regression model allowed an estimation of the fitness cost. Growth kinetics experiments showed significantly prolonged lag phases in five of six adapted isolates while there was not a direct correlation in the maximum growth rates compared to the wild-type isolates.

Conclusions: The results of the study showed that a stepwise adaptation of *Campylobacter* to organic acids is possible, but at the detriment of changes in growth behaviour and reduced fitness.

Significance and Impact of the Study: The study contributes to the understanding of adaptive responses of *Campylobacter* to organic acids treatments, for example, as part of risk mitigation strategies.

Introduction

Campylobacteriosis continues to be the most frequently reported zoonotic gastrointestinal infection in humans in the European Union and the number of cases has even increased in the last 5 years (EFSA and ECDC 2019). Of the cases where species differentiation was carried out, 83·9% were caused by the species *Campylobacter jejuni* and 10·3% by *Campylobacter coli*. The most common sources of food-borne campylobacteriosis outbreaks over the last years have been broiler meat and milk (EFSA and ECDC 2019). It is estimated that the handling, preparation and consumption of poultry meat account for 20–30% of human infections with *Campylobacter*, while 50–80% can be attributed to the entire poultry reservoir including laying hens and broilers (EFSA 2011). The presence of *Campylobacter* as commensals in chicken intestines and the contamination of poultry carcasses during slaughter and processing are of significant importance for the infection route (Hue *et al.* 2010). As the pathogen cannot be easily eliminated from poultry flocks, combined intervention strategies targeting different stages of the food chain appear to be most promising to reduce the burden of food-borne illness (Klein *et al.* 2015; Alter

2017). Organic acids seem to be an interesting alternative. The use of organic acids offers the advantage of a wide range of applications, as various studies have shown that organic acids are effective in reducing the concentration of *Campylobacter* at pre-harvest (Solis de Los Santos *et al.* 2008; Skånseng *et al.* 2010; Guyard-Nicodème *et al.* 2016), at harvest (Rasschaert *et al.* 2013) and at post-harvest in the broiler meat production chain (Cosansu and Ayhan 2009; Birk *et al.* 2010). Despite extensive investigations into the antimicrobial activities of organic acids against *Campylobacter* and although the ability to develop increased tolerance to organic acids has been demonstrated in different bacterial species and yeasts (Jarboe *et al.* 2013; Mira and Teixeira 2013), very little is known about possible adaptive responses in *Campylobacter*. One *in vitro* study indicated that *C. jejuni* may be able to develop increased tolerance to organic acids because the number of *C. jejuni* cells initially increased during treatment with fumaric acid or benzoic acid *in vitro* (Molatová *et al.* 2010). The proposed reason for this finding was that those cells might have acquired partial tolerance to the organic acids (Molatová *et al.* 2010). However, no further investigation of this observation was conducted.

Therefore, the aim of the present study was to investigate the development and stability of an adaptive response of *C. jejuni* and *C. coli* field isolates to two organic acids, propionic acid and sorbic acid. Subsequently, the fitness of the adapted variants was investigated in single culture growth kinetics experiments as well as in direct growth competition experiments, co-cultured with their wild-type isolates. A linear regression model was used to estimate the fitness costs of adapted variants in growth competition experiments.

Materials and methods

Bacterial isolates and growth conditions

For the experiments, two *C. jejuni* (Cj5, Cj18) and one *C. coli* field isolate (Cc7) were used. The isolates were selected based on the results of previous *in vitro* susceptibility testing of 20 *C. jejuni* and 10 *C. coli* isolates against propionic acid and sorbic acid (Peh *et al.* 2020). Two isolates with relatively low minimal inhibitory concentration (MIC) values of 2 mmol l^{-1} for sorbic acid and 32 mmol l^{-1} for propionic acid (isolates Cj5 and Cj18) and one isolate with relatively high MIC values of 4 mmol l^{-1} for sorbic acid and 64 mmol l^{-1} for propionic acid (isolate Cc7) compared to the other isolates were selected (Peh *et al.* 2020). The isolates were chosen from a panel of *Campylobacter* isolates which had been collected from poultry farms in January 2018 on the basis of epidemiological unrelatedness determined by macrorestriction analysis (unpublished data). Species identification was performed by MALDI-TOF mass spectrometry (Bruker Daltoniks GmbH, Bremen, Germany). Isolates were kept in cryotubes (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) at −80°C until use. Bacterial cultures were resuscitated by plating them onto Columbia agar supplemented with sheep blood (Oxoid Deutschland GmbH, Wesel, Germany) and were then incubated at 42 ± 1°C for 48 h under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2) in an incubator.

Campylobacter adaptation experiments

Campylobacter isolates were adapted to sorbic acid or propionic acid by successive subcultures on agar plates containing increasing levels of the respective organic acid. Stock solutions were prepared for propionic acid (3000 mmol l^{-1}) and sorbic acid (1000 mmol l^{-1}) in cation adjusted Mueller-Hinton broth (CAMHB) and adjusted to pH 7·3 using 2 and 8 mol l^{-1} sodium hydroxide. The agar plates were prepared so that they contained the maximal concentrations of organic acids at which growth of the respective isolate was not adversely affected. This resulted in the following initial concentrations: 2·5 mmol l^{-1} sorbic acid and 62 mmol l^{-1} propionic acid were used for isolate Cj18, 2·5 mmol l^{-1} sorbic acid and 58 mmol l^{-1} propionic acid for the isolate Cj5, and 4 mmol l^{-1} and sorbic acid and 66 mmol l^{-1} propionic acid for isolate Cc7. After incubation for 24–48 h at 42 ± 1°C under microaerobic conditions, subcultures were transferred to Mueller-Hinton (MH) agar plates containing increasing concentrations of sorbic acid (concentration steps of 0·5–2 mmol l^{-1}) or propionic acid (concentration steps of 2 mmol l^{-1}). This procedure was repeated 14–51 times until the achieved concentration of the organic acid completely inhibited the growth of the respective *Campylobacter* isolate.

Susceptibility testing of *Campylobacter* isolates to propionic acid or sorbic acid

The MIC values of the *Campylobacter* isolates were determined prior to and after adaptation to organic acids by the broth microdilution method (Peh *et al.* 2020). Procedures regarding growth medium, inoculum density, incubation time and conditions were performed in accordance with the recommendations given in the Clinical and Laboratory Standards Institute (CLSI) document VET01-A4 (CLSI 2013). Briefly, stock solutions of the organic acids were prepared in CAMHB and adjusted to pH 7·3 with 2 and 8 mol l^{-1} sodium hydroxide using a pH meter (Mettler Toledo AG, Schwerzenbach, Switzerland). Twofold serial dilutions of 0·5–512 mmol l^{-1} for

propionic acid (Carl Roth GmbH + Co. KG) and of 0·063–64 mmol l^{-1} for sorbic acid (Carl Roth GmbH + Co. KG) were prepared. A volume of 50 μl of each double-concentrated dilution was inoculated into the wells of U-shaped bottom 96-well plates (Sarstedt AG Co. KG, Nümbrecht, Germany). Bacterial colonies taken from overnight cultures were suspended in sodium chloride until a turbidity equivalent to a 0·5 McFarland standard was achieved. Then, the adjusted culture was diluted 1 : 100 in CAMHB (Carl Roth GmbH + Co.). A volume of 50 μl of this suspension was dispensed into each well containing 50 μl of the supplemented CAMHB to achieve a final bacterial concentration of 5×10^5 CFU per ml. The microtiter plates were incubated for 48 h at 42 ± 1°C in an incubator under microaerobic conditions. The MIC value was defined as the lowest concentration of organic acid that inhibited visible growth of the bacteria. MIC values were determined in triplicate.

Stability of the organic acid tolerant phenotype

The phenotypic stability of the decreased susceptibility to propionic acid or sorbic acid was tested in accordance with Rensch *et al.* (2013). For this, colonies from overnight cultures of the adapted *Campylobacter* isolates were subcultured daily for 10 successive days on MHA plates without supplementation with organic acids. Agar plates were incubated at 42 ± 1°C under microaerophilic conditions. MIC values were immediately determined after 10 subcultures using the broth microdilution method as described above. For isolates showing increased MIC values after 10 successive subcultures, the subculturing was repeated, and the MIC values were additionally determined after five, six, seven, eight and nine transfers, respectively. Experiments were repeated in triplicate.

Growth competition experiments in co-cultures and mathematical modelling

Growth competition experiments were performed as described by Rensch *et al.* (2015) with minor changes. For this purpose, the respective *Campylobacter* wild-type isolates were co-cultured together with their adapted variants. Briefly, the respective colonies taken from overnight cultures were adjusted to a McFarland standard of 0·5 in sodium chloride and diluted 1 : 10 in sodium chloride. For the co-cultivation of isolates, a volume of 100 μl of the diluted suspensions was inoculated into Erlenmeyer flasks containing 18·8 ml CAMHB supplemented with 1·0 ml foetal bovine serum to achieve a bacterial concentration of approximately 5×10^4 CFU per ml for each isolate. The cultures were incubated at 42 ± 1°C under microaerobic conditions on a shaking platform at 130 rev min^{-1}. Every 12 h (Cj5, Cc7) or every 24 h (Cj18), a volume of 100 μl was diluted 1 : 10 in saline solution and 200 μl of this dilution was transferred to a fresh flask containing 18·8 ml CAMBH and 1·0 ml foetal bovine serum to complete one cycle of competitive growth. Bacterial concentrations (CFU per ml) of the wild-type isolates and the adapted variants were determined every 24 h (isolates Cj5, C7) or every 48 h (isolate Cj18) due to the slower growth of the isolate. For that, 100 μl of tenfold serial dilutions was plated in duplicate on non-selective MHA plates and MHA plates supplemented with either 7 mmol l^{-1} (Cj5, Cj18) or 18 mmol l^{-1} (Cc7) sorbic acid or 90 mmol l^{-1} (Cj5, Cj18) and 115 mmol l^{-1} (Cc7) propionic acid, respectively. The bacterial counts of the wild-type isolates were calculated from the difference in colony counts between non-selective agar plates (growth of wild-type isolates and adapted variants) and agar plates supplemented with sorbic acid or propionic acid (only growth of adapted variants). Experiments were performed in triplicate.

Statistical analysis of the changes in log ratios of the wild-type isolates and their adapted variants was performed using a linear regression model in accordance with Rensch *et al.* (2015). Briefly, the $\log_{10}$ differences (*LD*) were calculated by subtracting the $\log_{10}$ concentrations of the adapted variants from the $\log_{10}$ concentrations of the respective wild-type isolates. The *LD* values were calculated for each incubation time of the three independent growth competition experiments conducted for each adapted variant. The observed *LD* values were fitted to a linear regression model with intercept fixed as zero to set the concentrations of the wild-type isolates and their adapted variants equal at time zero. The estimated regression parameter K_c can be considered as the difference between the growth rate constant of the wild-type isolate minus the growth rate constant of the adapted variant (Rensch *et al.* 2015). Furthermore, 95% uncertainty intervals were determined for the estimated parameter K_c, for the predicted time-dependent mean *LD* (referred to as 'confidence intervals') as well as for the predicted individual observations of *LD* for each *Campylobacter* isolate (referred as 'prediction intervals') (Rensch *et al.* 2015). The linear regression models were performed using the software R (R Core Team 2019).

Growth kinetics experiments of single cultures and statistical evaluation

Bacterial growth kinetics of the three wild-type isolates and the six adapted variants in single cultures was investigated via optical density (OD) absorbance measurements at 600 nm (OD_{600}) using a Tecan Spark automatic microplate reader (Tecan Austria GmbH, Grödig,

Austria) with an integrated Gas Control Module and F-shaped bottom 48-well plates (Sarstedt AG & Co. KG). Experiments were performed (i) in pure medium and (ii) in medium supplemented with subinhibitory concentrations (0·5 × MIC value of the wild-type isolate) of propionic acid or sorbic acid. The bacterial inocula were prepared in the same way as for the broth microdilution method described above. Wells of the microtiter plate were filled with 200 μl of the bacterial suspension and 200 μl aliquots of pure CAMHB or CAMHB supplemented with the respective organic acid (0·5 x MIC value of the wild-type isolates), reaching final bacterial concentrations of 5×10^5 CFU per ml. Plates were stirred and incubated for 37 h at 42 ± 0·5°C under microaerobic conditions (5% O_2, 10% CO_2). The measured absorbance was subtracted from the absorbance of the prepared plates prior to incubation. Experiments were performed in triplicate. Statistical analysis was performed using the software R. The maximum growth rates [μ_{max} (abs^{-1})] and the lag phases [t_{lag} (h)] were estimated using the Gompertz functions implemented in the R package growth-rates, available for download at Comprehensive R Archive Network (CRAN) (Petzold 2019). Statistical significance was evaluated using the non-parametric Mann–Whitney *U* test. Differences were considered significant when $P < 0{\cdot}05$.

Results

Increased tolerance of adapted variants to propionic acid or sorbic acid

After stepwise adaptation, the respective variants of the *C. jejuni* wild-type isolates Cj5 and Cj18 showed growth on MH agar plates supplemented with up to 12 mmol l^{-1} sorbic acid or 104 mmol l^{-1} propionic acid while the variants of the *C. coli* isolate Cc7 adapted to sorbic acid or propionic acid showed growth up to concentrations of 36 mmol l^{-1} sorbic acid or 148 mmol l^{-1} propionic acid, respectively.

Subsequent to the gradual adaptation, the MIC values were fourfold higher compared to the wild-type isolates both for the Cj18 and Cj5 variants adapted to sorbic acid (8 mmol l^{-1}) and propionic acid (128 mmol l^{-1}) (Table 1). The variant of Cc7 adapted to sorbic acid yielded a fourfold higher MIC value (16 mmol l^{-1}) than the wild-type isolate Cc7 while the MIC value of the Cc7 variant adapted to propionic acid (64 mmol l^{-1}) was twofold higher compared to the wild-type isolate. The changes in MIC values were confirmed in three independent replications and in comparison to the wild-type isolates.

Elevated MIC values of organic acids were stable in five of six adapted variants after 10 successive subcultures on MH agar plates without supplementation with organic acids. For the propionic acid-adapted variant of *C. jejuni* isolate Cj18, the MIC values were either equal or one dilution step lower after 6–10 subcultures compared to the MIC value of the adapted variant of Cj18 before subculturing in non-supplemented medium.

Adapted variants showed lower bacterial concentrations in growth competition experiments compared to the wild-type isolates

Parallel incubation of the wild-type isolate and its adapted variant in growth competition experiments without selection pressure showed a decrease in the proportion of adapted variants for all isolates used (Figs 1–3, A2 and B2). After an initial increase in bacterial counts, which was observed in five of six adapted *Campylobacter* variants, concentrations decreased gradually with successive competition cycles.

Table 1 Minimal inhibitory concentration (MIC) values and stability of increased MIC values of two *Campylobacter jejuni* variants and one *Campylobacter coli* adapted to sorbic acid or propionic acid in comparison to the wild-type isolates

Wild-type Isolate	Variant adapted to	Wild-type MIC (mmol l^{-1})	No. of subcultures to induce tolerance)*	Adapted variant MIC (mmol l^{-1})	Stability of tolerance (No. subcultures without decrease in MIC)†
C. jejuni Cj5	Sorbic acid	2	14	8	≥10
	Propionic acid	32	30	128	≥10
C. jejuni Cj18	Sorbic acid	2	14	8	≥10
	Propionic acid	32	30	128	5
C. coli Cc7	Sorbic acid	4	22	16	≥10
	Propionic acid	64	51	128	≥10

*Wild-type isolates were serially transferred on Mueller–Hinton (MH) agar plates supplemented with increasing concentrations of sorbic acid or propionic acid up to a concentration that completely inhibited growth.

†Stability of tolerance after serial subcultures in the absence of organic acids. MIC values were determined after ten subcultures. If MIC values decreased after 10 subcultures, MIC values were additionally determined after five, six, seven, eight and nine subcultures, respectively.

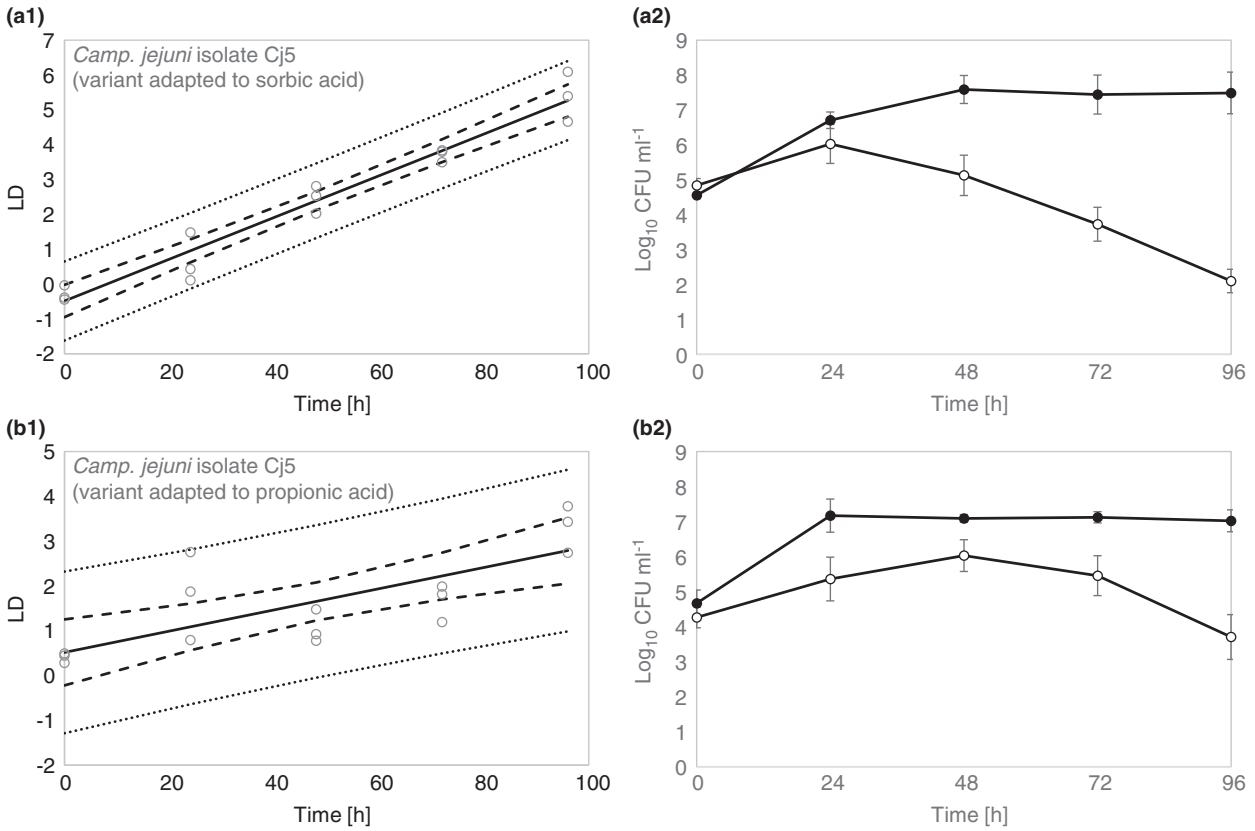

Figure 1 Log10 difference (*LD*) values between the *Camplyopacter jejuni* wild-type isolate Cj5 and its variants adapted to sorbic acid or propionic acid (log10 concentration of wild-type isolate minus log10 concentration of its variant adapted to sorbic acid or propionic acid) plotted against the incubation time (*t*) and fitted linear model (solid line) with 95% confidence intervals for expected *LD* values (dashed lines) and 95% prediction intervals (dotted lines) (A1, B1). Results of the *in vitro* growth competition experiments of the *C. jejuni* wild-type isolate Cj5 (●) and its variants adapted to sorbic acid (A2, ●) or propionic acid (B2, ○) in pure medium as determined by CFU per ml counting (A2, B2).

After 96 h of incubation (or 192 h in the case of the *C. jejuni* variant Cj18 adapted to propionic acid), bacterial concentrations of the adapted variants were 3·3–8·1 $\log_{10}$ CFU per ml lower than their wild-type isolates except for isolate Cj18 adapted to sorbic acid. For this variant, bacterial concentrations were below the detection limit after a 48-h incubation period (Fig. 2, A2).

The $\log_{10}$ differences (*LD*) increased linearly during the growth competition experiments for all tested *Campylobacter* isolates (Figs 1–3, A1 and B1). Therefore, a linear regression model was applied. As shown in Table 2, all estimated regression coefficients (K_c values) were significantly greater than zero and ranged between 0·024 for *C. jejuni* isolate Cj5 (variant adapted to propionic acid) and 0·082 for *C. coli* isolate Cc7 (variant adapted to sorbic acid). The linear regression models explained between 52·8% (*C. jejuni* wild-type isolate Cj5, variant adapted to propionic acid) and 95·3% (*C. coli* wild-type isolate Cc7, variant adapted to sorbic acid) of the variation in *LD* values.

Adapted variants showed increased lag phases during growth kinetics experiments in single cultures

When the isolates were cultured individually in CAMHB without supplementation with organic acids, five of six adapted variants showed significantly longer lag phases compared to their wild-type isolate (Table 3, Fig. 4a–c). The variants of isolate Cj5 adapted to sorbic acid (t_{lag} = 22·4 h) or adapted to propionic acid (t_{lag} = 18·5 h) revealed the most prolonged lag phases compared to their wild-type isolate Cj5 (t_{lag} = 12·4 h). In contrast, the lag phase of the variant of isolate Cj18 adapted to propionic acid did not differ significantly from that of the wild-type isolate. The maximum growth rates of the adapted variants did not show a uniform pattern. The values were significantly lower (adapted variants of isolate Cj5 and sorbic acid adapted variant of isolate Cj18), higher (adapted variants of isolate Cc7) or not significantly different (propionic acid adapted variant of isolate Cj18) from those of their wild-type isolates.

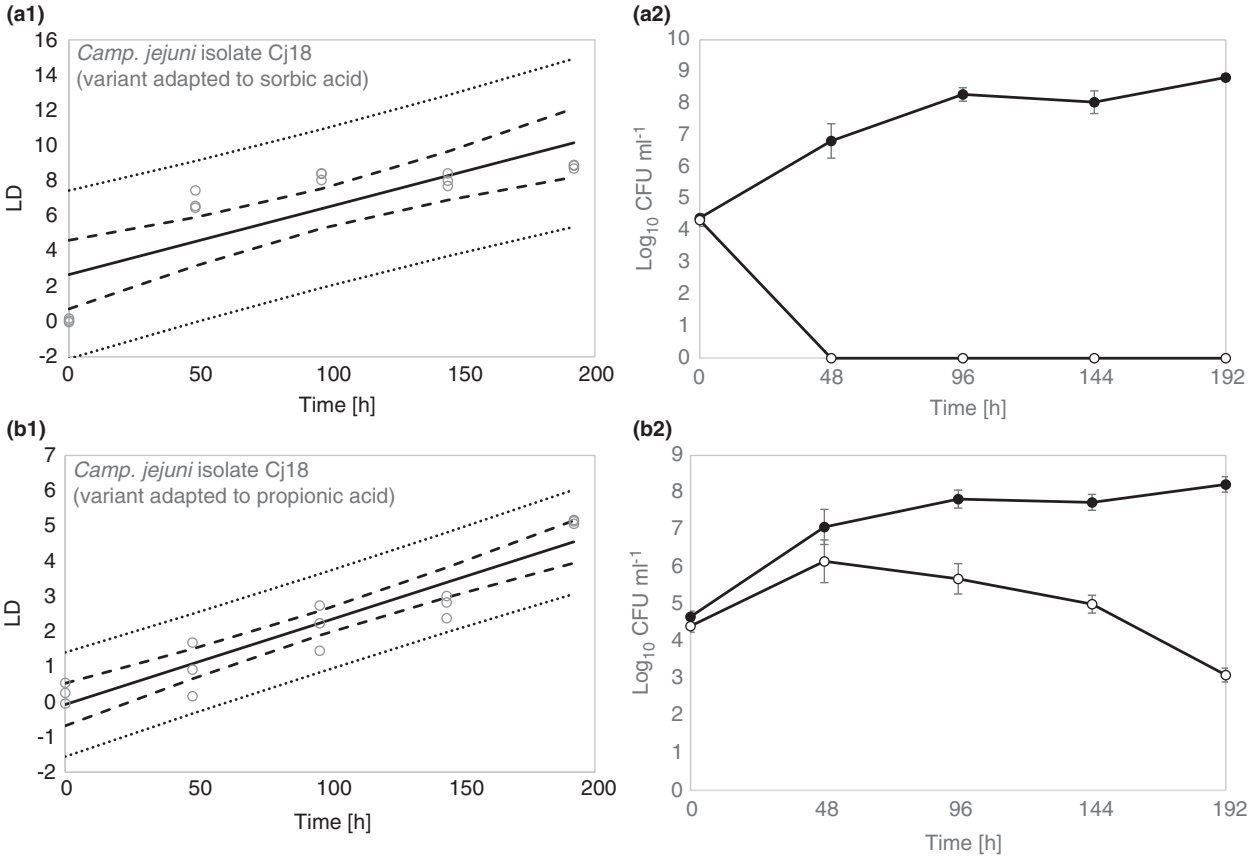

Figure 2 Log10 difference (*LD*) values between the *Campylobacter jejuni* wild-type isolate Cj18 and its variants adapted to sorbic acid or propionic acid plotted against the incubation time (*t*) and fitted linear model (solid line) with 95% confidence intervals for expected *LD* values (dashed lines) and 95% prediction intervals (dotted lines) (A1, B1). Results of the *in vitro* growth competition experiments of the *C. jejuni* wild-type isolate Cj18 (●) and its variants adapted to sorbic acid (A2, ●) or propionic acid (B2, ○) in pure medium as determined by CFU per ml counting (A2, B2).

In a further experimental step, the growth kinetics experiments were performed in the presence of 0·5× the MIC value of the organic acids. It was observed that lag phases were significantly longer for the variants of isolates Cc7, Cj5 and Cj18 adapted to sorbic acid and the variant of Cc7 adapted to propionic acid compared to their wild-type isolates. The lag phase of the variant of isolate Cj5 adapted to propionic acid did not differ significantly from that of the wild-type isolate. The variant of isolate Cj18 adapted to propionic acid showed a significantly lower lag phase (t_{lag} = 14·8 h) and a higher maximum growth rate (μ_{max} = 0·15 abs^{-1}) than its wild-type isolate (t_{lag} = 24·3 h, μ_{max} = 0·12 abs^{-1}). In the presence of organic acids, the maximum growth rates of adapted variants varied accordingly. The values were significantly higher (variants of isolates Cc7 and Cj18 adapted to propionic acid), significantly lower (variants of isolates Cj5 and Cj18 adapted to sorbic acid) or did not differ significantly (variant of isolate Cc7 adapted to sorbic acid and variant of isolate Cj5 adapted to propionic acid) from those of their wild-type isolates.

Discussion

The present study demonstrated for two *C. jejuni* field isolates and one *C. coli* field isolate that *Campylobacter* can adapt to increasing concentrations of sorbic acid or propionic acid, two organic acids that are considered as candidates for reducing *Campylobacter* colonization in poultry (Grilli *et al.* 2013). The growth behaviour of all isolates was investigated in growth kinetics experiments in single cultures and in direct growth competition experiments, co-cultured with their wild-type isolates. The results suggested that the enhanced tolerance of the isolates decreased in the absence of organic acids. This enhanced tolerance was also associated with fitness costs so that the isolates were significantly less able to compete with the wild-type isolates.

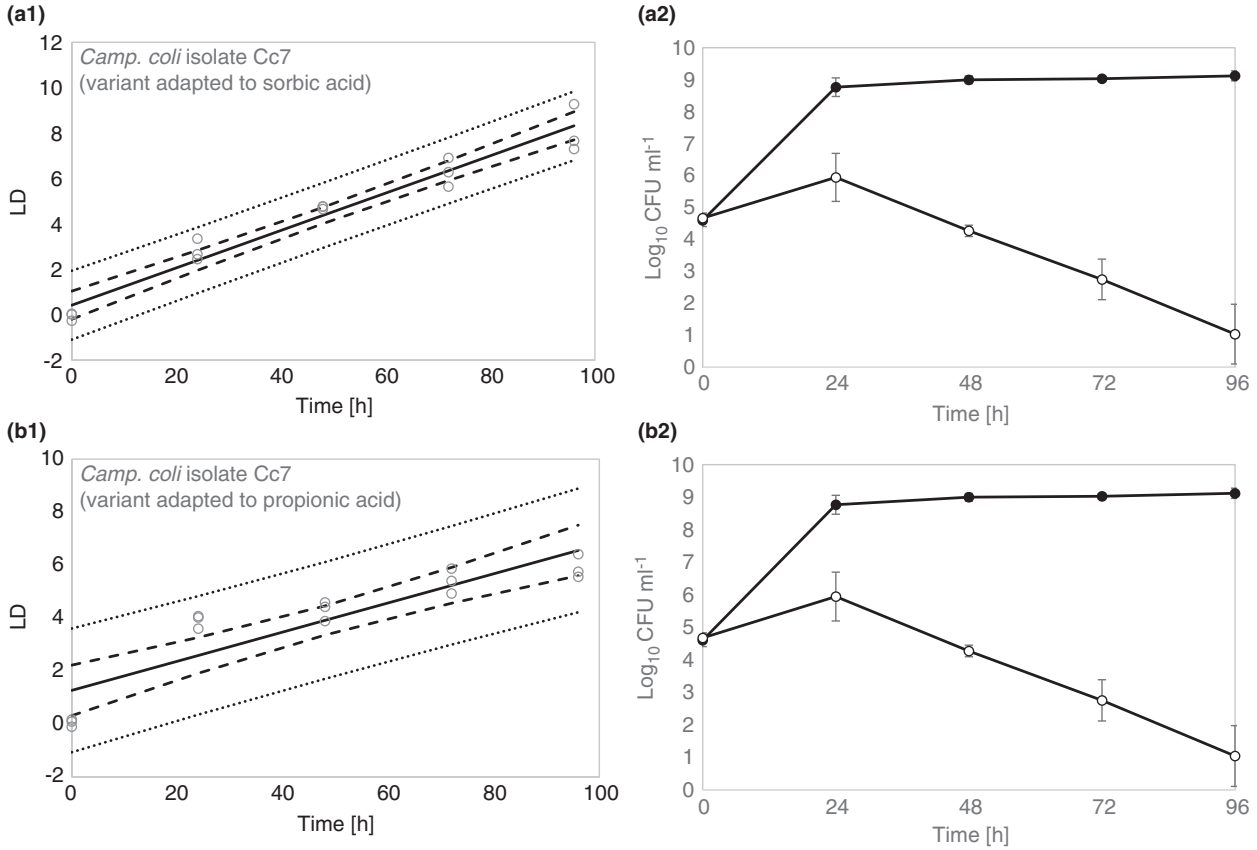

Figure 3 Log10 difference (*LD*) values between the *Campylobacter coli* wild-type isolate Cc7 and its variants adapted to sorbic acid or propionic acid (log10 concentration of wild-type isolate minus log10 concentration of its variant adapted to sorbic acid or propionic acid) plotted against the incubation time (*t*) and fitted linear model (solid line) with 95% confidence intervals for expected *LD* values (dashed lines) and 95% prediction intervals (dotted lines) (A1, B1). Results of the *in vitro* growth competition experiments of the *C. coli* wild-type isolate Cc7 (●) and its variants adapted to sorbic acid (A2, ●) or propionic acid (B2, ○) in pure medium as determined by CFU per ml counting (A2, B2).

Repeated exposure of two *C. jejuni* and one *C. coli* isolate to subinhibitory concentrations of propionic acid or sorbic acid, which were gradually increased, resulted in twofold to fourfold increased MIC values. Similarly, preexposure of different yeasts to benzoic acid at subinhibitory concentrations was shown to cause a 1·4- to 2·2-fold increase in MIC values (Warth 1988). Additionally, *Zygosaccharomyces bailii* grown in the presence of sorbic acid acquired resistance to sorbic acid (Warth 1977), whereas Moir and Eyles (1992) observed little or no adaptation of four different bacterial species (*Listeria monocytogenes*, *Pseudomonas putida*, *Yersinia enterocolitica*, *Aeromonas hydrophila*) when exposed to subinhibitory concentrations of methyl p-hydroxybenzoate or potassium sorbate. In the present study, the organic acid-tolerant phenotype was stable in five of six *Campylobacter* isolates after 10 subcultures in media without supplementation with organic acids. Similar results were observed in previous studies investigating the stability of induced tolerance in *Campylobacter* and *Salmonella enterica* to biocides (Mavri and Smole Mozina 2013; Rensch *et al.* 2013). In the case of antibiotics, stability of decreased susceptibility indicates that acquired resistance results from mutations or incorporation of genetic material (Fernandez and Hancock 2012). Accordingly, the observed stability of adaptation in five of six *Campylobacter* isolates might suggest that decreased susceptibilities resulted from genetic mutations. However, the number of subculture transfers might be insufficient to demonstrate stability of adaptation. Adaptive tolerance in *Pseudomonas aeruginosa* to different biocides was found to be lost after 20 subcultures (Méchin *et al.* 1999) while stability of macrolide resistance in *Campylobacter* was investigated for even 55 subcultures (Gibreel *et al.* 2005). In contrast, a temporary decrease in susceptibility generally refers to phenotypic adaptation due to alterations in gene and/or protein expression, for example caused by subinhibitory levels of antibiotics or biocides (Meyer and Cookson 2010; Fernandez and Hancock 2012). According to these definitions, the underlying mechanism of enhanced tolerance

Table 2 Results of linear regression models of the $\log_{10}$ differences (LD) between concentrations of the wild-type isolates and their variants adapted to sorbic acid or propionic acid as function of the incubation time

Wild-type Isolate	Variant adapted to	Coefficient K_c (standard error)	*P* value	Adjusted R^2	LD (mean values)	Time (h)
Campylobacter jejuni Cj5	Sorbic acid	0·060 (0·0036)	4·51e-10	0·950	−0·49	0
					0·95	24
					2·39	48
					3·83	72
					5·27	96
	Propionic acid	0·024 (0·0058)	1·57e-7	0·528	0·52	0
					1·08	24
					1·65	48
					2·22	72
					2·79	96
Campylobacter jejuni Cj18	Sorbic acid	0·078 (0·0154)	2·10e-4	0·639	2·66	0
					4·54	48
					6·41	96
					8·28	144
					10·15	192
	Propionic acid	0·048 (0·0048)	1·57e-7	0·878	−0·08	0
					1·08	48
					2·23	96
					3·39	144
					4·55	192
Campylobacter coli Cc7	Sorbic acid	0·082 (0·0048)	3·21e-10	0·953	0·42	0
					2·39	24
					4·37	48
					6·34	72
					8·32	96
	Propionic acid	0·055 (0·0075)	5·73e-6	0·790	1·27	0
					2·59	24
					3·91	48
					5·23	72
					6·55	96

in the *C. jejuni* isolate Cj18 adapted to propionic acid was more likely a phenotypic adaptation, as the decreased susceptibility was found to be unstable. Induction of genes related to efflux pumps or changes in the composition of the outer membrane has been frequently reported to cause enhanced tolerance to organic acids in different bacteria and yeasts (Simões *et al.* 2006; Beek *et al.* 2008; Mollapour and Piper 2012). Further research would be necessary to assess the underlying mechanisms of the decreased susceptibilities observed in the present study.

Growth kinetics of adapted variants were investigated both in single cultures and pairwise, co-cultured with their wild-type isolates in the same medium but in the absence of organic acids. The aim of the experiments was to estimate the competitive ability of adapted variants in a bacterial population. Results of the growth competition experiments were analysed using a linear regression model. The proportions of all adapted variants were shown to decrease over time, as indicated by K_c values greater than zero. One possible reason for the findings is that the tolerance-mediating adaptation was associated with a loss of bacterial fitness, resulting in wild-type isolates outcompeting their adapted variants as stated in previous studies (Zhang *et al.* 2006; Luangtongkum *et al.* 2012; Rensch *et al.* 2015). Mutations conferring resistance to antibiotics often impose fitness costs, as many antibiotics target important biological functions in the cell and resistance to them may either disrupt those processes or lead to increased energy consumption (Melnyk *et al.* 2015). Accordingly, enhanced tolerance to organic acids was observed to be energy-intensive in *Saccharomyces cerevisiae* (Holyoak *et al.* 1996; Bracey *et al.* 1998). As a result, resistance in bacteria is often associated with impairment of different growth phases such as the lag phase (Linkevicius *et al.* 2013; Lofton *et al.* 2014). Consistently, five of six adapted variants showed significantly prolonged lag phases during the growth kinetics experiments in single cultures in pure medium in the present study. The maximum growth rates of adapted variants varied, as the values were higher, similar or lower

Table 3 Growth kinetics parameters of the wild-type isolates and their adapted variants in single cultures estimated using the Gompertz function

Isolate	Supplementation of medium	Lag phase [t_{lag} (h)]	Maximum growth rates [μ_{max} (abs^{-1})]
Campylobacter jejuni Cj5			
Wild type	None	12·4a,b	0·17k,l
	Sorbic acid*	16·8c	0·16m
	Propionic acid†	18·7	0·14
Adapted to sorbic acid	None	22·4a	0·10k
	Sorbic acid	26·2c	0·11m
Adapted to propionic acid	None	18·5b	0·15l
	Propionic acid	19·5	0·14
Campylobacter jejuni Cj18			
Wild type	None	16·5d	0·14n
	Sorbic acid	18·8e	0·09o
	Propionic acid	24·3f	0·12p
Adapted to sorbic acid	None	19·5d	0·12n
	Sorbic acid	23·6e	0·09o
Adapted to propionic acid	None	17·4	0·14
	Propionic acid	14·8f	0·15p
Campylobacter coli Cc7			
Wild type	None	6·5g,h	0·11q,r
	Sorbic acid	8·9i	0·15
	Propionic acid	13·1j	0·12s
Adapted to sorbic acid	None	11·9g	0·14q
	Sorbic acid	12·8i	0·14
Adapted to propionic acid	None	8·7h	0·15r
	Propionic acid	14·9j	0·14s

"a–s" values differ significantly between the wild-type isolate and the respective adapted variant ($P < 0·05$).

*Supplemented with sorbic acid at a concentration of 0·5× the MIC value of the wild-type isolate.

†Supplemented with propionic acid at a concentration of 0·5× the MIC value of the wild-type isolate.

compared to their wild-type isolates in our study. Thus, the results of the present study suggest that the extended lag phase might have impacted the outcome of the growth competition experiments more than the individual maximum growth rates under given experimental conditions. This may have been influenced by the relatively short growth cycles used. In contrast, reduced growth rates were observed to be the main factor decreasing the fitness of resistant bacteria in previous studies (Han *et al.* 2009; Guo *et al.* 2012; Luangtongkum *et al.* 2012; Rensch *et al.* 2013). Interestingly, in the present study, the variant of isolate Cj18 adapted to propionic acid showed decreased proportions during the growth competition experiments, although the growth kinetics experiment in single culture revealed similar lag phases and significantly higher OD_{600} values compared to the wild-type isolate after a 37-h incubation period (Fig. 4b). A possible explanation for this finding might be that the enhanced tolerance decreased over time, as the MIC values of this adapted variant partially decreased after six successive transfers on MHA plates without supplementation with propionic acid. To verify this thesis, the stability of the tolerance in this adapted variant was further investigated in a similar manner to a previous study (Roch *et al.* 2017). Briefly, bacterial growth of isolate Cj18 adapted to propionic acid was monitored in accordance with the experimental design of the growth competition experiments, but without the addition of the wild-type isolate. After 144 and 192 h of incubation, bacterial counts on MHA plates supplemented with propionic acid were up to 4-log units lower than those on MHA plates without organic acid (Fig. S1). Thus, results suggest that reversion to a susceptible phenotype might have caused the decline in the concentration of the variant of isolate Cj18 adapted to propionic acid during growth competition experiments. Accordingly, apart from a decreased fitness, reversion of a resistant to a susceptible phenotype is another mechanism causing loss of resistance in a bacterial population (Andersson and Hughes 2011; Roch *et al.* 2017). Interestingly, even in the presence of subinhibitory concentrations of organic acids, four adapted variants showed significantly longer lag phases while there was no consistent trend regarding maximum growth rates compared to their wild-type isolates. As previously discussed, the lag phases might have a greater impact on the outcome of growth competition experiments than the maximum growth rates under the given experimental conditions. Thus, these results suggest for the four adapted variants that they might have been outcompeted by their wild-type isolates even under selective pressure at subinhibitory concentrations. In contrast, the variant of isolate Cj18 adapted to propionic acid showed both a significantly lower lag phase and a higher maximum growth rate than its wild-type isolate. However, as stated above, stability tests suggested that the enhanced tolerance of this adapted variant might be unstable in the absence of selective pressure. Consequently, the results of the growth kinetics experiments of the six adapted isolates in single cultures indicate that the rise of a stable organic acid-tolerant population is rather unlikely both at subinhibitory concentrations of organic acids and after removal of the selection pressure.

However, it should be noted that the effects of long-term treatment with organic acids on the emergence of an organic acid-tolerant *Campylobacter* population remain unclear, especially when administered at concentrations above the MIC values. For example, it has been shown for *S. enterica* that mutations conferring resistance to streptomycin differed after exposure to sub-MIC levels compared to treatment with lethal antibiotic concentrations (Wistrand-Yuen *et al.* 2018). The impact of

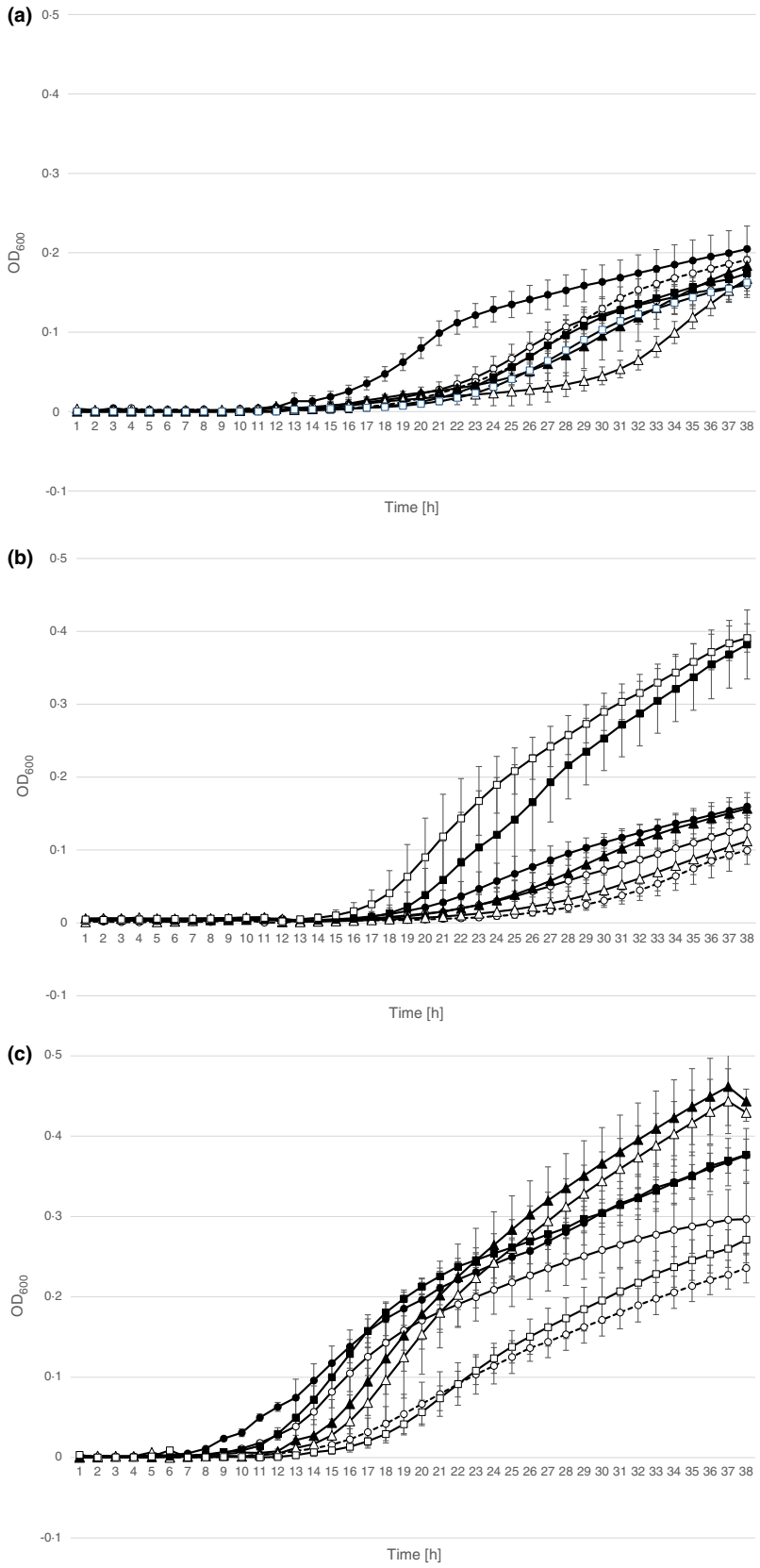

Figure 4 Growth curves of *Campylobacter jejuni* wild-type isolates Cj5 (a), Cj18 (b) and *C. coli* wild-type isolate Cc7 (c) and their variants adapted to sorbic acid or propionic acid in pure medium or at subinhibitory concentrations of organic acids (0·5 × MIC value of the wild-type isolate) determined by OD600 measurements. (a) Cj5 wild-type (●), Cj5 wild-type (0·5 × MIC sorbic acid) (○), Cj5 wild-type (0·5 × MIC propionic acid) (◇), Cj5 variant adapted to sorbic acid (▲), Cj5 variant adapted to sorbic acid (0·5 × MIC sorbic acid) (Δ), Cj5 variant adapted to propionic acid (■), Cj5 variant adapted to propionic acid (0·5 × MIC propionic acid) (□). (b) Cj18 wild-type (●), Cj18 wild-type (0·5 × MIC sorbic acid) (○), Cj18 wild-type (0·5 × MIC propionic acid) (◇), Cj18 variant adapted to sorbic acid (▲), Cj18 variant adapted to sorbic acid (0·5 × MIC sorbic acid) (Δ), Cj18 adapted to propionic acid (■), Cj18 adapted to propionic acid (0·5 × MIC propionic acid (□). (C): Cc7 wild-type (●), Cc7 wild-type (0·5 × MIC sorbic acid) (○), Cc7 wild-type (0·5 × MIC propionic acid) (◇), Cc7 variant adapted to sorbic acid (▲), Cc7 variant adapted to sorbic acid (0·5 × MIC sorbic acid) (Δ), Cc7 variant adapted to propionic acid (■), Cc7 variant adapted to propionic acid (0·5 × MIC propionic acid) (□). The values represent the means of at least three measurements, including the standard deviations. [Colour figure can be viewed at wileyonlinelibrary.com]

different resistance mutations on bacterial fitness was observed to vary widely depending on the particular mutation, as some mutations caused fitness costs, while others occasionally even lead to increased bacterial fitness (Komp Lindgren *et al.* 2005; Marcusson *et al.* 2009). In contrast to the conclusions drawn from growth kinetics experiments at subinhibitory concentrations in the course of this study, a previous study could show that *S. enterica* and *Escherichia coli* strains resistant to three different classes of antibiotics were selected even at antibiotic concentrations several 100-fold below the MIC values during growth competition experiments (Gullberg *et al.* 2011). Accordingly, Andersson and Hughes (2011) proposed that there is a selective window at subinhibitory concentrations of antibiotics where the resistant strain outcompetes the susceptible strain. However, it should be noted that growth kinetics assays in single cultures provide limited evidence about the relative fitness of isolates, as factors such as toxin production may not be considered (Melnyk *et al.* 2015).

The present *in vitro* study indicates that long-term treatment with subinhibitory concentrations of organic acids might increase the risk for adaptive responses in a *Campylobacter* population. However, experimentally induced enhanced tolerance to organic acids was observed to be either associated with reduced fitness in adapted variants or with a decrease in tolerance in the absence of selective pressure. Therefore, results indicated that increased tolerances might occur only temporarily in a competitive environment. Further research is therefore needed to examine the occurrence and characteristics of adaptation responses in a *Campylobacter* population under varying conditions, for example during long-term treatment or after exposure to lethal concentrations of organic acids.

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Conflict of Interest

We declare no conflict of interest.

Authors' contribution

Conceived the study: CK. Designed the experiments: EP, SK, DS and CK. Performed the experiments: EP. Analysed the data: EP, SK, DS, AV and CK. Statistical calculations: AV. Writing – original draft: EP and CK. Writing – review and editing: EP, SK, DS, AV and CK.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Results of the *in vitro* growth experiment of the variant of the *Campylobacter jejuni* isolate Cj18 adapted to propionic acid in non-supplemented medium to determine the stability of the organic acid tolerant phenotype.