



# Antimicrobial effect of a drinking water additive comprising four organic acids on *Campylobacter* load in broilers and monitoring of bacterial susceptibility

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**ABSTRACT** Application of organic acids via feed or drinking water is under discussion as a possible intervention strategy to reduce *Campylobacter* (*C.*) load in primary poultry production. A previous in vitro study showed that reduced concentrations of sorbic acid, benzoic acid, propionic acid, and acetic acid were required for antibacterial activity against *Campylobacter* when using a mixture of these 4 acids compared to when using the single acids. The present study aimed at determining the antibacterial efficiency of this combination in vivo as a drinking water additive for reducing shedding and intestinal *C. jejuni* colonization in broilers. Furthermore, we assessed whether the inoculated *C. jejuni* strain BfR-CA-14430 adapted

in vivo to the applied organic acids. Results of this study showed that adding the organic acids consistently reduced *Campylobacter* loads in cloacal swabs. While significant reductions were observed within the entire study period, a maximum 2 log reduction occurred at an age of 18 d. However, after dissection at the end of the trial, no significant differences were detected in *Campylobacter* loads of cecal and colon contents compared to the control group. Susceptibility testing of re-isolates from cloacal swabs and cecal content revealed equal minimum inhibitory concentration (MIC) values compared to the inoculated test strain, suggesting that *C. jejuni* remained susceptible throughout the trial.

**Key words:** mitigation, colonization, resistance, adaptation, in vivo

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## INTRODUCTION

Campylobacteriosis was the most frequently reported foodborne gastrointestinal infection in the European Union (EU) in 2020 (EFSA, 2021) and poses a serious health risk to humans (Zautner et al., 2014). Broiler meat is considered to be the most important source for human infection, *Campylobacter* (*C.*) *jejuni* being the most frequently reported causative species (EFSA, 2021). Both intestinal colonization and external contamination of feathers and skin with *Campylobacter* have been shown to be sources for *Campylobacter* contaminating broiler carcasses during slaughter (Smith et al., 2007; Seliwiorstow et al., 2015a; Seliwiorstow et al., 2016). Recently, the European Food Safety Authority (EFSA) updated its previously published opinion on control options for *Campylobacter*. Interventions targeting

*Campylobacter* at farm level that reduce intestinal *Campylobacter* concentrations by 2 log units (log<sub>10</sub>) colony-forming units (cfu) were estimated to reduce the public health risk by 42% compared to an estimated risk reduction of 76 to 98 % in a previous opinion from 2011 (EFSA, 2020). However, strategies applied at the beginning of the food chain offer the important advantage that their use in primary production can be easily combined with other measures during subsequent steps of the food production chain. Accordingly, combinations of control options targeting different stages of the food chain in a multiple-hurdle approach have been proposed to be more promising than the use of single measures (Klein et al., 2015; Alter and Klein, 2017; Kittler et al., 2021a). In the past years, research has focused on different *Campylobacter* mitigation strategies, such as bacteriophages, bacteriocins, or vaccines, with promising results in some studies (Stern et al., 2005; Neal-McKinney et al., 2014; Robyn et al., 2015; Meunier et al., 2017; Richards et al., 2019; Kittler et al., 2021b). A previous study conducted by Neal-McKinney et al. (2014) investigated the protective effect of *Campylobacter* antibodies after vaccination based on recombinant surface-exposed proteins. The

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authors observed a 2 log<sub>10</sub> reduction in *Campylobacter* counts (Neal-McKinney et al., 2014). Wagle et al. (2017) tested the use of the phytochemical b-resorcylic acid in an in vivo study. In this former study, the application significantly reduced cecal *Campylobacter* concentrations by ~2.5 and 1.7 log<sub>10</sub> cfu/g. However, there are as yet no approved products authorized for industrial use in poultry flocks (EFSA, 2020). In contrast, organic acids, such as sorbic acid or propionic acid are directly applicable, as they have already been approved as feed and drinking water additives in animal production (Jansen et al., 2014; Guyard-Nicodème et al., 2016; European Commission 2019). In addition, organic acid treatment is relatively inexpensive and can easily be administered via feed or drinking water (Mani-López et al., 2012; Meunier et al., 2016).

Previous in vivo studies investigated the antibacterial effect of organic acid supplements for feed or water on *Campylobacter* reduction, but results are contradictory (Solis de los Santos et al., 2008; Van Deun et al., 2008; Skånseng et al., 2010; Metcalf et al., 2011; Hermans et al., 2012). There is some evidence that the use of combined organic acids might be advantageous compared to the treatment with single organic acids. In fact, previous in vitro studies showed that combined organic acids exhibited synergistic activities against *Campylobacter* and *Escherichia coli* (Chaveerach et al., 2002; Kim and Rhee, 2013; Peh et al., 2020). Furthermore, adding a combination of formic acid and potassium sorbate to feed was shown to prevent *C. jejuni* colonization in broilers, whereas treatment with a single organic acid failed (Skånseng et al., 2010). Nonetheless, to our knowledge, in vivo studies investigating the antibacterial effect of systematically developed combinations of organic acids have not yet been published. Moreover, there are no published data on in vivo adaptive responses of *Campylobacter* although the ability to develop enhanced tolerances to organic acids has been shown in in vitro studies (Birk et al., 2012; Peh et al., 2021). New insights into the occurrence and development of decreased susceptibility of *Campylobacter* to organic acids might contribute to improved application schemes.

Therefore, the aim of the present study was to investigate the suitability of organic acids as a future component in a multiple-hurdle approach to reduce *Campylobacter* in broiler flocks. The present study focused 1) on the in vivo antibacterial effect of a systematically developed drinking water additive against *Campylobacter* colonization in broilers, and 2) on the monitoring of adaptive responses of *Campylobacter* during the in vivo animal experiment.

## MATERIALS AND METHODS

### Ethics

This study was carried out in accordance with the National Animal Protection Guidelines. The protocol was reviewed and approved by the German Animal Ethics Committee for the Protection of Animals of the Regional Office for Health and Social Affairs Berlin (“Landesamt

für Gesundheit und Soziales”, LAGeSo, registration number G 0098/18). All applicable national and institutional guidelines of the Freie Universität Berlin for the care and use of animals were followed. Animal treatments approved by LAGeSO were classified as being of minor distress (minor pain with short duration).

### Study Design

The animal trials were performed in the experimental facility of the Center for Infection Medicine of the Department for Veterinary Medicine of the Freie Universität Berlin, Germany. In total, 180 broiler hatching eggs (aerosol disinfected with formalin) of breed *Ross 308* were received from a commercial hatchery in Germany. Immediately after arrival, the eggs were disinfected again using WESSOCLEAN K 50 Gold Line containing 2.37% hydrogen peroxide and 0.015% peracetic acid (Wesso AG, Hersbruck, Germany). Afterwards, the eggs were incubated in a hatching incubator (Easy 250; J. Hemel Brutgeräte GmbH & Co. KG, Verl, Germany) for 21 d until hatching. Facilities for animal keeping were cleaned, disinfected using evaporated H<sub>2</sub>O<sub>2</sub>, and tested for the absence of *Campylobacter* as described by Szott et al. (2020). Hatched broilers of both sexes (n = 180) were randomly selected and housed in 2 separate experimental rooms. Each group consisted of 90 chickens: a positive control group (challenged with *C. jejuni*, receiving drinking water without supplementation) and an experimental organic acid group (challenged with *C. jejuni*, receiving drinking water supplemented with a drinking water additive comprising 4 organic acids). Within these 2 groups, the 90 broiler chickens were randomly assigned to one of the following categories: 1) seeder (n = 18), 2) sentinels (n = 36), and 3) stocking density broilers (n = 36). The affiliation of the chickens to the respective category was ensured by attaching an individual sequential number (individual tagging). Each pen in the experimental facility was supplied with filtered air using an HEPA filter and equipped with a temperature control maintained by an electronic thermometer sensor, and a programmable light regimen. Aiming to imitate a commercial broiler husbandry environment, broilers were placed in the barn with fresh litter at a stocking density of 39 kg/m<sup>2</sup>. Commercial broiler feed and filtered water from the municipal water supply were provided ad libitum during the entire study period. Water samples were routinely obtained every 4 to 8 wk to check the water quality. The results of the external testing laboratory showed that the water was of drinking water quality. Feed was offered in commercially available poultry troughs, and filtered water was given via nipple drinkers and changed twice a day. The organic acids were added to the drinking water of the experimental organic acid group as described below. The feed comprised a commercial standard 3-phase feeding program for broilers as shown in Table 1.

Animal health parameters and weight gain were monitored daily. At the end of the trial, broilers were euthanized,

and samples from cecal and colonic contents of the sentinels were collected for enumeration of *Campylobacter*.

### Bacterial Strain and Broiler Challenge

The *C. jejuni* strain BfR-CA-14430, originally isolated from chicken breast, was provided by the Federal Institute for Risk Assessment (BfR) and used for experimental inoculation. The strain was stored and prepared for experimental challenge as described by Szott et al. (2020). Each seeder was orally inoculated with 500  $\mu\text{L}$  containing approximately  $2.2 \times 10^4$  cfu of the *C. jejuni* strain BfR-CA-14430 10 d post hatch. For control purposes, the concentration of the inoculum was determined before and immediately after oral inoculation of the seeders. For this, 10-fold dilutions were plated on modified *Campylobacter*-selective charcoal cefoperazone deoxycholate agar (mCCDA) plates prepared from *Campylobacter* blood-free selective agar base (CM0739; Oxoid Deutschland GmbH, Wesel, Germany) and CCDA selective supplement (SR0155; Oxoid Deutschland GmbH). After a 48-h incubation period at 37°C under microaerobic conditions (85% N<sub>2</sub>, 10% CO<sub>2</sub>, 5% O<sub>2</sub>), colonies on plates containing 30 to 300 colonies were counted for *Campylobacter* enumeration.

### Combination of Organic Acids Used as a Water Additive

Based on the results of a previous in vitro study (Peh et al., 2020), a combination of sorbic acid, benzoic acid, propionic acid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), and acetic acid (E. Merck KG, Darmstadt, Germany) was selected for this study. All organic acids included are listed as authorized feed additives in the European Union (European Commission, 2019). A stock solution was prepared in autoclaved tap water at a total organic acid concentration of 480 mmol/L. The combination of organic acids was stored for up to 3 wk during the experiments. During storage and application, regular macroscopic checks were made to ensure that no precipitation of the organic acids occurred. Previous experiments showed that the MIC values of the organic acid mixture were constant during 4 wk of storage (data not shown), suggesting a stable antibacterial activity for the storage period of the present in vivo experiment. In order to

achieve constant concentrations of organic acids in the drinking water, the application procedure was standardized. The water was freshly prepared and changed every 12 h. Before dosing, the stock solution was shaken vigorously before being added to the water and the required volumes were measured precisely using volumetric flasks. The organic acids were administered at a dilution of 1:30 via drinking water to achieve final concentrations of 6.4 mmol/L for sorbic acid, 4.8 mmol/L for benzoic acid, 3.2 mmol/L for propionic acid, and 1.6 mmol/L for acetic acid. Adding the acids to the drinking water adjusted the water to pH 6.0.

For susceptibility testing of re-isolates, *Campylobacter* colonies were isolated from cloacal swabs or cecal content during the animal experiment; 2 stock solutions of the organic acids in combination were prepared in cation-adjusted Mueller Hinton broth (CAMH, Carl Roth GmbH + Co. KG). The total organic acid concentration was 64 mmol/L, comprising 25.6 mmol/L sorbic acid, 19.2 mmol/L benzoic acid, 12.8 mmol/L propionic acid, and 6.4 mmol/L acetic acid. Stock solutions were adjusted to pH 7.3 or pH 6.0 using 2 mol/L and 8 mol/L sodium hydroxide, and a total of 11 two-fold serial dilutions were prepared in CAMH broth.

### Sampling Design and Sample Analysis

Prior to oral inoculation of the seeders, at the fourth day post hatch, absence of *Campylobacter* was confirmed by cloacal swabbing (Sarstedt AG & Co. KG, Nümbrecht, Germany) of all 180 broilers. Seeders were verified to excrete *C. jejuni* 2 days postinoculation (dpi) (12 d post hatch) by qualitative analysis of cloacal swabs.

Throughout the study, *Campylobacter* colonization of the sentinels was determined by semiquantitative analysis of cloacal swabs. At the end of the trial, *Campylobacter* load in cecal and colonic contents of the broiler chickens was determined by semiquantitative analysis.

Semiquantitative analysis of *Campylobacter* was conducted using cloacal swabs which were taken at defined time points: 3 and 4 dpi, and subsequently twice a week (equivalent to 8, 11, 16, and 18 dpi) until necropsy. To ensure comparability of results, the same 36 sentinels (noninoculated, but naturally colonized with *C. jejuni* through contact with the seeders) were sampled in both groups. Cloacal swabs were analyzed semiquantitatively in accordance with DIN EN ISO 10272-3. Briefly, the standardized sampling procedure was as follows: cloacal swabs were inserted in the cloaca, rotated 5 times, removed, and immediately transferred to 3.0 mL Preston broth. Thereafter, cloacal swabs were homogenized for 3 s using a vortex shaker (VWR International GmbH, Darmstadt, Germany), allowing the fecal material to detach and evenly disperse in the medium. Afterward, cloacal swabs were serially diluted 10-fold in Preston broth (up to 10<sup>-8</sup>), incubated 24 h at 37°C under microaerophilic conditions, and then streaked out on quartered mCCDA plates using 10  $\mu\text{L}$  inoculation loops (Sarstedt AG & Co. KG). Plates were incubated

**Table 1.** Ingredients and nutrient contents of the experimental diets.

Components per kg	Starter diet (0–8 days)	Grower diet (9–26 days)	Finisher diet (27–33 days)
Crude protein (%)	21.5	21.0	20.0
Crude lipids (%)	4.9	6.4	5.5
Crude fiber (%)	2.9	3.4	3.3
Crude ash (%)	5.3	5.1	4.9
ME, kcal/kg	2,961.7	2,961.7	2,961.7
Calcium (%)	0.9	0.8	0.8
Phosphorous (%)	0.6	0.6	0.5
Sodium (%)	0.1	0.1	0.1
Methionine (%)	0.6	0.5	0.5
Lysine (%)	1.3	1.2	1.1



for another 48 h at 37°C under microaerophilic conditions and examined for *C. jejuni* growth. Presumptive colonies were examined microscopically for morphology and motility. Additionally, colonies were subcultured onto 5% sheep Columbia blood agar (Fisher Scientific, Germany) and then incubated for 24 h at 37°C under microaerophilic conditions. Afterward, colonies were analyzed using a Bruker Microflex system for matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The highest evaluable dilution on mCCDA plates with confirmed *Campylobacter* growth was then used to calculate the MPN (most probable number) value using an MPN table modified according to ISO/TS 10272-3:2010/Cor.1:2011(E).

At the end of the growth period (as defined by an average bird's weight of 2.0 kg) at d 23 postinfection, all 36 sentinels per group were euthanized using ZKS poultry pliers (Corstechnology UG, Neerstedt, the Netherlands) after confirming deep anesthesia as indicated by muscle relaxation, absence of the corneal reflex, and absence of the eyelash reflex. The animals were dissected and cecal and colonic contents were collected for subsequent *C. jejuni* enumeration. For semiquantitative analysis, the intestinal contents were removed aseptically, diluted 1:8 in Preston broth, homogenized, and 10-fold serially diluted in Preston broth (up to  $10^{-9}$ ). For enrichment, dilutions were incubated for 24 h at 37°C under microaerobic conditions. Approximately 2  $\mu$ L of each dilution was streaked out on quartered mCCDA plates using an inoculation loop. After incubation for 48 h at 37°C under microaerobic atmosphere, the highest dilution showing bacterial growth was used for calculating the most probable number of bacterial counts.

### Susceptibility Testing of Re-isolates In Vitro

A total of 90 *Campylobacter* re-isolates were collected during the animal trial to determine their susceptibility to the previously administered organic acids. Briefly, the re-isolates were selected from the mCCDA plates used for *Campylobacter* quantification as follows: 1) 18 presumptive *Campylobacter* colonies were isolated from cloacal swabs of each seeder bird (sampled 2 dpi), 1) 36 *Campylobacter* colonies were isolated from cloacal swabs of each sentinel bird (sampled 11 dpi), and 3) 36 colonies from the cecal content of each sentinel bird were collected during necropsy (sampled 23 dpi). Colonies were transferred to tubes containing 1 mL of skimmed milk and stored at  $-80^{\circ}\text{C}$  as described earlier (Kittler et al., 2013, 2014). Prior to susceptibility testing, re-isolates were plated out on Columbia agar supplemented with sheep blood and incubated for 48 h at  $42 \pm 1^{\circ}\text{C}$  under microaerobic conditions.

The minimum inhibitory concentration (MIC) values were determined as described earlier (Peh et al., 2020). In brief, susceptibility tests were performed using U-shaped bottom 96-well microtiter plates (Sarstedt AG & Co. KG). Fifty  $\mu$ L of the bacterial inocula at concentrations

of  $1 \times 10^6$  cfu/mL were dispensed into wells containing 50  $\mu$ L of the double concentrated organic acid mixture to achieve final bacterial concentrations of  $5 \times 10^5$  cfu/mL. After 48 h of incubation at  $42 \pm 1^{\circ}\text{C}$  under microaerobic conditions, the lowest concentration that inhibited visible growth of bacteria was assessed. The susceptibility of all 90 re-isolates was tested at pH 7.3. Additionally, MIC values of 18 randomly selected re-isolates collected 20 d post-hatch and isolated from cecal content during dissection were determined at pH 6.0.

### Statistical Analysis

The experimental data were analyzed using SPSS software version 25.0 for Windows (SPSS, Inc., Chicago, IL). Data were analyzed for normal distribution using the Shapiro-Wilk Test. As data were not normally distributed, we used the non-parametric Mann-Whitney U test. *Campylobacter* counts were logarithmically transformed ( $\log_{10}$ ) and then analyzed for significant differences using the non-parametric Mann-Whitney U test. *P*-values below 0.05 were regarded as statistically significant. To ensure an alpha error of 0.05, a beta error of 0.18, and power of 0.80, a total of 90 animals per group were included in the present study. In order to determine statistically significant differences, 36 animals were sampled during the experiment, and the differences calculated by using a biologically relevant difference of  $\Delta = 1$  log unit between *Campylobacter* counts of the groups and assuming a standard deviation of 1 log unit.

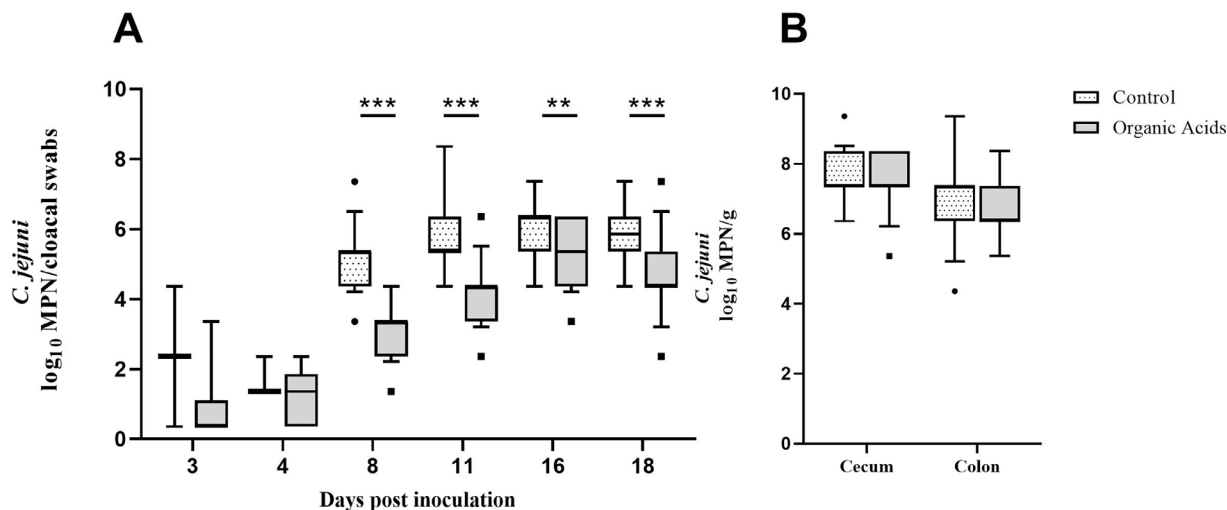
## RESULTS

### In Vivo Effect of Organic Acids on *Campylobacter* Colonization

Four days post hatch, broilers were confirmed to be *Campylobacter* free by microbial analysis of swabs. Eight days postinoculation, *Campylobacter* was detected in samples of all 72 sentinels.

Significantly reduced *Campylobacter* counts were detected in cloacal swabs of the experimental group receiving the organic acids compared to the control group at d 8, 11, 16, and 18 postinoculation ( $P \leq 0.003$ ; Figure 1A). A maximum 2.0-log ( $P < 0.0001$ ;  $r = 0.81$ ) and 1.0-log reduction ( $P < 0.0001$ ;  $r = 0.7$ ) in *C. jejuni* counts were detected in the experimental group ( $Md = 3.36$  and  $4.36 \log_{10}$  MPN/cloacal swabs) 8 and 11 dpi in comparison to the control group ( $Md = 5.36$  and  $5.36 \log_{10}$  MPN/cloacal swabs). Slightly lower 1.0 and 1.5-log reductions (16 dpi  $p = 0.003$ ;  $r = 0.35$  and 18 dpi  $P < 0.0001$ ;  $r = 0.65$ ) were observed 16 and 18 dpi in the experimental group ( $Md = 5.36$  and  $4.36 \log_{10}$  MPN/cloacal swabs) compared to the control group ( $Md = 6.36$  and  $5.86 \log_{10}$  MPN/cloacal swabs).

No reduction in *Campylobacter* counts was observed in cecal content sampled 23 dpi ( $P > 0.05$ ; Figure 1B).



**Figure 1.** *Campylobacter* (*C. jejuni*) colonization of 36 sentinels per group determined by semiquantitative analysis (min to max). (A) *C. jejuni* counts in log<sub>10</sub> most probable number (MPN) in cloacal swabs derived from sentinels confirmed to shed *C. jejuni* at distinct time points after oral inoculation of the seeders at d 10. Three and four days postinoculation (dpi), 2 (3 dpi) and 3 (4 dpi) sentinels of the control group and 6 (3 dpi) and 21 (4 dpi) sentinels of the experimental group shed *C. jejuni*. From eight dpi onwards, samples of all 36 sentinels were *Campylobacter*-positive. (B) *C. jejuni* counts in log<sub>10</sub> MPN per gram in intestinal content of 36 sentinels per group upon necropsy (23 dpi). White dotted boxes represent the control group (broilers challenged with *C. jejuni* and not treated with a combination of organic acids); gray boxes represent the experimental group challenged with *C. jejuni* and treated with a combination of organic acids. Medians (bold line) and significance levels (*P* values) determined by the Mann-Whitney U test are indicated. \* (*P* < 0.05), \*\* (*P* < 0.01), \*\*\* (*P* < 0.001).

### ***Campylobacter* Re-isolates Exhibited Minimum Inhibitory Concentration Values Equal to the Inoculated Test Strain**

A total of 90 *Campylobacter* re-isolates collected at 3 different time points from samples of the animal experiment were tested for their susceptibility to the drinking water additive and its individual components. All tested re-isolates showed MIC values equal to those of the initially inoculated *C. jejuni* strain BfR-CA-14430 both at pH 7.3 and pH 6.0. At pH 7.3, MIC values of 1.6 mmol/L for sorbic acid, 1.2 mmol/L for benzoic acid, 0.8 mmol/L for propionic acid, and 0.4 mmol/L for acetic acid were determined. At pH 6.0, both re-isolates and the test strain exhibited MIC values of 0.4 mmol/L for sorbic acid, 0.3 mmol/L for benzoic acid, 0.2 mmol/L for propionic acid, and 0.1 mmol/L for acetic acid.

### **Application of Organic Acids Showed No Adverse Effects on Broiler Growth Performance**

At the end of the trial, no significant difference in the mean final body weight of sentinel birds of the experimental (1.84 kg) and the control group (1.87 kg) were observed (*P* > 0.05).

## **DISCUSSION**

In this study, we examined the in vivo efficacy of a drinking water additive to reduce the intestinal *Campylobacter* colonization in broilers. The mixture consisting of sorbic acid, benzoic acid, propionic acid, and acetic acid was supplied during the entire growth period until slaughter age. By using a seeder bird model, we

aimed to use an experimental set-up that imitated the natural spread of *Campylobacter* colonization in conventional broiler flocks as far as possible. Moreover, we included investigations on adaptive responses of *Campylobacter* to the applied organic acids. For this purpose, we assessed the MIC values of the administered organic acids in *C. jejuni* re-isolates after an intestinal passage in broilers.

The selection and proportions of organic acids as well as their final concentrations in the broilers' drinking water were chosen based on results of a systematic approach from a previous in vitro study (Peh et al., 2020). When tested on a panel of 20 *C. jejuni* isolates, the selected combination of organic acids showed synergistic activities against 5 isolates, including the *C. jejuni* strain BfR-CA-14430 used in the present in vivo study. Furthermore, the MIC values of the organic acids decreased 2.5- (sorbic acid) to 160-fold (acetic acid) compared to those determined at pH 7.3 for the individual substances on the test strain (Peh et al., 2020). In this in vivo study, we decided to administer a drinking water additive containing the organic acids at final concentrations four-fold higher than the MIC values determined for *C. jejuni* strain BfR-CA-14430 at pH 7.3 (16-fold higher than the MIC values at pH 6.0). All of the organic acids were applied at concentrations lower than those indicated to cause adverse effects on broiler performance (Metcalf et al., 2011). Similar to the present study, organic acids and botanicals were administered at concentrations 2- to 8-fold higher than their MIC values for testing their antifungal and antibacterial effects in other in vivo studies (Chami et al., 2005; Grilli et al., 2013; Mousavi et al., 2020).

The results of the present in vivo study are encouraging, since the *Campylobacter* shedding was consistently reduced during the third and fourth fattening week,

reaching a maximum 2-log reduction at d 8 postinoculation. However, the drinking water additive failed to diminish *Campylobacter* colonization in the intestinal colonic and cecal contents at the end of the trial. The reason for this finding remains unclear.

One possible explanation for the limited effectiveness of the organic acids might be due to decreased concentrations of the organic acids in the course of the intestinal tract. Similar effects have been shown in previous studies that were caused by different absorption and metabolization processes (Hume et al. 1993; Thompson and Hinton 1997; Hermans et al. 2012). In contrast to these results, several studies detected reduced *Campylobacter* concentrations in the cecum after administering organic acids (Solis de Los Santos et al. 2008; Skånseng et al. 2010; Jansen et al. 2014). If we assume that in our experiment only low or very low concentrations of the supplemented acids reach the cecum, the indirect effects on *Campylobacter* load might be an interesting factor. While we did not include any analysis on the immune status of the chickens, in other studies, organic acids induced the formation of immunoglobulin Y (IgY) (Park et al. 2009), which was reported to induce inhibition of *Campylobacter* colonization (Vandeputte et al. 2019; Nothaft et al. 2021).

Another explanation for the results is that the in vivo efficacy of organic acids might have decreased over an extended period of time. This would be in agreement with results of a previous in vivo study where different commercially available feed additives were administered during the entire rearing period (Guyard-Nicodème et al., 2016). Three dpi, the authors of the aforementioned study observed that 4 of 5 organic acids blends significantly decreased cecal *Campylobacter* counts, whereas after 24 and 31 dpi, only one mixture remained significantly efficient (Guyard-Nicodème et al., 2016). Similarly, Ren et al. (2021) found no sustained reduction in *Campylobacter* counts after fortifying the drinking water of broiler chickens with malic acid for three weeks during rearing. This observation might be explained by the development of an increased tolerance in the test strain to the administered organic acids over time, similar to the emergence of resistant *Campylobacter* during treatment with antibiotics (McDermott et al., 2002; Luo et al., 2005; Ladely et al., 2007; Lin et al., 2007). Similarly, a previous in vitro study demonstrated a stepwise adaptation to propionic acid and sorbic acid for 2 *C. jejuni* field isolates, resulting in 2-fold higher MIC values compared to the wild-type isolates (Peh et al., 2021). However, susceptibility testing of 90 re-isolates collected during the animal trial showed no evidence of an organic acid-tolerant *Campylobacter* population. It is, therefore, rather unlikely that the missing efficacy in cecal and colonic contents was due to adaptive responses in *Campylobacter*.

A previous study showed that *Campylobacter* counts may differ significantly between sample types (Seliwiorstow et al., 2015b). While quantitative analysis of intestinal content or droppings are the gold standard, selective sampling of sentinels is required for analysis of natural colonization models as used in our study. Fecal

or cloacal sampling cannot be conducted in certain animals unless dissection or isolation of animals is used. This would require huge animal numbers or remove the desired practical conditions. Isolating seeder and sentinel broilers for a considerable time would have interfered with the experimental seeder bird model, aiming at a natural intestinal colonization and keeping conditions close to commercial poultry farming. Due to these considerations, we chose to use cloacal swabs for sampling and a semiquantitative approach for the enumeration of *Campylobacter*. This type of sampling ensured the sampling of “naturally” colonized sentinels and thus the examination of the individual course of each of the 36 sentinels (by assigning the samples to the tag-number). Furthermore, it allowed us to include a large sample size in our study. To overcome the issue of varying amounts of feces adhering to the swab, a standardized sampling and processing procedure was used to obtain comparable and reproducible data. The reproducibility and precision of the presented data are satisfactory, as in the control group, the *Campylobacter* counts in cloacal swabs were consistently homogeneous regardless of the sampling time (11, 16, and 18 days after inoculation). In agreement, no statistical difference was observed between enumeration by the semi-quantitative and quantitative technique for comparable concentrations of thermotolerant *Campylobacter* ( $P = 0.104$ ) (Rosenquist et al., 2007). Perdoncini et al. (2022) also observed no significant differences ( $P > 0.05$ ) in the detection and quantification of *Campylobacter* for either the source of isolation (cloacal swabs, carcasses, water) or the technique used (direct plating, MPN technique, and qPCR). Similarly, another research group found no significant differences ( $P > 0.05$ ) between results obtained by direct plating of carcass rinse samples and an MPN procedure (Line et al., 2001). Likewise, Scherer et al. (2006) found a highly positive correlation coefficient of 0.9 between direct plating and the MPN technique.

Regarding the risk of foodborne infections, *Campylobacter* load in the intestinal segments of cecum/colon, and cloaca need to be considered, as previous studies could show that both colonized fecal shedding and/or intestinal content can result in *Campylobacter*-contaminated broiler carcasses. For example, Rosenquist et al. (2006) and Reich et al. (2008) found that *Campylobacter* counts on broiler carcasses correlated significantly with bacterial concentrations in cecal contents. Russell (2003) observed that a cecal cut occurred in 0 to 8% of 200 investigated broiler carcasses in one processing plant. Leakage of intestinal contents usually takes place when processing machines are not programmed to detect carcass size deviations among broiler batches. In contrast, other studies did not observe a significant correlation between cecal *Campylobacter* counts and carcass contamination (Seliwiorstow et al., 2015a, 2016), whereas the external contamination of feathers and skin with *Campylobacter* was shown to be an important source of carcass contamination during the slaughter process (Smith et al., 2007; Seliwiorstow et al., 2015a, 2016). Furthermore, a



previous study demonstrated that the shedding of *Campylobacter* in feces from the cloaca during slaughter led to contamination of broiler carcasses during defeathering (Berrang et al., 2001). Thus, although there was no effect on colonic and cecal concentrations in our study, reduced *Campylobacter* levels in the feces might contribute to lower contamination levels of broiler carcasses during the slaughter process. However, a strict implementation of biosecurity measures and HACCP during slaughter and processing are necessary to avoid cross contamination between different slaughter batches.

In conclusion, the present study showed that a combination of sorbic acid, benzoic acid, propionic acid, and acetic acid applied via drinking water significantly reduced cloacal *Campylobacter* concentration in broilers in vivo, which might contribute to reduced entry of *Campylobacter* into the food chain. However, the drinking water additive failed to reduce *Campylobacter* concentrations in the cecum and colon of 33-day-old broilers at the end of the trial. Susceptibility testing of re-isolates collected at different stages of the animal experiment revealed no evidence of an organic-acid tolerant *Campylobacter* population during the long-term treatment with organic acids. Further research is needed to evaluate the effect of the organic acids in large-scale field studies and multiple-hurdle approaches.

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Authors’ contributions: VS and EP performed the experiments, collected, analyzed, interpreted the data, and drafted the manuscript and figures with critical evaluation and support from all authors. SK, AF, UR, CK, and MP conceived and designed the experimental study as well as critically revising the manuscript. All authors approved the final version to be published.

## DISCLOSURES

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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