Aus dem Institut für Tier- und Umwelthygiene
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Non-biosecurity based intervention measures against *Campylobacter* spp. in broiler production

Inaugural-Dissertation
zur Erlangung des Grades eines
Doctor of Philosophy (PhD)
in Biomedical Sciences
an der
Freien Universität Berlin

vorgelegt von
Vanessa Sylvana Szott
Tierärztin aus Berlin

Berlin 2023
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Für meine Familie

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III. List of Abbreviations

BfR	German Federal Institute for Risk Assessment
BMBF	Federal Ministry for Education and Research
C. coli	*Campylobacter coli*
C. fetus	*Campylobacter fetus*
C. jejuni	*Campylobacter jejuni*
C. lari	*Campylobacter lari*
CE	competitive exclusion
CFU	colony forming units
dpi	days post inoculation
e.g.	exempli gratia (for example)
EFSA	European Food Safety Authority
EU	European Union
g	gram
GIT	gastrointestinal tract
i.e.	id est (that is)
ISO	International Organization for Standardization
LAGeSo	Regional Office for Health and Social Affairs Berlin
MALDI-TOF MS	matrix-assisted laser desorption ionization-time of flight mass spectroscopy
mCCDA	modified charcoal cefoperazone deoxycholate agar
MCFA	medium chain fatty acids
ml	milliliters
MPN	most probable number
p	significance
PB	Preston broth

PFU	plaque forming units
SCFA	short-chain fatty acids
spp.	species
TiHo	University of Veterinary Medicine Hannover
WHO	World Health Organization

1. Introduction

Food-borne diseases causing severe gastrointestinal symptoms are an important cause of human morbidity and mortality worldwide. To be precise, the World Health Organization (WHO) reported that unsafe food causes 600 million cases of food-borne diseases and 420,000 deaths, particularly affecting children and elderly people (WHO 2015, 2020b). Globally, *Campylobacter* is one of the leading causes of diarrheal disease along with *Escherichia coli* and *Salmonella*. It is also considered the most common bacterial cause of human gastroenteritis (WHO 2020a). Campylobacteriosis in humans is caused predominantly by the two bacterial species *Campylobacter* (*C.*) *jejuni* and *C. coli*. The disease is strongly associated with poultry and poultry meat. In particular, broiler meat is considered a significant source of human infection (Humphrey et al. 2007). According to the European Food Safety Authority (EFSA), about 60–80% of global campylobacteriosis cases are due to the consumption of poultry products (EFSA 2015).

Regardless of causing severe infections in humans, *C. jejuni* can also colonize the intestinal tract of various livestock and wild animals as a commensal (Gölz et al. 2014; Awad et al. 2016; Connerton et al. 2018). In particular, it preferentially occupies the intestine of agriculturally relevant poultry and a great diversity of wild birds (Sahin et al. 2015). Numerous studies attempted to intervene and eliminate *Campylobacter* from the poultry chain but demonstrated insufficient success. So far, no suitable strategies have been implemented and complete elimination of *Campylobacter* is not attainable. Considering this information, the focus should be on establishing control strategies aimed at reducing the incidence and levels of *Campylobacter* in animals along the food chain (Alter and Reich 2021). In general, the application of control strategies on farm level is most effective, especially when a well-established general and personal hygiene protocol is also followed (van de Giessen et al. 1998; Gibbens et al. 2001). In particular, a 3 $\log_{10}$ reduction of *Campylobacter* in the broilers' ceca can lower the relative risk of human campylobacteriosis by 58% in the European Union (EU) (EFSA 2020).

Therefore, the aims of the project were as follows:

i. Developing an experimental seeder-bird model that can imitate natural *C. jejuni* colonization and spread in a conventional broiler chicken rearing facility
ii. Evaluating different non-biosecurity based intervention measures and their ability to reduce *C. jejuni* colonization in the intestine of broiler chickens

The work described here was conducted in the frame of the zoonoses research consortium "Preventing and combating *Campylobacter* infections: on track towards a One Health approach (PAC-CAMPY)", funded by the Federal Ministry for Education and Research (BMBF) (grant no. 01KI1725A).

1.1. *Campylobacter jejuni* a public health concern

Campylobacteriosis cases caused by bacteria of the genus *Campylobacter* spp. have progressively increased over the past two decades and have become a significant public health burden to humans worldwide (Newell et al. 2017; Burnham and Hendrixson 2018; Lackner et al. 2019). The EFSA reported, that associated costs to public health systems in the EU amount to approximately €2.4 billion per year (EFSA 2014). On a global scale, 8.4% of global diarrheal diseases are attributed to *Campylobac*ter (Connerton and Connerton 2017). Pathogenic zoonotic *Campylobacte*r species are mainly *C. jejuni* and *C. coli*. In percentage terms, 90% of infections are due to *C. jejuni* and the vast majority of the remaining infections are due to *C. coli* (Gillespie et al. 2002; Wilson et al. 2008). In 2020, in Europe, there were 120,946 confirmed cases of human campylobacteriosis (EFSA 2021). Of these, 55,831 cases were notified in Germany (Banerji et al. 2021).

In humans, the main routes of infection correspond to the ingestion of contaminated meat products, especially poultry, raw milk and water (Bull et al. 2006; Smith et al. 2020; Heimesaat, Backert, et al. 2021). Oftentimes, ingestion of very small amounts of *Campylobacter* (a few hundred bacteria) is sufficient to cause illness in consumers (Igwaran and Okoh 2019). In general, clinical symptoms of human *Campylobacter* infections include fever and mild, non-inflammatory and self-limiting diarrhea. Occasionally, the disease manifests itself as severe, inflammatory, bloody diarrhea with intense abdominal pain. In addition, *Campylobacter* infections can also lead to severe neurological late sequelae, including reactive arthritis, Guillain-Barré syndrome, irritable bowel syndrome, Miller-Fisher syndrome and inflammatory bowel disease (Keithlin et al. 2014; Saint-Cyr et al. 2016; Lackner et al. 2019; Vandeputte et al. 2019). If therapy becomes necessary, the patient is primarily relieved with fluid and electrolyte replacement. Nevertheless, if the symptoms do not improve, antibiotics are of significant choice for C*ampylobacter* therapy (Guarino et al. 2014).

As antibiotics use increases, however, *Campylobacter* resistance is also an emerging global burden, which may entail limited treatment options (de Vries et al. 2018; Igwaran and Okoh 2019). A study conducted in Belgium between 2006 and 2015 showed that of 199 clinical *C. jejuni* isolates, 56.3% were resistant to nalidixic acid, 55.8% to ciprofloxacin and 49.7% to tetracycline (Elhadidy et al. 2020). *Campylobacter* spp. isolated from conventional and organic turkey meat at retail in Germany demonstrated high resistance rates to ciprofloxacin, nalidixic

acid and tetracycline. Especially, those isolates from conventional meat revealed higher resistance rates (78.9% of the isolates were resistant to fluoroquinolones and 60.9% to tetracyclines) (Tenhagen et al. 2020).

1.2. Characteristics of *Campylobacter jejuni*

C. jejuni is a member of the genus *Campylobacter* belonging to the family of *Campylobacteriaceae* and class *Epsilonproteobacteria*. Among 22 other species, *C. jejuni* and *C. coli* are not only the best known but are also the main causative agents of campylobacteriosis in humans (Fitzgerald and Nachamkin 2011; Smith et al. 2020; Heimesaat, Backert, et al. 2021). *C. jejuni* is a gram-negative, non-spore-forming, slender and curved or spiral-shaped bacterium, with a length between 0.5 and 5 µm and a width between 0.2 and 0.9 µm (Percival and Williams 2013; Lastovica et al. 2014; Facciolà et al. 2017). Since they are quite fastidious bacteria, they prefer microaerophilic conditions with O_2 concentrations between 5 and 10% for their growth (Lastovica et al. 2014). Moreover, the bacteria are well adapted to temperatures between 37°C and 42°C (Stingl et al. 2012). *C. jejuni* has a polar flagellum that allows mobility (Facciolà et al. 2017) and facilitates colonization in their host. For its metabolism, *C. jejuni* derives its energy from amino acids or intermediates of the tricarboxylic acid cycle and is therefore not dependent on the utilization of sugars (Debruyne et al. 2008; Jeon et al. 2010). Under unfavorable conditions, *C. jejuni* can enter a viable but non-culturable state (Portner et al. 2007), in which it is not able to proliferate in culture media but still exhibit metabolic activity and membrane integrity (Ramamurthy et al. 2014). Whether viable but non-culturable bacteria can be revitalized in the gut after oral ingestion is still under debate (Li et al. 2014; Zhao et al. 2017).

1.3. Prevalence, introduction and colonization of *Campylobacter jejuni* in commercial poultry flocks

Due to its ubiquitous occurrence, *Campylobacter* is a common commensal in commercial poultry farms (Burnham and Hendrixson 2018). In Europe, prevalence varies from 2—100% (with variations between flocks) with a mean prevalence of 71.2% (EFSA 2010). In 2018, the EFSA reported a prevalence of 26% in broiler chickens in Europe (EFSA 2019). In 2019, the German Federal Institute for Risk Assessment (BfR) stated a *Campylobacter* prevalence of 47.5% for broiler chickens in Germany (BfR 2019). Additionally, there is a clear seasonal pattern showing *Campylobacter* prevalence peaks in summer and fall (Meldrum et al. 2005; Meldrum and Wilson 2007; Hartnack et al. 2009; Jorgensen et al. 2011; Sahin et al. 2015; Smith et al. 2019). Furthermore, *Campylobacter* prevalence in poultry flocks varies greatly by production system (organic or conventional) and region (Bahrndorff et al. 2013; Murphy et al. 2018). Geographically, in terms of Europe, *Campylobacter* prevalence is significantly lower in

the Northern European countries than in the rest of Europe (Newell and Fearnley 2003; Sibanda et al. 2018).

Different sources are under debate as the cause for the spread of *Campylobacter*, e.g., contaminated fixtures (drinkers, feeders), contaminated clothes of farm workers, pets or other livestock animals as well as insects and rodents (Newell and Fearnley 2003; Ellis-Iversen et al. 2009; Newell et al. 2011). However, to date, the sources from which *Campylobacter* is introduced into poultry flocks are not fully known or understood (Bull et al. 2006; Romdhane and Merle 2021). Nevertheless, it is agreed that vertical transmission between hen and egg is negligible since day-old chickens are usually *Campylobacter*-negative after hatching or arrival at the farm. More likely, chickens become colonized with *Campylobacter* during fattening through contact with the aforementioned sources (Bull et al. 2006; Newell et al. 2011). *Campylobacter* colonization naturally occurs in two- or three-week-old chickens and is associated with the progressive decrease of maternal antibodies (Sahin et al. 2003; Cawthraw and Newell 2010). Nevertheless, it is hypothesized that although chickens are protected by maternal antibodies, these do not necessarily preclude infection. Rather, chickens can be infected at any time during the rearing period but *Campylobacter* only multiply to the point where they are detectable and efficiently transmissible when chickens are more than 2 weeks old (Conlan et al. 2011; Connerton et al. 2018). However, ingestion of minimal doses of *C. jejuni* is sufficient to establish stable intestinal colonization (Hermans, Van Deun, Martel, et al. 2011). Most problematically, once a chicken is infected, the bacterium spreads rapidly and colonizes the entire flock via the fecal-oral route within a few days with a prevalence of nearly 100% (Stern et al. 2001; Hermans, Pasmans, et al. 2011; Awad et al. 2018). After ingestion, *C. jejuni* predominantly inhabits the intestine, especially the cecum of broiler chickens with high concentrations between 10^6 and 10^8 colony forming units (CFU)/g but is also carried in the crop and with partial high loads on the plumage (Rosenquist et al. 2006; Hazards 2010; Hermans, Van Deun, Martel, et al. 2011). High loads in the cecum are decisive for the risk of human infection as broilers remain colonized until slaughter and *Campylobacter* concentrations on broiler carcasses at the abattoir have been shown to correlate significantly with concentrations found in intestinal contents (Hermans et al. 2010; Jorgensen et al. 2011; Ridley et al. 2011; Sahin et al. 2015). Further, slaughter batches that carry high loads of the bacterium contribute to cross-contamination at various stages along the slaughter line. Once contamination of meat has occurred, even thoroughly implemented hygiene measures at the slaughterhouse are not able to ensure that the final product that ends up at retail is free of *Campylobacter*. Therefore, humans are at risk of *Campylobacter* infection when they consume inadequately cooked chicken products or handle contaminated poultry meat (Slader et al. 2002; Scherer et al. 2006; Lee et al. 2017; Romdhane and Merle 2021).

1.4. General detection methods for *Campylobacter*

In the PAC-CAMPY project, a hierarchical approach was applied to isolate and quantify *C. jejuni*. The first step included the cultivation of *C. jejuni* following ISO/TS 10272-3:2010, which describes a horizontal method for the semi-quantitative determination of *Campylobacter* spp. If results were inconclusive, suspicious colonies were subcultured onto blood agar plates and verified using matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF MS). The methods used for the investigation of "Non-biosecurity based intervention measures against *Campylobacter* spp. in broiler production" are described briefly in the next section.

1.4.1. Culture-based methods

C. jejuni is a fastidious bacterium with metabolic heterogeneity that hamper isolation and identification, especially regarding cultivation-based methods (Ricke et al. 2019). However, the use of reliable cultural methods for the detection, identification and enumeration of *Campylobacter* on the species level is paramount, especially for clinical, food and veterinary laboratories. Culture-based methods for *Campylobacter* detection are cost-efficient and simple (Corry et al. 2003). Its elusive nature and specific growth requirements concerning temperature, microaerophilic atmosphere and antibiotic susceptibility demand culture-dependent procedures which follow either direct plating or enrichment (in case of low *Campylobacter* numbers or high concentrations of accompanying flora) and plating (Moore et al. 2005; Jasson et al. 2009; Jokinen et al. 2012).

1.4.2. Quantitative detection method

Campylobacter cultivation is usually carried out using the ISO standard protocol for *Campylobacter* spp. isolation 10272. To be specific, DIN EN ISO 10272-1 defines the procedures for detection while DIN EN ISO 10272-2 specifies the procedure for enumeration.

For the cultivation of food and fecal samples, the ISO 10272 recommends the use of Bolton or Preston broth (PB) as a selective enrichment medium, including a pre-enrichment step of 24h at 37°C to suppress competitive flora or rather revive sublethal damaged cells (Hazeleger et al. 2016; Anonymous 2017). The general approach proposes a composition for microaerophilic incubation as follows: 5 ± 2% O_2, 10 ± 3% CO_2, ≤10% H_2 (optional), saturating the rest of the gas mixture with 75–85% N_2 (Anonymous 2017). Besides, it is further recommended to isolate *Campylobacters* on modified charcoal cefoperazone deoxycholate agar (mCCDA) as a blood-free alternative with a subsequent incubation for another 48h at 37°C under microaerophilic conditions (Hutchinson and Bolton 1984; Corry and Atabay 2012; Oyarzabal and Carrillo 2017). The major advantage of culturing on mCCDA is that it provides an ideal background for

visualization and differentiation of otherwise translucent *Campylobacter* colonies. (Gharst et al. 2013; Oyarzabal and Carrillo 2017). On mCCDA, *Campylobacter* colonies appear grey, flat, swarming, and irregular in shape (Hansson et al. 2004; Eberle and Kiess 2012).

1.4.3. Matrix-assisted laser desorption ionization-time of flight mass spectroscopy (MALDI-TOF MS)

MALDI-TOF MS is a powerful diagnostic tool to identify microorganisms quickly and reliably. Using unique highly conserved protein patterns from intact bacterial cells, MALDI-TOF MS identifies and differentiates between bacterial genera and species. A major advantage is a corresponding database that contains a wide range of microorganisms for appropriate identification. A previous study, comparing the diagnostic potential of two major commercially available MALDI-TOF MS platforms (VITEK MS; Biotyper MALDI-TOF) demonstrated that regardless of the system used, isolates were identified as *C. jejuni, C. coli, C. lari, and C. fetus* with high confidence of up to 100% (Hsieh et al. 2018). This is also in agreement with other studies (Mandrell et al. 2005; Kolínská et al. 2008), in which MALDI-TOF-MS was shown to provide rapid and reasonably accurate species-level identifications. Although MALDI-TOF MS provides reliable results, the identification of mixed cultures is a major challenge that may contribute to the inability to obtain adequate results (Bessède et al. 2011). Unfortunately, analysis of bacteria previously grown on mCCDA agar may compromise the spectral integrity of the mass spectroscopy. Therefore, it is suggested to perform an additional subcultivation step on non-selective agar such as Columbia blood agar. Compared with conventional biochemical assays, MALDI-TOF MS proved to be superior. In addition, compared to whole genome sequencing, MALDI-TOF MS is a rapid and reliable method for identifying *Campylobacter* of different origins down to the species level (Lawton et al. 2018).

1.5. Non-biosecurity based intervention measures against *Campylobacter* at the farm level

To minimize human exposure to *Campylobacter*, the quantitative *Campylobacter* load in broiler chickens needs to be decreased. In general, there is a wide range of strategies available. These fundamentally encompass, first of all, on-farm management options that are designed to produce broiler flocks that either carry less *Campylobacter* in their intestine or, rather, are *Campylobacter*-free (Wagenaar et al. 2006; Connerton et al. 2008; Lin 2009; Alter and Reich 2021). This category mainly includes biosecurity (proper clothing, strictly implemented hygiene regime, instructed personnel, thoroughly cleaned and disinfected equipment and vehicles, rodent and insect control) (Gibbens et al. 2001; Hald et al. 2007; Newell et al. 2011) and non-biosecurity based measures (feed and water additives, application of bacteriophages, competitive exclusion (CE) cultures, probiotics or bacteriocins and the use of slow-growing

breeds) (Meunier et al. 2016; Sima et al. 2018; Ricke 2021). Secondly, strict implementation of biosecurity measures at the abattoir is essential to prevent cross-contamination during the slaughter process (including logistic slaughter) and interventions measures at different processing steps (Musgrove et al. 1997; Bashor et al. 2004; Berrang and Bailey 2009; Lehner et al. 2014; Musavian et al. 2014; Wang et al. 2018). Finally, at the retail level, there is the possibility to reduce *Campylobacter* concentrations in the final product. Worth mentioning is acidification, drying, salting, freezing, the composition of different atmospheric inert gases (Boysen et al. 2007; Rajkovic et al. 2010), UV irradiation (Haughton et al. 2012), high-temperature application (Moore and Madden 2000) or storage at low temperatures (Blankenship and Craven 1982; Bhaduri and Cottrell 2004).

On-farm strict biosecurity has been implemented in numerous countries to control *Campylobacter* colonization of poultry. However, this alone does not diminish *Campylobacter* from poultry. For this reason, major endeavors have been made to develop other intervention strategies to reduce *Campylobacter* colonization of poultry (Newell et al. 2011). Given that, notably, the reduction of *Campylobacter* intestinal colonization farm level appears to be a reasonable and effective strategy to reduce campylobacteriosis in humans (Meunier et al. 2016; Soro et al. 2020; Abd El-Hack et al. 2021), possible non-biosecurity based intervention measures are discussed in detail in this section.

1.5.1. Feed and water additives

In principle, additives added as antimicrobials are considered to inhibit pathogenic bacteria either directly in the feed or water or in the gastrointestinal tract (GIT) after the additive has been consumed (Dittoe et al. 2018). Desirably, both of the aforementioned mechanisms occur simultaneously (Wales et al. 2010).

In the past few years, there has been increasing focus on the use of water and feed additives to control *Campylobacter* carriage in broilers. Various products or substances of different origins (herbal or chemical) have been administered to chickens to minimize horizontal transmission of *Campylobacter*, as fecal-contaminated water or feed have been previously reported as a potential source of infection or reinfection (Pearson et al. 1993; Gibbens et al. 2001; Jones 2001; Newell et al. 2001). Regarding persistence, it has been described that *Campylobacter* can survive especially in water for a prolonged period (Rollins and Colwell 1986). Accordingly, a wide range of studies reported a beneficial effect when acids (organic or fatty acids) were fed alone or as a main active ingredient of commercial or non-commercial feed or water additives.

In the past decade, several commercial acidifying water additives have been tested. Recently, a commercially available water additive (Selko 4Health®) based on short-chain organic acids

and medium-chain fatty acids (MCFA) was added at a concentration of 0.075% to drinking water and reduced *Campylobacter* load in naturally colonized broilers significantly at day 42 in two out of three trials. Nevertheless, *Campylobacter* contamination of carcasses was not minimized after carcass processing (Jansen et al. 2014). Similar observations were made by Chaveerach et al. (Chaveerach et al. 2004) after adding Selko DWB ® to broilers' drinking water. The water additive reduced their cecal carriage by 0.5—2 $\log_{10}$ CFU, dependent on the initial experimental inoculation dose used. In contrast, although effective *in vitro*, Haughton et al. (Haughton et al. 2013) did not observe a reducing effect *in vivo* by acidifying the drinking water of broilers with a commercially available acidified water treatment (PWT (Jones and Hamilton, Walbridge, Ohio, USA)). Even different administration programs (addition of PWT to broiler drinking water for the first seven days, two days before and two days after each feed change and when feed was withdrawn before slaughter or only after feed withdrawal) did not achieve a cecal reduction.

Nevertheless, it should not be neglected that commercially available feed additives have also been tested. For instance, a study conducted by van Gerwe et al. (van Gerwe et al. 2010) determined the effect of feed supplementation with a mixture of MCFA (1% Lodestar™ C8–10) on the susceptibility of broilers for *Campylobacter* intestinal colonization. Remarkably, the authors found the number of *C. jejuni* bacteria required to colonize half of the inoculated broilers was estimated to be 200 times higher in broilers fed with supplemented feed. Conversely, a recently published *in vivo* study, using a commercially available organic acid feed additive, failed to diminish *C. coli* in experimental challenged Cobb-500 broiler chickens at 42 days of age (Mortada et al. 2020). Neither of the two treatments (organic acids only or a combined treatment consisting of probiotics and organic acids) yielded a significant cecal load reduction.

As mentioned earlier, some studies have also been conducted to determine the efficacy of single acids or an individual selected composition (consisting of multiple components) to decrease *Campylobacter* colonization, when added to broiler feed or drinking water.

Recently, Ren et al. (Ren et al. 2021) found malic acid being effective to reduce *Campylobacter* cecal carriage by 1.56 log when added to the drinking water for five consecutive days before slaughter. Interestingly, this reducing effect was not observed when malic acid was administered over a total period of three weeks. The authors concluded that prolonged administration may stimulate the intestinal tract of chickens to self-regulate or induce *Campylobacter* tolerance mechanisms, thereby limiting the decontamination effect. Further, another study demonstrated that acidification of the drinking water using lactic acid, significantly lowered *Campylobacter* concentrations (about 20%) from recovered crop samples

when applied during feed deprivation at the end of fattening (Byrd et al. 2001). Likewise, the addition of monocaprin, the 1-monoglyceride of capric acid, to broilers drinking water and feed proved successful when mature broilers were treated three days before slaughter (Hilmarsson et al. 2006). Solis de Santos et al. (de los Santos et al. 2008; Solis de los Santos et al. 2008; Solis de los Santos et al. 2009; Solís de los Santos et al. 2010) after having carried out four consecutive studies, found a MCFA administered to the feed as a potential treatment to diminish *Campylobacter* colonization. Interestingly, the lower doses were more efficient than the higher doses. Moreover, when 0.7% caprylic acid was supplemented three days before slaughter and feed was withdrawn from respective broiler chickens, *Campylobacter* carriage was also reduced. However varying results were found between trials. Recurring inconsistencies were also observed by Hermans et al. (Hermans et al. 2010; Hermans, Pasmans, et al. 2011; Hermans et al. 2012). The authors concluded that MCFA-supplemented drinking water contributes to broilers being less susceptible to *Campylobacter* colonization when applied before an oral *Campylobacter* challenge. However, results were not reproducible using a seeder bird model, highlighting the difficulty of animal trial standardization. Nevertheless, at least, MCFA was demonstrated to rapidly kill *C. jejuni* in MCFA-treated water, therefore, excluding it as a possible source for *C. jejuni* to enter and colonize a flock (Hermans et al. 2012).

In contrast, Gracia et al. (Gracia et al. 2016) supplemented different additives (including mixtures of essential oils, MCFA, monoglycerides and organic acids to a finishing diet for broilers at slaughter age (21 to 42 days of age) and demonstrated that two of the tested feed additives, namely MCFA and monoglycerides of MCFA, successfully reduced *Campylobacter* carriage. After an experimental challenge at 14 days of age, a blend of MCFA and monoglycerides of MCFA revealed to have a significantly diminishing effect on *Campylobacter* colonization at 21 days post inoculation (dpi) (>0.9 $\log_{10}$ CFU/g). Furthermore, monoglycerides of MCFA also significantly reduced *Campylobacter* prevalence at 28 dpi by 0.88 $\log_{10}$ CFU/g.

A similar approach was carried out by Guyard-Nicodéme et al. (Guyard-Nicodème et al. 2016). The researchers evaluated the ability of different feed additives to reduce *Campylobacter* shedding if fed during a whole fattening period. In total twelve feed additives were tested, covering a wide range of ingredients (organic acids, fatty acids, monoglycerides, plant extracts, prebiotics or probiotics). Again, cecal colonization results varied widely between the different treatment groups. In addition, at 42 days of age, only one feed additive containing short-chain fatty acids (SCFA) (Adimix® Precision) significantly decreased *Campylobacter* load with a mean reduction > 2 $\log_{10}$ CFU/g. Further, a recently published *in vivo* study by Wagle et al. (Wagle et al. 2017) revealed phytophenolic compound, ß-resorcylic acid as a potential antimicrobial feed additive for broilers. ß-resorcylic acid was offered to the experimental diet in

various doses for 14 days. Chickens of breed Cobb500 were experimentally challenged with a four-strain mixture of *C. jejuni* on day 7. At 14 days of age, a dosage of 0.5 and 1% ß-resorcylic acid respectively led to a significant reduction in *Campylobacter* cecal load (by 2.5 and 1.7 log CFU/g).

Likewise, the use of alternative antimicrobials as feed supplements to reduce the intestinal population and distribution rate of *Campylobacte*r in broilers is an emerging area of interest in the poultry industry (Micciche et al. 2019). Numerous studies determined the strong bactericidal activity and thus the efficacy of essential oils against *Campylobacter* (Kollanoor Johny et al. 2010; Hermans, Martel, et al. 2011; van Alphen et al. 2012; Kurekci et al. 2013; Arsi et al. 2014). In an *in vivo* experiment with naturally colonized broilers, Kelly et al. (Kelly et al. 2017) demonstrated the beneficial impact of carvacrol, an essential oil, on *Campylobacter* spp. carriage if added to the broilers' feed in three different concentrations (120, 200 and 300 mg/kg of diet). Carvacrol feed supplementation delayed *Campylobacter* spp. colonization as the presence of *Campylobacter* was only detectable at 35 days of age whereas in the control group *Campylobacter* was detectable already at 21 days of age. The researchers suggested that carvacrol could stimulate an increase of probiotic bacteria in the ceca leading to a reduced *Campylobacter* spp. presence. However, carvacrol supplementation did not lower *Campylobacter* cecal counts.

In fact, due to their different mechanism of action, the combination of organic acids and essential oils seems to have a beneficial synergistic effect, resulting in a better efficacy (Hinton 2006; Grilli et al. 2013). More recently, Sima et al. (Sima et al. 2018) demonstrated a novel phenolic antimicrobial, Auranta 3001 (a mixture of organic acids and plant extracts), as a potential natural strategy to control *Campylobacter.* In their *in vivo* trials, the blend added to drinking water reduced broiler cecal colonization in three replicate experiments from log 8 CFU/ml to log 2 CFU/ml for the used *C. jejuni* strain. However, since the broilers' age (18 days) did not correspond to the usual slaughter age it remains unclear whether the blend achieves the same results in older chickens.

For completeness, new and innovative approaches dealing with other substances should also be mentioned. Just recently, Alrubaye et al. (Alrubaye et al. 2019) successfully pointed out the ability of a secondary bile acid, namely deoxycholic acid to reduce *Campylobacter* carriage in broiler chickens if supplemented to the feed in a concentration of 1.5 g/kg throughout an entire fattening period. In particular, the cecal colonization of *C. jejuni* strain 81-176 was attenuated below the limit of detection at 16 and 28 days of age. Similar results were observed after an experimental challenge with *C. jejuni* strain AR101. Deoxycholic acid effectively reduced *C. jejuni* AR-101 colonization in chickens (2.06×10^6 vs. 2.39×10^7). Interestingly they also

observed an altered microbiome in chickens fed with deoxycholic acid, suggesting a bidirectional interaction between microbiota and microbial metabolites. An *in vivo* approach conducted by Khattak et al. (Khattak et al. 2018) demonstrated a beneficial effect of TYPLEX® Chelate (ferric tyrosine) on *Campylobacter* prevalence. Added to the feed throughout the experiment, TYPLEX® Chelate reduced intestinal colonization up to 2.1 $\log_{10}$ per gram of cecal sample after an artificial challenge with seeded litter. Likewise, Currie et al. (Currie et al. 2018) observed a significant effect of ferric tyrosine on *Campylobacter* cecal load at slaughter age. Overall, ferric tyrosine decreased *Campylobacter* load in naturally colonized broilers by 2–3 $\log_{10}$. In addition, ferric tyrosine revealed to have an advantageous effect on the broilers' weight gain in both of the mentioned approaches (Currie et al. 2018; Khattak et al. 2018).

However, in terms of feed or water additives, it is most challenging to achieve similar results in field studies as observed in experimental studies (Huneau-Salaün et al. 2018). The researchers conducted a randomized control trial, evaluating the decreasing effect of a patented feed additive (ion-exchanged compound) on *Campylobacter* contamination in broilers reared under commercial conditions. The administration of the finisher feed did not have a significant impact on *Campylobacter* load in the chicken ceca, highlighting the need to verify reproducibility under commercial conditions.

1.5.2. Competitive Exclusion-cultures

The gut microbiota composition of broiler chickens has a major impact on host health, growth performance and productivity but also plays a key role in combating the invasion of pathogens, for the proper functionality of the host immune system and for the physiological development of chickens (Yeoman et al. 2012; Clavijo and Flórez 2018; Diaz Carrasco et al. 2019; Takeshita et al. 2021). In fact, the gut microbiota can be exploited as the most valuable biomarker for disease prevention and therapy (Cong and Zhang 2018). Thus, a valuable concept to control *Campylobacter* in poultry flocks is the concept of CE. CE is the administration of non-pathogenic intestinal bacteria (CE culture) from adult chickens to newly hatched chickens to ensure an early development of mature adult-type microflora which reduces the number of pathogenic bacteria and thus improves animal health (Nurmi et al. 1992; Mead 2000).

This concept has proven to be effective to control *Salmonella* in poultry but showed inconsistent potential to lower *Campylobacter* colonization (Aho et al. 1992; Schoeni and Doyle 1992; Schoeni and Wong 1994; Stern 1994; Mead et al. 1996; Mead 2002; Wagenaar et al. 2006). Recently, Ty et al. (Ty et al. 2022) observed reduced *C. jejuni* colonization in Ross 708 broilers 39 days post-hatching, following a single administration of the commercially available CE product Aviguard® via drinking water on the first day of life. Likewise, a research group in Finland demonstrated the efficacy of another commercially available CE product (Broilact®) in

preventing *Campylobacter* colonization in broilers in a 5-week pilot-scale study (Schneitz and Hakkinen 2016). In this study, newly hatched chickens were treated with Broilact® and subsequently introduced to seeder chickens, which had been experimentally challenged with *C. jejuni*. Within the first two weeks, the number of chickens being *Campylobacter* positive as well as their respective cecal colonization was reduced. At slaughter age, Broilact® treatment reduced *Campylobacter* colonization significantly in cecal contents (by 1.4 $\log_{10}$ CFU/g). A similar reduction of cecal carriage using Broilact® was observed previously, as well (Hakkinen and Schneitz 1999).

Schoeni and Wong (Schoeni and Wong 1994) determined a CE culture preparation containing *Citrobacter diversus*, *Klebsiella pneumoniae and Escherichia coli*, originally received from *Campylobacter*-free laying hens, as the most effective treatment. In addition, administering the CE culture with mannose on day one (prevention treatment) led to a 62% decrease in the colonization rate and a high protection factor (>13.2). These results emphasize, that the efficacy of CE culture might be enhanced if administered in a combination with prebiotics (e.g., mannose). In an *in vitro* study Zhang et al. (Zhang et al. 2007) identified *Lactobacillus salivarus*, isolated from CE donor chickens most resistant to *C. jejuni*, as strongly inhibitory to *C. jejuni*.

Interestingly, Laisney et al. (Laisney et al. 2004) indicated that CE efficacy was highly dependent on the respective bird strain. Surprisingly, CE cultures initially received from layer hens prevented *Campylobacter* colonization in layer hens of the same strain (ISA Brown), but the same procedure was ineffective when repeated with broiler chickens (JA957). In this trial, neither CE material from layer hens nor from broiler chickens was able to prevent *Campylobacter* colonization in broiler chickens.

1.5.3. Bacteriophages

Another promising strategy is the use of *Campylobacter*-specific bacteriophages (referred to hereafter as phages) to control *Campylobacter* intestinal carriage in poultry. In biology, phages occupy a truly unique position as they represent the absolute majority of all organisms in the biosphere (Hatfull and Hendrix 2011). Briefly, phages are bacterial viruses that can specifically infect and lyse certain bacterial cells (Kutter 2009; Janež and Loc-Carrillo 2013). Their potential to mitigate *Campylobacter* colonization in chickens has been shown in several trials (experimental as well as field trials) (Loc Carrillo et al. 2005; Wagenaar et al. 2005; El-Shibiny et al. 2009; Carvalho et al. 2010; Fischer et al. 2013; Kittler et al. 2013). Phages can either be applied by using a single or by using multiple phage types (phage cocktail), with phage cocktails most commonly used to mitigate pathogenic bacteria (Abedon 2009; Chan et al. 2013). In fact, using a phage cocktail seems to be the more effective choice. Indeed, the use of phages of different virus types targeting different receptors may decrease the potential risk

of phage resistance (Chan et al. 2013). The latter is a crucial issue as *Campylobacter* may counter phage attack by using different strategies of bacterial defense (Atterbury et al. 2005; Scott et al. 2007; Labrie et al. 2010; Kittler et al. 2014). Such an adaption or resistance formation of *Campylobacter* may not only emerge very quickly but may also compromise the efficacy of phage therapy. The main reason is its unique nature, which allows *Campylobacter* to respond quickly to environmental stimuli (Lee and Newell 2006; Dai et al. 2020). For this reason, it seems reasonable to limit the treatment to a short period (Loc Carrillo et al. 2005; El-Shibiny et al. 2009). Phage specificity provides the ability to accurately target pathogenic bacteria without interfering with the surrounding benign microbiome (Abedon 2009; Galtier et al. 2016; Richards, Connerton, et al. 2019). Recently, Richards et al. (Richards, Connerton, et al. 2019) found a phage cocktail containing two specific virulent *Campylobacter* phages (CP20 and CP30A) as effective to reduce *C. jejuni* cecal colonization in experimentally challenged broiler chickens. In particular, the highest reduction (2.4 $\log_{10}$ CFU $g^{-}1$) of the cecal load was determined two days post-treatment. However, proper phage selection and time are critical for the outcome of the therapy. Consistently, sequential treatment of experimental challenged Vrolix chickens using a group II (CP68) and a group III phage (CP14) yielded the highest reduction in *Campylobacter* counts in fecal samples whereas a concurrent administration of two group III phages (CP14, CP 81) did not yield a significant reduction in the cecum (Hammerl et al. 2014). Wagenaar et al. (Wagenaar et al. 2005) observed reduced *Campylobacter* cecal counts in artificially challenged broiler chickens after offering phages preventively as well as therapeutically. However, the outcome may differ when phages are applied in commercial broiler production. Even though a phage cocktail, administered to broiler chickens via drinking water a few days before slaughter, significantly reduced *Campylobacter* cecal counts in one trial (> 3 $\log_{10}$ units), the same reduction was not achieved in two other replicates (Kittler et al. 2013).

To overcome varying potential of phages as well as *Campyobacter* host cell resistance, the use of novel non-phage technologies such as isolation of phage-derived enzymes (endolysins) has been proposed (Olson et al. 2021). Endolysins are phage-encoded enzymes that degrade peptidoglycan, leading to osmotic imbalance and cell lysis. For example, innolysins targeting *C. jejuni* were recently shown to reduce *C. jejuni* cells on chicken skin by an average of 1.4 log units (Zampara et al. 2021). However, to date, their application is currently more appropriate for gram-positive bacteria, since gram-negative bacteria feature an outer membrane that inhibits entry of endolysins to the peptidoglycan layer (Gutiérrez and Briers 2021).

1.5.4. Breed and stocking density

Breed and stocking density play a major role in animal health. The use of intensive genetic traits (fast-growing broiler breeds) and keeping broiler chickens at high stocking densities have not only been shown to deteriorate their health and welfare (Bessei 2006; Ghosh et al. 2012; Hartcher and Lum 2020) but also to increase the burden of unfavorable bacteria populations in their intestine (Law et al. 2019) thereby promoting the horizontal spread of infectious pathogens. The principle of genetic variation for disease control exploits the lower susceptibility and superior evolutionary potential of certain hosts. Consequently, using selected broiler breeds with a genetic predisposition (e.g., lower *Campylobacter* susceptibility) may contribute to reduce both, *Campylobacter* colonization in commercial poultry and campylobacteriosis cases in humans (Han et al. 2016). Interestingly, such heritable resistance to *C. jejuni* colonization has already been identified in miscellaneous chicken lines (Li et al. 2010; Li et al. 2011; Li et al. 2012; Bailey et al. 2018). Broiler breeds and production systems vary widely between countries. Depending on which production system is used, a specific broiler breed is selected, i.e., Hubbard ISA-JA-757 is currently the most common slow-growing chicken line used for organic farming in Germany and Austria (Ökolandbau.de 2021). Chickens reared in such systems are kept at lower densities than chickens reared under commercial conditions (Williams et al. 2013). Previously, flock size and intensive farming have been found to increase the risk of a flock becoming colonized with *Campylobacter* (Barrios et al. 2006; Guerin et al. 2007; Borck Høg et al. 2016; Seman et al. 2020). Whether, on the other hand, breed or growth profile affects *C. jejuni* colonization is still under debate (Georgiev et al. 2017; Babacan et al. 2020). Slow-growing breeds are thought to carry less *Campylobacter* in their ceca than standard conventional fast-growing breeds, indicating breed-dependent colonization (Bull et al. 2008; Georgiev et al. 2017). In turn, Gormley et al. (Gormley et al. 2014) found no significant difference in cecal load between naturally colonized chickens of different genotypes both in mixed and single broiler breed genotypes. Based on these controversial findings, another research group suggested that protection or resistance might be associated with differences in the cecal microbiota (Chintoan-Uta et al. 2020). However, after conducting multiple trials with inbred chicken lines (resistant and susceptible) and homologous and heterologous cecal microbiota transplants, the cecal microbiome was not significantly altered. Nevertheless, the correlation between breed and colonization risk should be further explored, as factors other than chicken traits may be responsible (Georgiev et al. 2017). For example, Han et al. (Han et al. 2016) demonstrated that in addition to breed also feed composition can influence the outcome of *C. jejuni* colonization, immunity development and the gut microbiota in commercial hybrid layer and broiler-type birds.

1.5.5. Probiotics

Probiotics are live microorganisms that are commonly administered as feed additives to modulate the chickens' microbiome of the GIT. Proper supplementation may ensure favorable effects on the host's health by providing a propitious balance between commensal and pathogenic microbiota in the GIT and by increasing the digestibility and absorbability of nutrients (Smialek et al. 2018). Therefore, they act as a kind of beneficial barrier and antagonist against harmful pathogens by inhibiting their establishment (Van Immerseel et al. 2002; Hume 2011; Clavijo and Flórez 2018). Inhibition occurs both by competition for nutrients and niches (Abd El-Hack et al. 2021) and by altering quorum sensing. The latter thereby alters the virulence factors of pathogens (Medellin-Peña et al. 2007). Studies using microorganisms with anti-*Campylobacter* activity as a preventive strategy against *Campylobacter* colonization have shown large discrepancies concerning intestinal *Campylobacter* load (Meunier et al. 2016; Saint-Cyr et al. 2016). A recently published study postulated *Bacillus* spp. as a potential opponent to compete with *Campylobacter* for growth resources, especially mucin utilization (Shrestha et al. 2017). On the day of hatch, broiler chickens were orally treated with one of four *Bacillus* spp. isolates, grown in the presence or absence of mucin respectively, and later challenged with *C. jejuni*. At 14 days of age, researchers observed that two isolates (one isolate grown with mucin, the other without mucin) consistently reduced cecal *Campylobacter* counts (by 1.5–4 $\log_{10}$ CFU/g) in two independent trials. Similar results were observed by adding a mono-species probiotic product based on two *Bacillus* strains (Calsporin®) to the diet of broiler chickens throughout an entire rearing cycle. At 42 days of age, Calsporin® showed a significant mean reduction in *Campylobacter* counts of 1.70 $\log_{10}$ CFU/g (Guyard-Nicodème et al. 2016). In contrast, another study found that *B. subtilis* DSM 17299 did not significantly reduce cecal *Campylobacter* counts in broiler chickens at 21 or 28 days post-challenge when supplemented in a concentration of 0.05% to the finisher feed (Gracia et al. 2016). Another study using Light Sussex chickens reported that at 20 days of age, *Lactobacillus johnsonii* successfully reduced *C. jejuni* colonization and altered the microbial composition of the chicken gut if given in high doses ($1x10^9$ CFU/ml) directly after hatch and at 7 days of age. However, such a result was only observed in one out of two treatment groups (Mañes-Lázaro et al. 2017). After screening multiple *Lactobacillus* isolates *in vitro,* Dec et al. (Dec et al. 2018), determined seven *Lactobacillus salivarius* isolates as potential feed additives to eliminate *Campylobacter* in broiler chickens. Taha-Abdelaziz et al. (Taha-Abdelaziz et al. 2019) performed an *in vitro* study and found different probiotic lactobacilli species with anti-*Campylobacter* and immunomodulatory activities. In particular, the researchers observed inhibitory effects on *C. jejuni* growth and invasion, a reduced expression of virulence-related genes and an enhanced phagocytosis. Additionally, *Lactobacillus rhamnosus* was shown to

reduce the adhesion efficacy of *C. jejuni in vitro*, most significantly under co-culture conditions. The authors concluded that the supplementation of *Lactobacillus* spp. might reduce the prevalence and transmission of *Campylobacter* spp. in broiler chickens (Šikić Pogačar et al. 2020). Due to a lack of field studies, Smialek et al. (Smialek et al. 2018) evaluated the feasible use of the commercially available probiotic product Lavipan® to diminish *Campylobacter* spp. prevalence in broiler chickens. However, broilers receiving the product during the entire rearing cycle did not show a statistically significant decrease in *Campylobacter* numbers in their intestine at 37 days of age.

1.5.6. Bacteriocins

The use of Bacteriocins (ribosomally-synthesized antimicrobial peptides) produced by various bacterial species (e.g., lactic acid bacteria) (Lin 2009; Saint-Cyr et al. 2016; Hansson et al. 2018), is an auspicious treatment option to decrease the incidence of *Campylobacter* colonization (Johnson et al. 2017). Bacteriocins provide a less expensive alternative to the use of antibiotics (Reid and Friendship 2002; Patterson and Burkholder 2003; Zommiti et al. 2016) and can be easily applied through the broilers' drinking water a few days before slaughter (Svetoch and Stern 2010). They are expected to be involved in the mechanism of CE of probiotics (Abd El-Hack et al. 2021). Svetoch et al. (Svetoch et al. 2005) found different purified bacteriocins from strains of *Paenibacillus polymyxa* and *Bacillus circulans* with antagonistic activity against *Campylobacter* isolates from broiler chickens. The observation of an antagonistic activity is in line with a previous study (Stern et al. 2005a). Zommiti et al. (Zommiti et al. 2016) examined broiler chicken cecal contents for lactic acid bacteria and identified an active peptide (curvaticin DN317) produced by *Lactobacillus curvatus* with high antimicrobial activity against *C. jejuni* ATC 33560. Another approach revealed a possible link between multiresistant *Campylobacter* strains and their sensitivity to enterocins. Among 23 *Campylobacter* strains, Ent A inhibited 52%- while Ent 131 and Ent 9296 inhibited 48% of the *Campylobacter* strains. In total, *Campylobacter* spp. sensitivity to enterocins led to an inhibition activity of 100 antibody units/ml (Ščerbová and Lauková 2016).

With regard to progress toward commercial-scale trials, unfortunately no *in vivo* experiments were conducted in the last few years (Hansson et al. 2018). However, previous approaches showed encouraging results (Stern et al. 2005b; Cole et al. 2006; Stern et al. 2006; Line et al. 2008; Svetoch et al. 2008; Messaoudi et al. 2012).

2. Outline of the study

The trials described herein were conducted between 2018 and 2020 within the framework of the zoonoses research consortium “Preventing and combating *Campylobacter* infections: on track towards a One Health approach (PAC-CAMPY)”, funded by the BMBF (grant no. 01KI1725A). For two experimental groups (organic acids and phages), the data and results are based on samples that were taken and analyzed in cooperation with the University of Veterinary Medicine Hannover (TiHo).

2.1. Objective of the study

The main objective of the “PAC-CAMPY” subproject was to determine the efficacy of different non-biosecurity based intervention measures to reduce *Campylobacter* colonization in broiler chickens.

Therefore, the aims of the project were as follows:

i. Developing an experimental seeder-bird model that can imitate natural *C. jejuni* colonization and spread in a conventional broiler chicken-rearing facility
ii. Evaluating different non-biosecurity based intervention measures and their ability to reduce *C. jejuni* colonization in the intestine of broiler chickens

In our experimental studies, we investigated the effects of carvacrol, a complex CE culture, organic acids, phages and an alternative breed in combination with a reduced stocking density on *Campylobacter* colonization in broiler chickens in a seeder bird model. Three of the aforementioned measures are addressed in publications I-III. Publication I addresses the results of the administration of carvacrol in the broiler feed as a preventive measure if administered during an entire fattening period. Publication II deals with the potential efficacy of a complex CE culture when administered twice during the rearing period. Publication III provides results on the continuous administration of an organic acid cocktail via drinking water.

This work provides an overview of the main results from previous publications as well as previously unpublished data (remaining non-biosecurity based intervention measures).

2.2. Study design

In this study, a total of eight trials were carried out (two preliminary trials and six main trials). For the trials, broiler-hatching eggs of breed Ross 308 (except for one group where the breed was Ranger Gold) were obtained from a commercial hatchery and incubated for 21 days until hatch. Immediately after hatch, 90 (or 20 for the dose-finding experiments) chickens per group were placed on ground floor with litter at a stocking density of 39 kg/m^2 to imitate a commercial broiler chicken husbandry environment. The housing provided filtered air, temperature control

maintained by an electronic thermometer sensor and a programmable light regimen. Throughout the trials, broilers had *ad libitum* access to both, commercial broiler feed (starter, grower, and finisher) and water. Water was refreshed daily and offered via nipple drinkers. All chickens were examined for their general well-being, daily.

In each experimental group, 18 chickens were determined as seeders, 36 as sentinels and 54 as stocking density birds (seeder bird model). Prior, two preliminary trials were carried out to determine the lowest dose necessary to successfully colonize 20 broiler chickens within 2 days. Thus, broilers were orally inoculated with 10^3 and 10^4 CFU/500 µl of a well-characterized *C. jejuni* strain (BfR-CA-14430). Results revealed that a dose consisting of 10^4 CFU/500µl fulfilled the desired requirement and was therefore subsequently used as the inoculation dose for the main trials which were conducted in a seeder bird model.

To examine the effect of different non-biosecurity based intervention measures (specific preventive and therapeutical measures) on *C. jejuni* colonization a seeder bird model was established. One group served as a control group whilst five other groups received one of the following treatments: (i) carvacrol, (ii) a complex CE culture (Aviguard®), (iii) a blend of different organic acids and (iv) a combination of two phages. The last group did not receive any treatment but was conducted with an alternative slow-growing breed (Ranger Gold) in combination with a reduced stocking (25 kg/m^2). The exact treatments are specified in **Table 1**.

Table 1: Treatment groups, doses and application time of the respective treatment

Group	Treatment	Dose	Time of application*	Method
1	control	none	none	none
2	carvacrol[a]	120 mg/kg	4–33	feed
3	CE culture[a/b]	50 mg/ml	1	spray
		125 mg/l	25	water
4	organic acids[a]	16 mmol/l	1–33	water
5	Ranger Gold[a]	none	1–47	N/A
6	phages[b]	10^7 PFU**/ml	29–31	water

*corresponding to days of age; **plaque forming units; [a]preventive measure; [b]therapeutical measure.

In each group, broilers were determined to be *Campylobacter-free* on day 4 of life. Accordingly, *Campylobacter* challenge of the 18 seeders was performed on day 10 of life. At the end of the trial, according to day 33 of life (except Ranger Gold where it was day 47 of life), broiler chickens were euthanized and cecal and colon contents of the sentinels were collected for *Campylobacter* enumeration.

Sampling using cloacal swabs followed a predetermined scheme but deviated in some groups as an adaption was required depending on the appropriate preventive or therapeutic measure (**Table 2**). Fundamentally, cloacal swabs were taken 2, 3 and 4 dpi (equivalent to 12, 13 and 14 days of age), subsequently twice a week until necropsy.

Table 2: Cloacal swab sampling scheme following the corresponding measure

Group	Treatment	Sampling (day of life)
1	control	4, 12, 13 14, 18, 21, 25, 26, 27, 28, 29, 30, 31, 32
2	carvacrol	4, 12, 13, 14, 18, 21, 25, 28
3	CE culture	4, 12, 13, 14, 18, 21, 26, 28
4	organic acids	4, 12, 13, 14, 18, 21, 26, 28
5	Ranger Gold	4, 12, 13, 14, 18, 21, 25, 28, 32, 35, 41
6	phages	4, 12, 18, 29, 30, 31

Lilac: all 90 chickens; red: seeder; blue: seeder and sentinels; green; sentinels

2.3. Proceeding of samples using a semi-quantitative method

Sample analysis was performed according to DIN EN 10272-3 (horizontal method for detection and enumeration of *Campylobacter* spp. - Part 3: semi-quantitative method). The semi-quantitative method is based on qualitative detection in selected dilutions and is expressed in most probable number (MPN) values. To estimate concentration ranges that correspond to a serial dilution, the Poisson distribution is applied. *Campylobacter* counts were estimated using an MPN table modified according to ISO/TS 10272-3:2010/Cor.1:2011(E) (**Table 3**). The highest dilution with confirmed *Campylobacter* growth was used to determine the MPN value.

2.3.1. Cloacal swabs

For semi-quantitative analysis, cloacal swabs were transferred into sterile 5 mL tubes containing 3.0 mL PB, homogenized for 3 seconds and afterward serially diluted 1:10 in PB. Dilutions were incubated for 24 hours at 37°C under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) and then streaked out with 10 µL inoculation loops (Sarstedt, Numbrecht, Germany) on quartered mCCDA plates (with the addition of cefoperazone and amphotericin B selective supplement (Oxoid)) followed by an incubation of 48 hours under the same conditions. Afterward, the plates were examined for *C. jejuni* growth and enumerated. Suspicious colonies were also identified and confirmed using MALDI-TOF MS.

2.3.2. Intestinal samples

Intestinal samples (cecum and colon) were removed sterilely, weighed and diluted 1:8 in PB. Samples were homogenized thoroughly and a 10-fold dilution series was prepared in PB. For enrichment, dilutions were incubated 24h at 37°C under microaerophilic conditions and then

streaked out on quartered mCCDA plates using 10 µL inoculation loops. After an incubation of 48h, plates were examined and *Campylobacter* counts were determined.

Table 3: Result range for dilution series

Quantity (g)	Verified *Campylobacter* growth								
10^0	+	+	+	+	+	+	+	+	+
10^{-1}	-	+	+	+	+	+	+	+	+
10^{-2}	-	-	+	+	+	+	+	+	+
10^{-3}	-	-	-	+	+	+	+	+	+
10^{-4}	-	-	-	-	+	+	+	+	+
10^{-5}	-	-	-	-	-	+	+	+	+
10^{-6}	-	-	-	-	-	-	+	+	+
10^{-7}	-	-	-	-	-	-	-	+	+
10^{-8}	-	-	-	-	-	-	-	-	+
MPN/g	2.3	23	230	2300	23000	230000	2300000	23000000	230000000

+ *Campylobacter* growth; - no *Campylobacter* growth

2.4. Statistical Analysis

The experimental data were analyzed using SPSS software version 25.0 for Windows (SPSS, Inc., Chicago, IL). The data were analyzed for normal distribution using the Shapirow-Wilk Test. As the data was 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. Prior to the start of the study, a statistical report was provided by the Institute for Veterinary Epidemiology and Biostatistics of Freie Universität Berlin. To ensure alpha error of 0.05, beta error of 0.18 and power of 0.80, 90 animals per group were required in the present study. In order to determine statistically significant differences, 36 animals were sampled during the experiment and the differences were 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.

2.5. Ethics

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

3. Publications

3.1. Publication I

Szott, V.; Reichelt, B.; Alter, T.; Friese, A.; Roesler, U. (2020): ***In vivo* efficacy of carvacrol on *Campylobacter jejuni* prevalence in broiler chickens during an entire fattening period.** In: *European Journal of Microbiology and Immunology,* 10(3):131-138.

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ORIGINAL RESEARCH PAPER

ABSTRACT

Carvacrol, a primary constituent of plant essential oils (EOs), and its antimicrobial activity have been the subject of many *in vitro* studies. Due to an increasing demand for alternative antimicrobials and an emerging number of antibiotic resistant bacteria, the use of essential oils has played a major role in many recent approaches to reduce *Campylobacter* colonization in poultry before slaughter age. For that purpose, the reducing effect of carvacrol on *Campylobacter jejuni* prevalence in broilers was determined *in vivo* in an experimental broiler chicken model during an entire fattening period. Carvacrol was added to the feed in a concentration of 120 mg/kg feed four days post hatch until the end of the trial. In this study, we demonstrated a statistically significant decrease of *C. jejuni* counts by 1.17 decadic logarithm ($\log_{10}$) most probable number (MPN)/g in cloacal swabs during starter and grower periods (corresponding to a broilers age between 1 and 28 days). Similar results were observed for colon enumeration at the end of the trial where *C. jejuni* counts were significantly reduced by 1.25 $\log_{10}$ MPN/g. However, carvacrol did not successfully reduce *Campylobacter* cecal colonization in 33-day-old broilers.

KEYWORDS

Campylobacter jejuni, carvacrol, broiler, essential oils, feed additive, seeder bird, prevalence

INTRODUCTION

Campylobacter gastroenteritis in humans is an issue of major clinical importance worldwide [1]. Since 2005 *Campylobacter* is the most commonly reported zoonotic agent in the European Union (EU). In 2017, there were 246,158 reported human campylobacteriosis cases in the EU whereas *Campylobacter jejuni* (*C. jejuni*) was the most commonly isolated species and fresh broiler meat in turn the most frequent source of infection [2].

Campylobacter is highly prevalent in broiler flocks with 60–80 % of the flocks being affected [3–5]. The bacterium preferably colonizes the cecal and cloacal crypts as well as the colon with high prevalence up to 10^9 colony forming units (cfu)/g [6–8]. In broiler chickens, *C. jejuni* colonization is related to possible carcass contamination during the slaughtering process [3, 9, 10]. Even small amounts of cecal content suffice to contaminate poultry products [10]. As a result, already a 2 $\log_{10}$ reduction of *C. jejuni* counts on broiler carcasses is estimated to substantially decrease the risk of human campylobacteriosis [9–13].

Although there are many approaches to reduce *Campylobacter* prevalence in broilers (such as feed additives, pre- and probiotics, vaccination, bacteriocins and bacteriophages), none of them has proven to be sufficient so far [10, 12, 14–17], leading to a need for further studies. Even more concerning, a recently published report stated that in the EU *C. jejuni* shows increasing resistance levels to fluoroquinolones (57.7%) and tetracyclines (45.5%) in

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humans. These levels of resistance are classified as high [18] which supports and highlights the need to develop alternative antimicrobials.

Essential oils, especially their phenolic compounds, revealed to have an antimicrobial effect against various bacteria [19, 20]. Their antimicrobial activity can be explained by their hydrophobicity [21, 22] and their ability to disintegrate the outer membrane of bacteria [23–25]. Moreover, studies indicated that EOs could also alter the mucosal layer through microbiome modulation [26–29]. Therefore, the use of EOs has become a promising alternative to the conventional antimicrobials [29, 30].

Furthermore, previous studies indicate that *Campylobacter* prefers a characteristic amino acid pattern for its metabolism [31, 32] since it is not dependent on carbohydrate fermentation. Due to protein-rich diets the broilers' ceca contain large amounts of these specific amino acids facilitating *Campylobacter* colonization [29, 33–35]. It is suggested that EOs modulate and influence ileal amino acid absorption [36] which could confine an essential source of nutrients of *Campylobacter* [29, 37]. As a result, it seems possible that such lack of nutrients could effectively reduce cecal *Campylobacter* colonization. Moreover, supplementation of EOs has been described to have beneficial effects on body weight, growth rate and feed conversion [38–41]. A supplementation is able to improve feed digestibility [42] and nutrient absorption [29].

Carvacrol is a component of many different EOs, e.g. thyme and oregano oil, and its antimicrobial activity was examined against *Campylobacter* in various *in vitro* studies [15, 22, 43].

A previous *in vitro* study indicates that carvacrol is able to suppress *C. jejuni* in cecal content [20]. However, it has not been sufficiently established whether carvacrol can be used to reduce the *C. jejuni* prevalence in broiler chickens [13]. For this reason, an *in vivo* study with a seeder bird model was performed aiming to evaluate the ability of carvacrol to reduce *C. jejuni* colonization in broiler chickens if supplemented during an entire fattening period.

MATERIALS AND METHODS

Study design

The trials were carried out in the experimental animal facility of the Centre for Infection Medicine of the Department for Veterinary Medicine of Freie Universität Berlin. For the experiments, in total 180 broiler hatching eggs (aerosol disinfected with formaline) of breed Ross 308 were obtained from a commercial hatchery. The eggs were incubated for 21 days until hatch. Meanwhile all facilities were cleaned, disinfected (by evaporated H_2O_2) and tested for the absence of *Campylobacter* by taking various gauze swabs soaked in sterile phosphate-buffered saline (PBS; Oxoid, Wesel, Germany). Gauze swabs were processed according to DIN EN ISO 10272 and found to be free of *Campylobacter*.

Prior to the beginning of the trial, the 180 broiler chickens (males and females) were divided into two groups (n = 90 per group): a positive control group (challenged with *Campylobacter* and not treated – T1) and a carvacrol group (challenged with *Campylobacter* and treated with the experimental feed additive – T2). Immediately after hatch, 90 chickens per group were placed on ground floor with litter at a stocking density of 39 kg/m^2 in order to imitate a commercial broiler chicken husbandry environment. The experimental facility provided filtered air, temperature control maintained by an electronic thermometer sensor and a programmable light regimen. Broilers had access to commercial broiler feed and water *ad libitum* throughout the study period.

On day 1 of age, each chick was randomly assigned with an individual consecutive number for distinguishing between seeders (n = 18), sentinels (n = 36) and stocking density broilers (n = 36).

On day 10 of age, the seeders were orally challenged with approximately 10^4 cfu/500 µL of *C. jejuni* aiming to reproduce a natural way of infection within the broilers, as the bacteria will distribute from the seeders to the contact animals (sentinels and stocking density broilers).

In order to examine the effect of carvacrol, broilers were fed a standard diet (starter, grower, finisher) until the end of fattening (average weight 2.0 kg) whereas the feed of the carvacrol group was supplemented with 120 mg/kg feed of carvacrol (Sigma-Aldrich, Munich, Germany), each.

During the trials, animal health parameters, feed intake and weight gain were monitored and recorded daily to observe possible carvacrol effects. At the end of the trial, broiler chickens were euthanized and cecal and colon contents of the sentinels were collected for *Campylobacter* enumeration.

Bacterial strain and broiler inoculation

The *C. jejuni* reference strain BfR-CA-14430 was isolated from poultry origin (chicken breast) and was provided by the Federal Institute for Risk Assessment (BfR). The strain is characterized by whole genome sequencing (WGS) and multilocus sequence typing (MLST). It belongs to the ST-21 complex. *Campylobacter* stock cultures were grown for 24 h at 37 °C under microaerophilic conditions (85% nitrogen, 10% carbon dioxide, 5% oxygen) in Preston Broth (PB) supplemented with Preston *Campylobacter* selective Supplement (SR0117; Oxoid, Wesel, Germany), Growth Supplement (SR0232; Oxoid, Wesel, Germany) and defibrinated horse blood (SR0050; Oxoid, Wesel, Germany) and then stored at −80 °C in microbank vials (Mast Diagnostica, Germany). 48 hours before inoculation the strain was freshly recovered from frozen stocks and streaked out on Columbia Blood Agar (CBA) with 5% sheep blood (Fisher Scientific, Germany). Plates were incubated at 37 °C under microaerophilic conditions in a tri-gas incubator (CB 160; Binder, Germany). After incubation, single colonies were resuspended in 4.0 mL Muller-Hinton Broth (MHB) (Oxoid, Wesel, Germany) to achieve an optical density of 0.4 at a

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wavelength of 600 nm (OD_{600}) in order to obtain an inoculum containing 1×10^6 cfu/mL. This suspension was diluted two times 1:10 in MHB to receive an inoculum amounting to approximately 10^4 cfu/mL. The inoculum was filled in 1 mL syringes and closed with plugs. Immediately after, seeders where orally challenged with 0.5 mL of the prepared bacteria suspension. The dose necessary for colonization was determined in previous dose-finding experiments (data not shown). For enumeration of *C. jejuni,* 10-fold dilutions were plated on modified *Campylobacter*-selective charcoal cefoperazone deoxycholate agar (CCDA) plates (CM0739; Oxoid, Wesel, Germany) supplemented with CCDA selective supplement (SR0155; Oxoid, Wesel, Germany) and incubated 48 h at 37 °C under microaerophilic conditions.

Experimental diets and admixture of carvacrol

A three-phase feeding program diet for broilers matching the commercial standard served as experimental diet as shown in Table 1. Starter diet was offered to the broilers up to day 8 of age, followed by a grower diet until one week before necropsy (day 9–26) and a finisher diet, which was fed from day 27–33. To examine the effect of carvacrol, the carvacrol group (T2) was fed, with 120 mg/kg feed of carvacrol with a purity of >98% beginning at day four of age. To ensure uniform mixing, the carvacrol was vaporized in a small amount of feed and then carefully mixed with the rest of the feed. In order to decrease destabilizing effects, 25.0 kg of the carvacrol-supplemented feed was prepared on demand and stored in airtight containers.

Sampling design and sampling preparation

On day 4 of age, all animals were monitored for *Campylobacter* by taking cloacal swabs (Sarstedt, Nümbrecht, Germany). For qualitative detection, the swabs were processed according to DIN EN ISO 10272. The swabs were transferred into sterile tubes containing 3.0 mL PB, incubated for 24 hours at 37 °C under microaerophilic conditions and then streaked out with 10 µL inoculation loops (Sarstedt, Nümbrecht, Germany) on mCCDA agar plates followed by a 48 hours incubation under the same conditions. Afterward, the plates were examined for the absence of *C. jejuni*. Suspicious *Campylobacter* colonies were analyzed using a Bruker Microflex™ system for matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

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
MJ ME[a]	12.4	12.4	12.4
Calcium (%)	0.9	0.8	0.8
Phosphorous (%)	0.6	0.55	0.5
Sodium (%)	0.14	0.14	0.14
Methionine (%)	0.55	0.50	0.50
Lysine (%)	1.25	1.15	1.05

[a]Megajoules of metabolizable energy.

In order to compare the results, the same 36 sentinels (untreated broilers) were sampled in both groups throughout the study by taking cloacal swabs at defined points in time: 48 hours, 72 hours and 96 hours post inoculation (equivalent to day 12, 13 and 14 of age), subsequently twice a week (equivalent to day 8, 11, 15 and 18 post inoculation, respectively) until necropsy. Successful *C. jejuni* colonization of the seeders was verified by taking cloacal swabs 48 hours after inoculation. Cloacal swabs were prepared as described above and analyzed semiquantitatively according to DIN EN ISO 10272-3 to determine levels of *Campylobacter* colonization and load. For semiquantitative analysis cloacal swabs were homogenized for 3 s in 3.0 mL PB using a vortex shaker (VWR, Darmstadt, Germany) and afterward serially diluted 1:10 in PB. Dilutions were incubated 24 h at 37 °C under microaerophilic conditions and then streaked out on mCCDA plates as described above.

On day 33 of age (average weight 2.0 kg) all 36 sentinels per group were euthanized using ZKS poultry pliers (Corstechnology, Neerstedt, Netherlands) after confirming deep anesthesia. The animals were dissected and intestinal content (cecum and colon) was collected for *C. jejuni* enumeration and determination. Necropsy samples were prepared to perform semiquantitative analysis according to DIN EN ISO 10272-3. For that purpose, intestinal contents were removed sterile and diluted 1:8 in PB. After homogenization, a 10-fold dilution series was prepared in PB. For enrichment, diluted intestinal samples were incubated 24 h at 37 °C under microaerophilic conditions, then streaked out on mCCDA plates and incubated as described above.

Statistical analysis

The experimental data was analyzed using SPSS software version 25.0 for Windows (SPSS, Inc., Chicago, IL, United States). The sample size of 36 animals was chosen to obtain statistical representative results. *Campylobacter* counts were logarithmically transformed ($\log_{10}$) and then analyzed for significant differences using the non-parametric Mann-Whitney U test. For each sampling, the mean obtained from the treated group was compared to the mean received from the control group. *P*-values below 0.05 were regarded statistically significant.

Ethics

All experimental animal procedures were approved in accordance with the German Animal Welfare Act by the State Office of Health and Social Affairs Berlin, Germany

(Landesamt für Gesundheit und Soziales Berlin, LAGeSo) under the registration number G 0098/18. The study was conducted according to the institutional guideline for animal welfare of the Freie Universität Berlin.

RESULTS

The semiquantitative results of the control and the carvacrol group are shown in Fig. 1. *Campylobacter* counts are presented in $\log_{10}$ most probable number (MPN)/g. Before oral inoculation all broilers were found to be free from *Campylobacter* as bacteriological analysis revealed no detectable *Campylobacter* growth. Given that the 36 sentinels in both groups were positive for *C. jejuni* 8 days post inoculation (pi) dietary treatment with carvacrol was not able to delay *C. jejuni* colonization. Nevertheless, *Campylobacter* counts in cloacal swabs were significantly and consistently reduced ($P \leq 0.02$) for the carvacrol group in comparison to the control group at any point in time (8, 11, 15 and 18 days pi) (Fig. 1A). Eight and 11 days pi the highest difference in *C. jejuni* counts between the treated group (mean value 4.2 and 4.6 $\log_{10}$ MPN/g) and the control group (mean value 5.2 and 5.8 $\log_{10}$ MPN/g) could be observed. These results correspond to a mean reduction of $\geq$1 $\log_{10}$ MPN/g (at 8d pi $P < 0.0001$; $r = 0.53$ and at 11d pi $P < 0.0001$; $r = 0.45$) respectively. 15 and 18 days pi carvacrol treated animals still had significantly decreased bacterial counts (5.1 and 5.4 $\log_{10}$ MPN/g) in comparison to the control group (5.8 and 5.97 $\log_{10}$ MPN/g). However, mean reduction was <1 $\log_{10}$ MPN/g (at 15d pi $P < 0.0001$; $r = 0.46$ and at 18d pi $P = 0.02$; $r = 0.28$).

Nevertheless, carvacrol feed supplementation failed to reduce *Campylobacter* cecal colonization (Fig. 1B). Comparing both groups, *C. jejuni* counts in the ceca showed no significant difference ($P > 0.05$). However, significantly reduced *C. jejuni* numbers in the colon of carvacrol treated animals could be observed ($P < 0.0001$; $r = 0.53$) in comparison to the control group (Fig. 1B).

In addition, carvacrol did not have an effect on the animals' growth performance. In comparison to the control group, the treated group showed no significant difference ($P > 0.05$) regarding weight gain and carcass weight (data not shown).

DISCUSSION

There are a few *in vivo* approaches which recently described the effects of carvacrol usage [13, 15, 41]. Nevertheless, to our knowledge none of these trials have been conducted in a seeder bird model and during an entire fattening period until slaughter age. In this study, we analyzed the ability of carvacrol to reduce *Campylobacter* carriage in broilers if supplemented to the feed in a concentration of 120 mg/kg throughout an entire fattening period. By using a seeder bird model, we aimed to achieve a better comparability with conventional animal husbandries. In particular, we aimed to evaluate the *in vivo* effect of carvacrol on *Campylobacter* prevalence on a herd and individual broiler level.

With regard to the applied dosage it is to note that carvacrol is so far only approved in one single commercial zootechnical feed additive for chicken fattening in an average amount of 5 mg/kg feed under Commission Implementing Regulation (EU) 2015/1490. However, instead of using this exact amount we decided to apply carvacrol in a 24 times higher concentration since this dosage has already shown promising reducing effects on *C. jejuni* colonization in a previous *in vitro* and *in vivo* study [41]. *In vitro* results yielded by using a gentamicin protection assay revealed that the presence of carvacrol (120 mg/kg feed) significantly reduced the adhesion and invasion of a highly virulent *C. jejuni* RC039 isolate to chicken intestinal primary cells. Obviously, there is a huge discrepancy between the amount used in this study and the minimum inhibitory

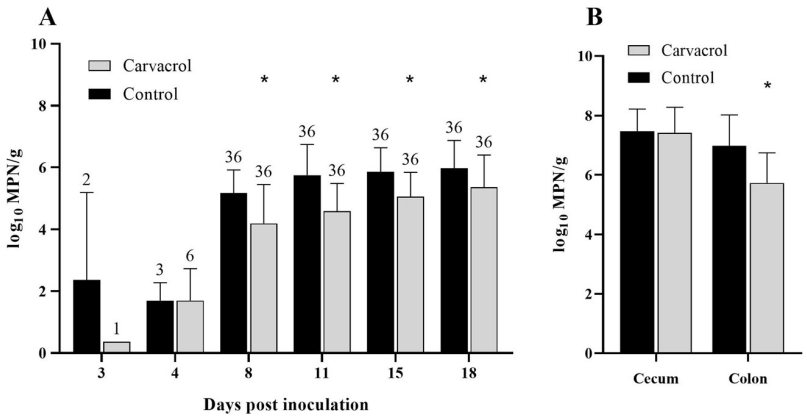

Fig. 1. (A) Mean $\log_{10}$ most probable number (MPN) of *C. jejuni* per gram in cloacal swabs at each point in time after inoculation. (B) Mean $\log_{10}$ MPN of *C. jejuni* per gram in intestinal content. Black bars represent the control group (broilers challenged with *C. jejuni* and not treated with carvacrol); gray bars represent broilers challenged with *C. jejuni* and treated with 120 mg/kg feed of carvacrol. The data presented was obtained from 36 broilers/group after necropsy. Bars marked by an asterisk differ significantly ($P < 0.05$).

concentration (MIC) of carvacrol against *C. jejuni* determined in previous *in vitro* studies [15, 44, 45]. However, the determined MIC values for carvacrol against *C. jejuni* vary considerably (0.006–0.2%) depending on the employed techniques [45] and the MIC definitions [46] used. Also, in practice, carvacrol can only be applied in relatively low concentrations, given that already dietary carvacrol in the amount of 200 mg/kg significantly lowers the broilers' feed intake and weight gain [47], highlighting that a MIC of carvacrol necessary to inhibit *C. jejuni* may compromise animal health as well as growth performance. Finally, Alphen et al. [48] demonstrated in an *in vitro* study that even sub-inhibitory concentrations (subMIC) of carvacrol (0.2 mM) which do not affect bacterial growth are able to attenuate *C. jejuni* virulence and protect against cellular infection.

The results of this *in vivo* study demonstrate that a supplementation with 120 mg/kg feed of carvacrol is able to reduce *C. jejuni* load in cloacal swabs significantly at a 0.6 $\log_{10}$ minimum. As McLendon et al. [49] enlightened in a recent study, the use of cloacal swabs is a reliable method to detect *Campylobacter*. Also, Glünder [50] reported a high correlation between the analysis of cloacal swabs and cecal content. Therefore, we assume that the bacteria prevalence determined by cloacal swabs is representative for cecal *C. jejuni* colonization. Moreover, we could observe a significant *C. jejuni* decrease in the broilers' colon at the end of the trial at necropsy. Since we could not only observe a consistent *C. jejuni* reduction in cloacal swabs during the entire fattening period but also in the colon at the end of the trial it is reasonable to assume that a carvacrol supplemented diet likely leads to a reduced fecal contamination with *C. jejuni* at slaughter and to a reduction of cecal colonization.

Nevertheless, the cecal counts showed no significant difference in 33-day-old broilers compared to the control group. A possible reason for the limited efficacy of carvacrol at the end of fattening may be the chosen dosage. Our results suggest that the selected dosage was able to decrease *C. jejuni* colonization during animal starter and grower periods but that at the end of fattening the dosage was insufficient. A study in which Arsi et al. [13] demonstrated that *Campylobacter* cecal counts were significantly reduced in 10-day-old broiler chicken (orally challenged with *C. jejuni* on day 3 with $1x10^7$ cfu/mL) if they were fed with 1% carvacrol or an EOs combination of 0.5% carvacrol and thymol supports this assumption. Another explanation for the limited effect of carvacrol at the end of the fattening period could be a feed uptake reduction consequently leading to a lower carvacrol uptake. Broiler chicken in their starter and grower periods show a higher individual feed consumption than broilers at the end of fattening [51]. Also, a feed uptake reduction could affect the stability of the EOs due to an extended residence time in the feed as EOs are volatile and thermolabile. Depending on their structure, EOs easily oxidize [52, 53] possibly leading to terpenoid deterioration [53]. However, Turek and Stintzing [53, 54] reported that EOs from thyme, including carvacrol tend to have a good storage stability.

It is also possible that components in the poultry diets itself limited the efficacy of carvacrol due to an altered cecal microbiome [15, 16]. Recent studies indicate a possible correlation between crude protein and *C. jejuni* colonization. Moreover, it was shown that diets based on corn or wheat containing different levels of crude protein were able to modify broiler gut viscosity and histomorphology [16] and as a result could reduce *C. jejuni* colonization [11]. In a previous study where layer hens and broiler were orally inoculated with *C. jejuni* 1 day post hatch and fed either with broiler or layer feed Han et al. [55] also demonstrated the possible role of crude protein. The diets differed in their composition with crude protein and fat levels in broiler feed being higher than in layer feed. It was observed that layer hens fed with broiler feed were higher colonized than layer hens fed with layer feed. It is our assumption that protein rich diets overload the carvacrol-enhanced ileal amino acid absorption capacity as described above. Moreover, the interaction of high crude proportion in poultry diets and lower feed and carvacrol uptake at the end of fattening could consequently decrease the inhibitory effect on *Campylobacter* cecal colonization and might therefore explain the limited effect of carvacrol at the end of fattening.

Interestingly, Kelly et al. [41] observed in an *in vivo* study in which naturally colonized broilers were fed with three different concentrations of carvacrol (120, 200 and 300 mg/kg of diet) that carvacrol feed supplementation delayed *Campylobacter* spp. colonization as the presence of *Campylobacter* was only detectable at day 35 of age whereas in the control group *Campylobacter* was detectable already at 21 days of age. Cecal content quantification showed that treated broilers did not have significantly lower *Campylobacter* cecal counts at 35 days of age, which correspond with our observations. However, we were not able to observe such delay in colonization. In fact, 8 days pi all sentinels were tested positive for *C. jejuni*. Other *in vivo* studies which examined the effect of different feed additives or a feed additive containing organic acids and botanicals could not observe a delay in *C. jejuni* colonization either [15, 17]. This may be due to the difference between the colonization models [56]. In our approach, we orally inoculated 18 seeders artificially. A previous mathematical model suggests that one seeder will colonize 1.04 broilers per day successfully [57]. According to the model 3 days pi all broilers should be colonized successfully. Considering the digestive time to process *C. jejuni* [56] it is very likely to determine the bacteria in the animals 6–8 days pi. In addition, seeders were tested positive for *C. jejuni* 48 hours pi, which suggests that carvacrol supplementation is not able to prevent colonization in artificially colonized broilers. As a result, it is unlikely to observe a colonization delay in sentinels since seeders already started shedding the bacterium.

For further experiments it could be beneficial to add microencapsulated EOs to the feed because this procedure prevents certain components of the EOs from being absorbed or enzymatically decomposed before reaching their destination [13, 29, 58] and may therefore improve their

efficacy [13, 29, 30]. In an *in vivo* attempt, Grilli et al. [15] administered an organic acid and EO combination micro-encapsulated to broiler chickens. Cecal content enumeration showed significantly reduced *C. jejuni* counts.

In addition, it appears that an enhanced *in vivo* efficacy of EOs may be achieved through synergistic effects [15, 29, 30]. Skånseng et al. [59] performed an *in vivo* study in which broilers were fed a feed additive containing a combination of two acids (sorbate and formic acid) and observed similar results. While a combination of these two acids was able to prevent *C. jejuni* colonization in cecal contents completely, this effect could not be achieved by adding a single acid to the feed.

CONCLUSION

To our knowledge, this is the first *in vivo* study which assessed the effect of carvacrol in a seeder bird model and during an entire fattening period simultaneously. The present study demonstrates promising effects of carvacrol on *C. jejuni* colonization. Carvacrol feed supplementation decreased *C. jejuni* counts during broiler starter and grower periods while this effect could also be observed in colon contents but not in cecal contents at the end of the trial. Therefore, it is reasonable to assume that carvacrol in a concentration lower than 120 mg/kg may fail to reduce *Campylobacter* cecal colonization *in vivo* which is in contrast to the currently approved amount of carvacrol as a feed additive. In order to achieve reduced *Campylobacter* numbers in the cecum there are two promising options which could be worthwhile for further investigation: on the one hand, supplementing carvacrol in a combination with organic acids or other efficient supplements, on the other hand, supplementing increasing levels of carvacrol over the fattening period in relation to the broilers' body weight and their feed uptake. At the same time however, adverse effects on growth performance should be taken into account. In addition, the described experimental approach should be carried out with a subsequent experimental slaughter, in which the *Campylobacter* numbers in the cecum, in the colon and on the carcasses are examined.

In conclusion, successful establishment of pre-harvest interventions measures requires further *in vivo* examinations on the effect of carvacrol, especially on its mode of action and physiological pathways within the broilers gut. Once its effects have been understood entirely, intervention measures could target and control *Campylobacter* successfully and subsequently may lead to a decreased risk of human infections. If future *in vivo* experiments or field studies confirmed the efficacy of carvacrol to diminish *Campylobacter* colonization it would be desirable to approve carvacrol as a feed additive in higher amounts. In addition, verification of the commercial suitability and economic efficiency of the use of carvacrol as a feed additive for the poultry industry is needed.

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Authors' Contributions: VS performed the experiments, collected, analyzed and interpreted the data, and drafted the manuscript and figures, with critical evaluation and support of all other authors. BR helped with the experiments and laboratory work. AF and UR conceived and designed the study, as well as critically revised the manuscript. All authors approved the final version to be published.

Conflicts of interest: The authors declare that they have no conflict of interest.

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3.2. Publication II

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Article

A Complex Competitive Exclusion Culture Reduces *Campylobacter jejuni* Colonization in Broiler Chickens at Slaughter Age In Vivo

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Abstract: Diminishing *Campylobacter* prevalence in poultry flocks has proven to be extremely challenging. To date, efficacious control measures to reduce *Campylobacter* prevalence are still missing. A potential approach to control *Campylobacter* in modern poultry productions is to occupy its niche in the mucosal layer by administering live intestinal microbiota from adult chickens to dayold-chicks (competitive exclusion (CE)). Therefore, this in vivo study investigates the efficacy of a complex CE culture to reduce *Campylobacter* (*C.*) *jejuni* colonization in broiler chickens. For this purpose, the complex CE culture was applied twice: once by spray application to day-old chicks immediately after hatching (on the 1st day of life) and subsequently by an additional application via drinking water on the 25th day of life. We observed a consistent and statistically significant reduction of *C. jejuni* counts in cloacal swabs throughout the entire fattening period. At the end of the trial after necropsy (at 33 days of age), *C. jejuni* cecal counts also showed a statistically significant decrease of 1 $\log_{10}$ MPN/g compared to the control group. Likewise, colon counts were reduced by 2.0 $\log_{10}$ MPN/g. These results suggest that CE cultures can be considered a practically relevant control strategy to reduce *C. jejuni* colonization in broiler chickens on poultry farms.

Keywords: *Campylobacter*; competitive exclusion; CE culture; broiler; control measure; microbiome; intervention strategy

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1. Introduction

Campylobacter is still considered a cause of concern in broiler production as it is the most frequently reported food-borne pathogen in the European Union (EU). In 2019 there were 220,682 confirmed cases of human campylobacteriosis. Although there are many approaches to reduce the burden of *Campylobacter* in poultry, the number of European campylobacteriosis cases in humans remained stable between 2015 and 2019 [1]. Since *Campylobacter* preferentially colonizes the poultry intestinal tract, the elimination in the poultry reservoir must be considered a key step to successfully combat the bacterium in the food chain [2].

In this context, preventing *Campylobacter* contamination in poultry farms remains a major challenge given its ubiquitous occurrence in the environment [3–6]. On farm, control strategies such as the establishment of well-implemented hygiene protocols have shown to lower the incidence of *Campylobacter* [7,8]. Nevertheless, strict adherence to biosecurity measures does not guarantee that broilers do not become colonized with *Campylobacter* during an entire fattening period [9]. For this reason, it is essential to introduce alternative control strategies to keep *Campylobacter* prevalence in poultry flocks as low as possible [10]. This is because any properly implemented control measure can reduce the likelihood of *Campylobacter* colonization of a poultry flock [11]. So far, effective and applicable measures are still missing or insufficient [10].

A feasible strategy to control *Campylobacter* in poultry flocks is the concept of competitive exclusion (CE). CE is the administration of non-pathogenic intestinal bacteria from adult-chickens to newly hatched-chickens to ensure an early development of mature adult-type microflora that improves animal health and as a result reduces the quantity of pathogenic bacteria [12–14]. CE culture treatment has proven effective against *Salmonella* colonization in young chickens [14–16] but showed inconsistent effects on *Campylobacter* colonization [17–23].

A complex mixture of viable commensal bacterial cultures (CE culture) originally isolated from the cecal microbiota of specific pathogen-free adult chickens was used for this study. The CE culture is applicable for chickens and turkeys either as spray treatment immediately after hatch or as drinking water application during the growth phase. The use as a spray aims to increase the broilers' resistance to subsequent infections caused by harmful bacteria. In addition, when used as a drinking water application after an antibiotic therapy, CE promotes the reestablishment of a balanced microbiota composition in the chicken intestine [12,14]. This second treatment during rearing is expected to boost the effect of CE and consequently contribute to reduced *Campylobacter* colonization [24].

The objective of this study was to determine the efficacy of a complex commercially available CE culture on *Campylobacter* (*C.*) *jejuni* colonization when administered both via spray application and via drinking water to broiler chickens in an in vivo experimental seeder bird model.

2. Materials and Methods

2.1. Ethics Statement

This study was carried out in accordance with the National Animal Protection Guidelines. The protocol was 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, permission number G 0098/18).

2.2. Experimental Animal Trial

The study was performed in the experimental facilities of the Centre for Infection Medicine of the Department for Veterinary Medicine of Freie Universität Berlin (biosafety level 2, law on genetic engineering). According to strict, established hygiene management, clothes and shoes were changed in an adjacent separate anteroom before entering the experimental animal facility.

Prior to the beginning of the trials, the experimental units, which had been previously cleaned and disinfected with hydrogen peroxide, were tested for the presence of *Campylobacter* by taking various gauze swabs soaked in sterile phosphate-buffered saline (PBS; Oxoid, Wesel, Germany). Gauze swabs were prepared to perform qualitative *Campylobacter* analysis according to DIN EN ISO 10272-1:2017-09. For this purpose, five selected areas of 10 × 10 cm were individually swabbed. The gauze swabs were transferred to sterile plastic seward stomacher filter bags (Norfolk, UK) containing 20 mL Preston broth (PB) supplemented with Preston *Campylobacter* selective supplement (SR0117; Oxoid, Wesel, Germany), growth supplement (SR0232; Oxoid, Wesel, Germany), and defibrinated horse blood (SR0050; Oxoid, Wesel, Germany). Gauze swabs were homogenized for 2 min at 200 rpm using a stomacher (Seward Stomacher 400 Lab System, Norfolk, UK) and afterwards incubated for 24 h at 37 °C under microaerophilic conditions (85% nitrogen, 10% carbon dioxide, 5% oxygen). One loop material per gauze swab was spread onto modified *Campylobacter*-selective charcoal cefoperazone deoxycholate agar (mCCDA) plates (CM0739; Oxoid, Wesel, Germany) supplemented with CCDA selective supplement (SR0155; Oxoid, Wesel, Germany) using 10 µL inoculation loops (Sarstedt, Nümbrecht, Germany). Plates were incubated for 48 h under microaerophilic atmosphere and examined for *Campylobacter* growth.

For the trials, 180 eggs of broiler breed Ross 308 (both aerosol disinfected with formalin and liquid disinfected with WESSOCLEAN® K 50 Gold Line (Wesso AG, Hersbruck, Germany) were received from a commercial poultry production and incubated for 21 days

until hatch. Newly hatched chickens of both sexes were then randomly assigned to two groups of 90 chickens each: either the positive control group (challenged by oral gavage with *Campylobacter* and not treated—T1) or the CE group (challenged by oral gavage with *Campylobacter* and treated with the complex CE culture—T2). In order to imitate a commercial broiler chicken husbandry, broiler chickens were housed in separate units at a stocking density of 39 kg/m^2 on ground floor with fresh litter (1 kg/m^2)—litter was neither removed nor added throughout the experimental period. Temperature, filtered air (ventilation and HEPA filtration of the exhaust air), and light were controlled throughout the entire study period and adjusted properly as to the broilers' age. Broilers had access to drinking water (tap water) and feed *ad libitum.* The chickens received a conventional three-phase diet as shown in Table 1. Fresh water was provided on a daily basis. On the 1st day of life, each chick was randomly tagged with a unique number to distinguish between seeders (orally inoculated with *C. jejuni*, n = 18), sentinels (repeatedly sampled non-inoculated contact animals, n = 36), and stocking density broilers (non-inoculated and non-sampled contact animals, n = 36). At the age of 10 days, the seeders were orally challenged with approximately 10^4 colony forming units (cfu)/500 µL of *C. jejuni* to assess natural transmission within the broilers, as *C. jejuni* will spread from the seeders to the non-infected contact animals. Consequently, the contact animals were naturally colonized with *C. jejuni*. For the determination of *C. jejuni* colonization, seeders and sentinels were sampled by means of cloacal swabs. Health and weight gain of the animals were supervised and documented daily. At the end of the trial, at 33 days of age (average weight 2.0 kg), animals were euthanized, dissected and *Campylobacter* counts were determined in the cecal and colon contents of the sentinels.

Table 1. Composition and analytical constituents of the experimental three-phase diet.

Ingredients, per kg	Starter Feed (Day 0–8)	Grower Feed (Day 9–26)	Finisher Feed (Day 27–33)
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
MJ ME [1]	12.4	12.4	12.4
Calcium (%)	0.9	0.8	0.8
Phosphorous (%)	0.6	0.55	0.5
Sodium (%)	0.14	0.14	0.14
Methionine (%)	0.55	0.50	0.50
Lysine (%)	1.25	1.15	1.05

[1] megajoules of metabolizable energy.

2.3. Application of the Complex Competitive Exclusion Product

The product used in the study was the competitive exclusion product Aviguard® (Lallemand, Worcestershire, UK). The compound contains the following bacterial species (approximately 10^9 cells per g, as specified by the manufacturer): *Escherichia coli*, *Citrobacter species*, *Enterococcus species* (*E. faecalis*, *E. faecium*), *Lactobacillus species* (*L. casei*, *L. plantarum*), *Bacteroides species*, *Clostridium species* (*C. sporogenes*), *Eubacterium species*, *Propionibacterium species*, *Fusobacterium species*, *Ruminococcus species* [25]. To examine the efficacy of the CE culture, broilers in the T2 group received the product twice: (i) via spray application and (ii) via drinking water according to manufacturer's specifications. Simultaneously with each administration, the CE culture was examined for the presence of *Campylobacter.* In brief, the CE culture suspensions were homogenized in sterile PBS (Oxoid, Wesel, Germany) and assayed in serial dilutions plated in 100-µL aliquots on mCCDA plates. The absence of *C. jejuni* growth was detected after the bacteria were grown for 48 h at 37 °C in a microaerophilic atmosphere.

The spray application was performed immediately after hatching. For this purpose, about 20 min before treatment, the entire sachet of the CE culture (25 g) was dissolved in

500 mL (amount for 2000 chickens) of tap water. From this homogenized total volume, 25 mL CE culture solution (amount for one hundred chickens) was taken and poured into three manual sprayer devices delivering coarse droplets (for 30 chickens each). We used the amount for one hundred chickens since there is always some residue in the spray bottle. In total, each chicken in the T2 group was treated with 0.25 mL of the prepared CE culture solution. To ensure an accurate application during spraying procedure and thus allow sufficient CE culture uptake for an early mature colonization, chickens were divided among three plastic boxes (each containing 30 chickens) and then treated simultaneously with the CE culture. After a short application time (five to ten minutes), broiler chickens were allocated to their corresponding pen. The administration via drinking water was performed one week before necropsy (at 25 days of age). In order to treat 90 chickens, of the total amount of 25 g (amount for 2000 chickens), 1.125 g of CE culture was dissolved in 1 L of tap water, thoroughly homogenized and then added to 8 L of drinking water (corresponding to the water consumption of 90 broiler chickens). The CE culture was provided to the broilers via nipple drinkers for six hours. Within this treatment period, the entire amount of water was consumed by the chickens. The drinking buckets were thoroughly rinsed out and refilled with fresh tap water.

2.4. C. jejuni Strain and Seeder Inoculation

Oral inoculation of the seeders was conducted using a comprehensively characterized *C. jejuni* reference strain BfR-CA-14430 (characterized by whole genome sequencing (WGS) and multilocus sequence typing (MLST), which was originally isolated from poultry (chicken breast). This particular strain belongs to the MLST clonal complex (CC)21, which on the one hand is one of the largest clonal complexes found so far [26] and on the other hand is highly prevalent in livestock and different environmental sources worldwide [27,28]. Indeed, *C. jejuni* genomes associated with CC21 are often found in chickens but also met the criteria for host-generalist lineages [29]. In addition, it is frequently associated with human disease cases [30].

For the experiments, an inoculum containing 3.4×10^4 cfu of *C. jejuni* was prepared and analyzed as described earlier [31]. Seeders were then orally inoculated individually into the crop with 0.5 mL of the prepared bacterial suspension.

2.5. Sampling Design and Microbiological Analysis

Prior to oral inoculation, all broilers were monitored for the presence of *Campylobacter* by taking cloacal swabs (Sarstedt, Nümbrecht, Germany) at four days of age.

Throughout the study, *C. jejuni* colonization of broilers was determined by semi-quantitative analysis of cloacal swabs. Cloacal swabs were collected in a standardized manner (time of sample collection, method of sample collection, sample processing). Cloacal swabs were taken as follows: on three consecutive days (namely 2, 3 and 4 days post inoculation (dpi)) (corresponds to day 12, 13, and 14 of age), then at least twice a week (8, 11, 16, and 18 dpi) (equivalent to day 18, 21, 26, and 28 of age). To ensure comparability of results, the same 36 sentinels (non-inoculated, but naturally colonized with *C. jejuni* through contact with the seeders) were sampled in both groups. Seeders were examined only once for *C. jejuni* excretion by collection of cloacal swabs 2 dpi.

Cloacal swabs were analyzed semi-quantitatively and adapted to International Organization for Standardization/Technical Specifications (ISO/TS) 10272-3:2010. Cloacal swabs were inserted in the cloaca, rotated five times, removed and immediately transferred to 3.0 mL PB. Thereafter, cloacal swabs were homogenized for three seconds using a vortex shaker (VWR. Darmstadt, Germany), allowing the fecal material to detach and evenly disperse in the medium. Afterward, cloacal swabs were 10-fold serially diluted in PB (up to 10^{-8}) and bacteria were grown for 24 h at 37 °C in a microaerophilic atmosphere. Dilutions were then streaked onto quartered mCCDA plates using 10 µL inoculation loops (Sarstedt, Nümbrecht, Germany). Plates were incubated for another 48 h under the same conditions and examined for *C. jejuni* growth. The highest dilution with confirmed *Campylobacter*

growth was used to determine the MPN (Most Probable Number) value. The result was determined using an MPN table modified according to ISO/TS 10272-3:2010/Cor.1:2011(E).

At 33 days of age (average weight 2.0 kg), all 36 sentinels per group were euthanized using ZKS poultry pliers (Corstechnology, Neerstedt, The Netherlands) after confirming deep anesthesia. The animals were dissected and intestinal contents (from cecum and colon) were collected for subsequent *C. jejuni* enumeration. Necropsy samples were prepared to perform semi-quantitative analysis also adapted to ISO/TS 10272-3:2010. Approximately 1 g of gut content was removed aseptically, diluted 1:8 in PB, thoroughly homogenized, and then ten-fold diluted in PB to 1×10^{-9}. Diluted intestinal samples were then processed as described above. After incubation for 48 h at 37 °C under microaerobic atmosphere, the highest dilution showing bacterial growth was assessed.

2.6. Statistical Analysis

Statistical analysis was carried out using SPSS software version 25.0 for Windows (SPSS, Inc., Chicago, IL, USA). Before statistical analysis, individual *Campylobacter* counts were transformed to $\log_{10}$ counts and then used as the experimental unit. The Shapiro–Wilk test was used to test the normal distribution of the data. Since our data did not meet criteria of normal distribution, we applied pairwise comparisons using the non-parametric Mann–Whitney *U*-test. To ensure alpha error of 0.05, β-error of 0.18 and power of 0.80, 90 animals per group were included in the present study. In order to determine statistically significant differences, 36 animals were sampled during the animal trial. Probability (p)-values below 0.05 were considered statistically significant.

3. Results

3.1. CE Culture

No *Campylobacter* spp. was cultivated from the CE culture used.

3.2. Effect on Colonization

The semi-quantitative results of both groups are presented in Figure 1. Prior to oral inoculation, *C. jejuni* was not detectable in any of the broilers. All seeders per group shed *C. jejuni* 2 dpi. The determined *Campylobacter* counts from cloacal swabs collected from seeder birds were similar in both groups (Md = 1.86 $\log_{10}$ MPN/cloacal swabs in the control group vs. Md = 2.36 $\log_{10}$ MPN/cloacal swabs in the CE group). Initial sampling of sentinels (3 dpi) showed that although the number of positive *Campylobacter* excretors in the control group was comparatively lower (2 sentinels in the control group vs. 8 sentinels in the CE group), the *C. jejuni* counts obtained from cloacal swabs were the same (Md = 2.36 $\log_{10}$ MPN/cloacal swabs). Likewise, 4 dpi fewer sentinels (n = 3) in the control group excreted *C. jejuni* than in the CE group (n = 21). *Campylobacter* counts determined 4 dpi from cloacal swabs were slightly lower in the control group (Md = 1.36 $\log_{10}$ MPN/cloacal swabs) than in the CE group (Md = 1.72 $\log_{10}$ MPN/cloacal swabs). All sampled sentinels were positive for *C. jejuni* 8 dpi. Comparing both groups, *Campylobacter* counts in cloacal swabs were significantly and consistently lower ($p < 0.0001$) in the CE group at 8, 11, 16, and 18 dpi (Figure 1A). At 8, 16, and 18 dpi, cloacal swabs from the CE group demonstrated the highest decrease in *C. jejuni* counts (Md = 3.36, 4.36, and 4.36 $\log_{10}$ MPN/cloacal swabs) in comparison to the control group (Md = 5.36, 6.36 and 6.36 $\log_{10}$ MPN/cloacal swabs). These results correspond to a log reduction of 2 $\log_{10}$ MPN/cloacal swabs (at 8 dpi $p < 0.0001$; $r = 0.67$, at 16 dpi $p < 0.0001$; $r = 0.63$ and at 18 dpi $p < 0.0001$; $r = 0.59$) respectively. Similar results could be observed at 11 dpi. Sentinels treated with the CE culture revealed to have significantly lower *Campylobacter* counts (Md = 4.36 $\log_{10}$ MPN/cloacal swabs) compared to the control group (Md = 5.36 $\log_{10}$ MPN/cloacal swabs), corresponding to a log reduction of 1 $\log_{10}$ MPN/cloacal swabs ($p < 0.0001$; $r = 0.68$). Furthermore, the analysis of cecal samples demonstrated a significant decrease ($p < 0.0001$; $r = 0.46$) of *Campylobacter* cecal colonization (Figure 1B) for the CE group (Md = 6.36 $\log_{10}$ MPN/g) compared to the control group

Md = 7.36 $\log_{10}$ MPN/g). The observed log reduction in *Campylobacter* cecal counts for the CE group was 1 $\log_{10}$ MPN/g. Equally, *C. jejuni* counts in the colon of CE culture treated broilers were significantly reduced (*Md* = 5.36 $\log_{10}$ MPN/g) ($p < 0.0001$; $r = 0.45$) in comparison to the control group (*Md* = 7.36 $\log_{10}$ MPN/g). The log reduction in *Campylobacter* colon counts of sentinels receiving the CE culture was 2 log MPN/g (Figure 1B). Individual *C. jejuni* counts of seeder and sentinels are presented in Tables S3 and S4.

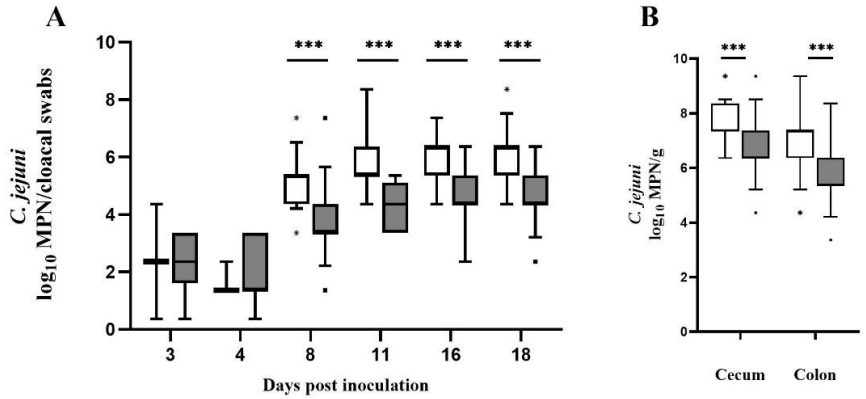

Figure 1. *C. jejuni* colonization of 36 sentinels per group determined by semi-quantitative analysis. *C. jejuni* counts in $\log_{10}$ most probable number (MPN) of 36 sentinels per group (**A**) in cloacal swabs at distinct time points after oral inoculation of the seeders on day 10 and (**B**) per gram in intestinal content upon necropsy (day 23 post inoculation). White boxes feature the control group (broilers challenged with *C. jejuni* and not treated with the CE culture); gray boxes feature broilers challenged with *C. jejuni* and treated with the CE culture on days 1 (via spray) and 25 (via the drinking water). The box plots show the 5th and 95th percentiles (whiskers) and outliers (shown as asterisk (*) for the control group and black square for the CE group). Medians (bold line) and significance levels (*p* values) determined by the Mann Whitney *U*-test are indicated. Time points showing a significant reduction ($p < 0.0001$) in *Campylobacter* counts compared to the control group are marked with three asterisks.

3.3. Effect on Broilers' Performance

CE culture treatment showed no effect on the animals' growth performance as presented in Tables S1 and S2. At the end of the trial, no significant differences in the final mean body weight were observed ($p > 0.05$) between sentinels of the experimental (1.98 kg) and sentinels of the control group (1.87 kg).

4. Discussion

Supporting the development of a mature and competitive microbiota by administering intestinal content of adult birds to newly hatched chickens is a promising approach to reduce *Campylobacter* cecal colonization. Previous research has indicated a profound mutual interdependence between *Campylobacter* and the present ubiquitous microbiome. On the one hand, the ability of *Campylobacter* to colonize the intestinal cecal crypts was influenceable by cecal microbiota composition. On the other hand, previous research demonstrated that *Campylobacter* colonization itself induces a shift in the intestinal microbiome, especially the beta-diversity (the variability in community composition within the same habitat) [32,33].

In this in vivo study, we examined whether a complex CE culture has the potential to reduce *Campylobacter* carriage in broiler chickens at slaughter age when administered via spray application and via drinking water. As far as we are aware, this is the first in vivo study to evaluate the efficacy of this complex CE culture in reducing *Campylobacter* colonization in broiler chickens using a practical setup that approximates commercial poultry farming, as two administration methods common to conventional poultry operations were used. This approach differs from earlier in vivo attempts, in which CE cultures were

mostly administered via an oral gavage into the broilers' crop [24,34–37] in order to ensure an accurate dosage per chicken [14]. However, these experimental setups are difficult to implement on poultry farms and do not conform to current administration methods. Therefore, especially regarding practicability, we chose a simpler experimental approach which has been shown to be as effective as direct gavage into broilers' crop [38]. This method was first introduced by Pivnick and Nurmi [39] and can be easily repeated in field studies or on poultry farms without subjecting the broilers to any undue strain or stress. In particular, spray application with coarse droplets is a proven method ensuring a quick ingestion because sparkling spray droplets on the feathers excite the day-old chickens to preen themselves [14] while being harmless and without any adverse effects [14,40]. Besides, an early administration of CE cultures shortly after hatch is advisable to rapidly induce the formation of a yet stable gut microbiota [41], as chicken cecal microbiota becomes diverse and stable with increasing age [42]. In addition, we administered the CE culture via broilers' drinking water, as this is a common administration method in commercial broiler production [43].

The results of this in vivo study are encouraging as the CE culture reduced *C. jejuni* load in cloacal swabs significantly and consistently throughout the fattening period (the maximum observed log reduction was 2 $\log_{10}$ MPN). Moreover, at the end of the trial after necropsy we determined a significantly decreased *C. jejuni* cecal load in 33-day-old broilers. In comparison to the control group, the cecum of broilers receiving the CE culture showed significantly reduced *Campylobacter* counts (log reduction of 1 $\log_{10}$ MPN/g). Likewise, colon counts were significantly lower (log reduction of about 2 $\log_{10}$ MPN/g). Based on the relationship between *Campylobacter* concentrations in the ceca and corresponding broiler carcass skin samples, a 2-$\log_{10}$ reduction in broiler cecal concentrations is estimated to reduce the relative risk of human campylobacteriosis in the EU by 42%, while a 3-$\log_{10}$ reduction in broiler cecal concentrations would reduce the risk by as much as 58% [11]. Although the effect of the CE culture on *Campylobacter* cecal colonization was modest (1 $\log_{10}$ MPN/g), it is important to note that any reduction in *Campylobacter* numbers in the cecal content may contribute to reduce the *Campylobacter* load on the broiler carcasses during processing [44,45].

One may argue that (i) cloacal swabs are an unreliable source for quantitative *Campylobacter* detection (varying or low amounts of feces adhering to the swab) and (ii) quantitative analysis of samples may have been more accurate. Indeed, quantitative analysis of samples where high *Campylobacter* counts are expected is considered the gold standard for the detection and quantification of *Campylobacter* and determination of *Campylobacter* concentrations via cloacal swabs is not the most reliable method available. However, selective sampling of sentinels is required for analysis of natural infection models such as those used in the present study. Indeed, *C. jejuni* enumeration of fecal samples, or in particular cecal droppings might have been more accurate to illustrate the cecal *Campylobacter* colonization of the broilers. However, the collection of cecal droppings of certain sentinels (as necessary in this study) was not feasible in our experimental setting for the following reasons: (i) broiler chickens excrete them infrequently [46] and (ii) the isolation of seeder and sentinel broilers for a prolonged period of time would have compromised the experimental seeder bird model, which targets natural infection and keeps conditions close to commercial poultry production. The use of cloacal swab ensured the sampling of "naturally" infected sentinels and thus the examination of the individual course of each of the 36 sentinels (by assigning samples to the tag number). Furthermore, this allowed us not only to include a large sample size in our study but also to assess *Campylobacter* reduction under real-life conditions. To address the varying amounts of feces adhering to the swab and the associated difficulties in quantification, we used the semi-quantitative method and a standardized sampling and sample processing procedure to obtain comparable and reproducible data. The reproducibility and accuracy of the data of the present approach are satisfactory, as in the control group the *Campylobacter* counts in cloacal swabs were consistently homogeneous regardless of the time of sampling (11, 16, and 18 days

post inoculation). In line, a previous study showed a similar isolation rate between direct culture on mCCDA and enrichment when pooled cecal samples from different slaughter batches were examined [47]. Likewise, another study found no statistical difference between enumeration by the semi-quantitative and quantitative technique for comparable concentrations of thermotolerant *Campylobacter* ($p = 0.104$) [48]. Similarly, no significant differences ($p > 0.05$) could be detected between results obtained by direct plating of carcass rinse samples on Campy-cefex agar and an MPN procedure [49]. In support, Scherer and colleagues observed a highly positive correlation coefficient of 0.9 between direct plating and MPN technique and therefore concluded both methods to be suitable for the detection and quantification of *Campylobacter* [50].

Although our results are auspicious, earlier attempts using CE cultures of different compositions showed varying potential to lower *Campylobacter* colonization [14,17–19,21,22]. Stern [51] found no reducing effect of a conventional CE preparation on *Campylobacter* colonization. A preparation made from cecal wall material (MCE culture), however, yielded lower *Campylobacter* cecal colonization (average reduction 2.01 log cfu/g cecal material) [35]. In addition, Hakkinen and Schneitz [34] displayed the efficacy of another commercially available CE product Broilact® (Orion Corporation, Espoo, Finland) against *Campylobacter jejuni* colonization in Ross I broiler chickens 12 days after oral challenge. Consistent with the results of the present study, Ty and colleagues [52] observed reduced *C. jejuni* colonization in Ross 708 broilers 39 days post hatching, following a single administration of Aviguard® via drinking water on the first day of life. To increase the protective effect of CE cultures, Mead et al. [14] contemplated that an extended time period between CE treatment and challenge might be beneficial which is why in this approach we defined a 10-day interval between CE culture administration and artificial *C. jejuni* challenge. Additionally, CE culture retreatment during rearing might boost the CE effect [24]. In accordance, Schoeni et al. [19] demonstrated the advantageous effect of an additional booster treatment on *Campylobacter* colonization in White Leghorn cockerel chickens. Accordingly, in this study we observed a consistent and significant reduction of *C. jejuni* in cloacal swabs after CE booster treatment, as both cloacal swabs taken 1 and 3 days after CE booster treatment (corresponding to 16 and 18 dpi) revealed lower *C. jejuni* counts (log reduction of 2 $\log_{10}$ MPN/cloacal swabs) as shown for the control group. Based on these observations in conjunction with the results after necropsy, it can be speculated that a CE booster treatment might contribute to reducing *C. jejuni* colonization. Whether the second treatment had a protective effect remains to be determined. In addition, the colonization of broilers with *C. jejuni* may naturally be subject to individual variation, and the present study cannot fully clarify whether or to what extent these natural variations may have affected our results.

With regard to the use of CE cultures, several other factors might affect their efficacy, namely rearing conditions, administration as well as challenge method, time of administration, CE culture composition (few bacterial strains or abundance of different bacteria), donor material, bird strain, stress, and rearing length [14,39,53]. Moreover, it should be considered that the composition of CE cultures may vary considerably between different batches. Reasons for this variability are that several parameters such as environmental (seasonal and geographical climate changes), host factors (genetics, gut development), increasing broiler age [54], hygiene, medication, housing and switch of feed type (starter, grower, finisher) may contribute to changes in microbiota abundance and diversity in donor chickens [42,55,56]. Consequently, the efficacy of the CE culture may be compromised if the number of bacterial strains that contribute significantly to CE activity is reduced [57]. Nevertheless, the content of the CE culture was not investigated in the present study, so we cannot be certain whether this product itself or only the content of this batch has an effect. This should be explored and needs to be verified in further studies.

It appears that commercially available CE products are effective due to the complexity and richness of naturally occurring elements of the normal microbiota [14] on the one hand, and the presence of facultative anaerobic bacteria on the other hand. Nevertheless, it should be mentioned that recent research has aimed at identifying potential defined

CE cultures (consisting of a small proportion of well-characterized bacteria) that show similar potential against *Campylobacter* colonization as has been observed for commercially available CE products. However, those studies yielded different competitive strains and outcomes [17–19,21,58].

Notwithstanding the efficacy of complex CE products, they have not yet been approved for poultry fattening in Germany. As early as in 1994, the World Health Organization (WHO) suggested to classify CE products as "normal gut flora", a classification provided for license simplification, since they do not fall precisely in one of the following categories: vaccine, feed additive, or veterinary medicinal product [59]. Indeed, due to their complex nature, the establishment of an appropriate regulatory framework for the placing on the market of CE cultures appears to be quite challenging. Many species of the intestinal microbiota have not yet been fully identified [57] nor have they ever been cultivated [60]. Despite the current scientific progress, it is still difficult to achieve a complete characterization of their contents as required by some legislation, for example the European Regulation on additives for use in animal nutrition (Regulation (EC) No 1831/2003). Moreover, CE cultures may be considered a potential source of pathogens [14] and may harbor transferable antimicrobial drug resistance or virulence genes [57] that could pose a risk to human health. Although a few European countries have their own framework which allows the approval of CE products, a harmonized EU regulatory framework is still missing [61]. In fact, in many countries around the world CE cultures are registered under national legislation either as veterinary medicines or as feed additives/probiotics. CE cultures have been and are still being used successfully in several European countries (e.g., UK, Sweden, Norway and Finland). Notably, the low incidence of *Campylobacter* in Finnish broiler flocks has been indicated to correlate with the consistent long-term use of CE cultures against *Salmonella* [24,62].

5. Conclusions

In conclusion, CE cultures can be considered a valuable concept for the control of *Campylobacter* on poultry farms although the entire mode of action is not yet clearly elucidated. At present, it is difficult to assess the extent to which CE cultures are effective and which specific factors are responsible for their effectiveness. As observed previously, it appears that both defined and complex CE products may diminish *Campylobacter* colonization in broiler chickens. The results of the present study are encouraging and can be considered of practical relevance in poultry production as conventional administration of the CE culture significantly reduced *Campylobacter* cecal colonization in broilers at slaughter age. However, in Germany full disclosure of the composition of CE products are required for authorization to ensure health safety of human consumers. For this reason, enlightening endeavors are needed to comply with this requirement. Since the chickens in this study were raised under favorable experimental conditions, it is also necessary to verify whether the results obtained can be reproduced with larger broiler flocks under commercial conditions. In order to draw careful conclusions, further studies are needed that advance microbiome analyses, especially through innovative and sophisticated sequence techniques. These outstanding tools are essential to gain important insights into the interplay between *Campylobacter* and the currently ubiquitous microbiome to adequately examine the suitability of CE cultures for commercial poultry productions. In addition, it should be investigated whether a reduction in *C. jejuni* colonization can be achieved by only one of the two CE treatments or whether the second application in the drinking water can actually contribute to a reduction in *C. jejuni* counts (increased protection). Furthermore, the simultaneous combination of CE cultures and other control strategies may be a promising approach for further reducing *Campylobacter* colonization in broiler chickens.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/vetsci9040181/s1. Table S1: Mean body weight (g) of broiler chickens in the control group during the animal trial. Ten randomized broiler chickens were weighed daily. Table S2: Mean body weight (g) of broiler chickens treated with the CE culture during the

animal trial. Ten randomized broiler chickens were weighed daily. Table S3: C. jejuni counts (log10 MPN) of seeder and sentinel broiler chickens at 2, 3, 4, 8, 11, 16, and 18 days after inoculation from cloacal swabs. Table S4: C. jejuni cecal and colon colonization of sentinel broiler chickens at 33 days of age after necropsy.

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Institutional Review Board Statement: This study was carried out in accordance with the National Animal Protection Guidelines. The protocol was 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, permission number G 0098/18).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are contained within the article and available in the Supplementary Materials.

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3.3. Publication III

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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 ($\log_{10}$) 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 $\log_{10}$ reduction in *Campylobacter* counts (Neal-McKinney et al., 2014). Wagle et al. (2017) tested the use of the phytochemical b-resorcyclic acid in an in vivo study. In this former study, the application significantly reduced cecal *Campylobacter* concentrations by ~2.5 and 1.7 $\log_{10}$ 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_2O_2, 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^2. 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 μL containing approximately 2.2×10^4 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_2, 10% CO_2, 5% O_2), 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.

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

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^{-8}), incubated 24 h at 37°C under microaerophilic conditions, and then streaked out on quartered mCCDA plates using 10 μL inoculation loops (Sarstedt AG & Co. KG). Plates were incubated

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 μL 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°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 ± 1°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 μL of the bacterial inocula at concentrations of 1×10^6 cfu/mL were dispensed into wells containing 50 μL of the double concentrated organic acid mixture to achieve final bacterial concentrations of 5×10^5 cfu/mL. After 48 h of incubation at 42 ± 1°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 Shapirow-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).

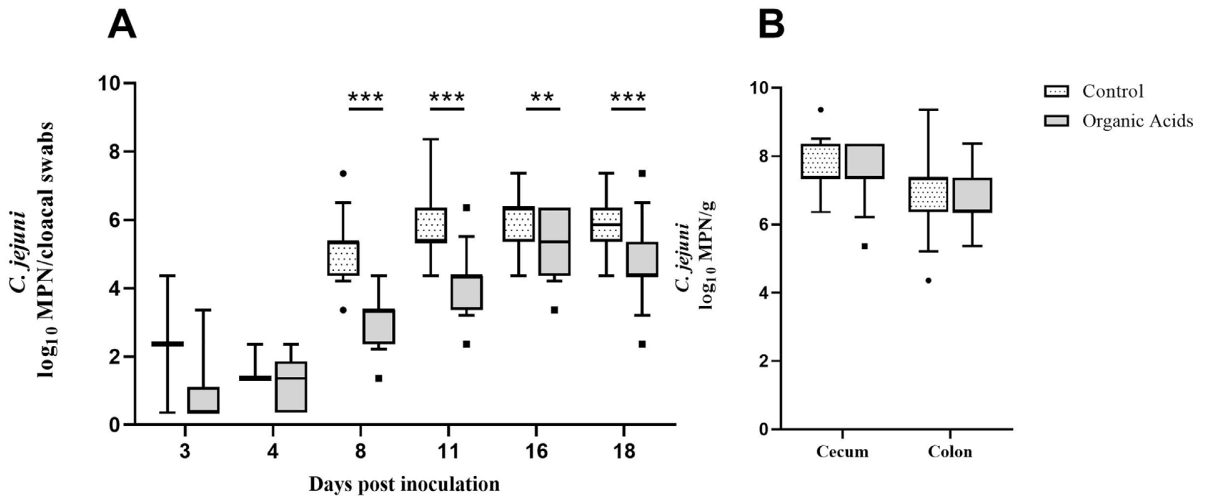

Figure 1. *Campylobacter* (*C.*) *jejuni* colonization of 36 sentinels per group determined by semiquantitative analysis (min to max). (A) *C. jejuni* counts in $\log_{10}$ most probable number (MPN) in cloacal swabs derived from sentinels confirmed to shed *C. jejuni* at distinct time points after oral 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 $\log_{10}$ 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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4. Unpublished data

In the publications presented, only three measures (carvacrol, CE culture and organic acids) were addressed. Consequently, in addition to the preliminary tests, the remaining measures (alternative breed and phages) that were not part of the respective publications are discussed in this section.

4.1. Preliminary trials (dose-finding)

Before conducting the main trials, we conducted two preliminary trials. Preliminary trials were carried out to determine (i) the lowest *C. jejuni* dose necessary to successfully colonize all 20 broiler chickens within 2 days and (ii) whether the semi-quantitative method achieves comparable results to the quantitative method. The first group received a dose consisting of 10^3 – whereas the second group received 10^4 CFU/500µl of *C. jejuni.* At 4 days of age, all 20 chickens were determined to be free from *Campylobacter.* The chickens were sampled using cloacal swabs 1, 2 and 3 dpi and then twice a week until the end of the trial. At 33 days of age, broiler chickens were euthanized and cecal and colon contents of all chickens were collected for *Campylobacter* enumeration.

The results of the preliminary trials are shown in **Figure 1–3**. Two days after oral inoculation with the lower dose consisting of 10^3 CFU (**Figure 1A**), 11 out of 20 chickens were excreting *C. jejuni* while in the other group all 20 broilers were already *Campylobacter* positive (**Figure 1B**). Both doses used were able to successfully colonize all 20 broiler chickens after 2 or 4 dpi, as determined by semi-quantitative method (**Figure 1A, 1B**). In a direct comparison between the two methods used, the semi-quantitative method was able to detect the colonization of broiler chickens with *C. jejuni* earlier (**Figure 1**). While the semi-quantitative method detected *C. jejuni* in 4/20 chickens the day after oral application of 10^4 CFU, the quantitative method detected one positive chicken 2 dpi (1/20), four positive chickens 3 dpi (4/20) and 12 positive chickens 4 dpi (12/20) (**Figure 1B**). In comparison, the semi-quantitative method detected all 20 animals as *C. jejuni* positive after 48h (**Figure 1B**). The results were similar in the group receiving the lower inoculation dose. The semi-quantitative method detected *C. jejuni* in 11/20 chickens, whereas the quantitative method detected *Campylobacter* only in 1/20 chickens at 2 dpi. At 3 dpi, the quantitative method failed to detect *Campylobacter* in any chicken (**Figure 1A**).

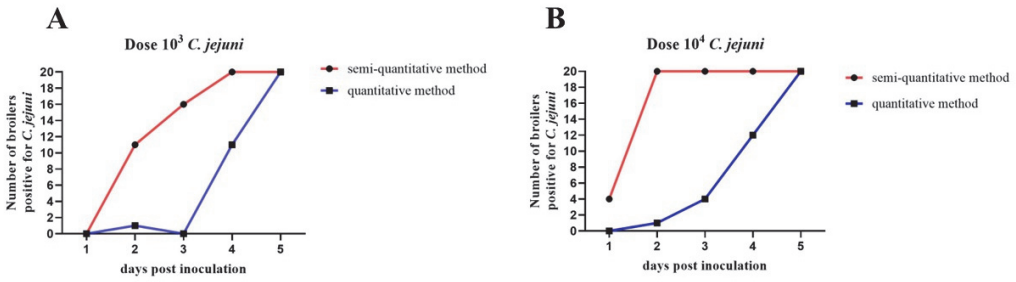

Figure 1: Number of positive animals (n = 20) after oral inoculation with 10^3 or 10^4 CFU/500µl *C. jejuni*. **(A)** Detection of positive animals after oral inoculation with 10^3 *C. jejuni*. **(B)** Detection of positive animals after oral inoculation with 10^4 *C. jejuni*. Red lines represent chickens detected with the semi-quantitative method and blue lines represent chickens detected by the quantitative method.

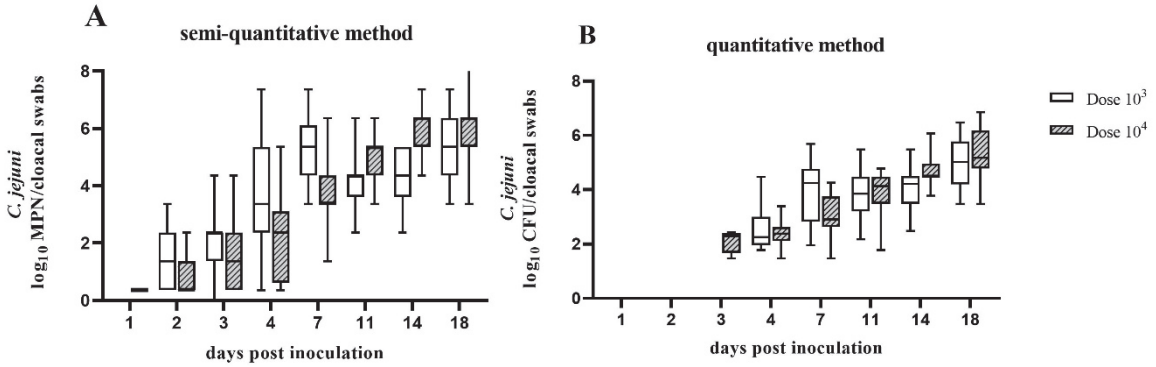

Figure 2: Cloacal swab results of both preliminary trials obtained by the semi-quantitative or quantitative method. **(A)** Mean $\log_{10}$ MPN of *C. jejuni* in cloacal swabs at each point in time after oral inoculation determined by semi-quantitative method. **(B)** Mean $\log_{10}$ CFU of *C. jejuni* in cloacal swabs determined using the quantitative method. White boxes represent the group orally challenged with 10^3 CFU of *C. jejuni*; gray striped boxes represent broilers challenged with 10^4 CFU of *C. jejuni*. The data presented were obtained from 20 chickens per group. The box plots show the 5th and 95th percentiles.

Figure 2 shows comparative results of cloacal swabs depending on the detection method and dose used. **Figure 2A** depicts the results of the semi-quantitative method while **Figure 2B** depicts the results received by the quantitative method. For the group receiving 10^3 CFU, the semi-quantitative method yielded mean *C. jejuni* counts in cloacal swabs ranging from 1.36 to 5.57 $\log_{10}$ MPN/g (**Figure 2A**). Similar *C. jejuni* mean counts were obtained by the quantitative method (ranging from 2.57 to 5.34 $\log_{10}$ CFU (**Figure 2B**).

For the group receiving 10^4 CFU, the semi-quantitative method revealed mean *C. jejuni* counts in cloacal swabs ranging from 0.71 to 6.11 $\log_{10}$ MPN/g (**Figure 2A**). Similar *C. jejuni* mean counts were obtained by the quantitative method, ranging from 2.12 to 5.3 $\log_{10}$ CFU (**Figure 2B**).

Comparing both methods, the determination of *C. jejuni* counts using the semi-quantitative method tended to yield higher counts.

The results after necropsy are shown in **Figure 3**. In both groups, chickens were highly colonized with *C. jejuni* in their intestines. Neither in the group receiving 10^4 CFU (p = 0.327) nor in the group receiving 10^3 CFU (p = 0.114) intestinal cecal colonization differed significantly when both methods were compared. In the group receiving the higher dose, mean counts of 7.51 $\log_{10}$ MPN/g were observed using the semi-quantitative method while the quantitative method yielded 7.66 $\log_{10}$ CFU/g (**Figure 3B**). In the group receiving the lower dose, mean counts of 7.66 $\log_{10}$ MPN/g were observed using the semi-quantitative method while the quantitative method yielded 7.46 $\log_{10}$ CFU/g (**Figure 3A**). Colon colonization differed significantly in the group receiving the lower dose (p = 0.043) when comparing the two methods (mean counts of 6.76 $\log_{10}$ MPN/g and mean counts of 7.23 $\log_{10}$ CFU/g (**Figure 3A**). The group receiving the higher dose showed similar colonization rates in the colon 6.76 $\log_{10}$ MPN/g and 7.18 $\log_{10}$ CFU/g (**Figure 3B**).

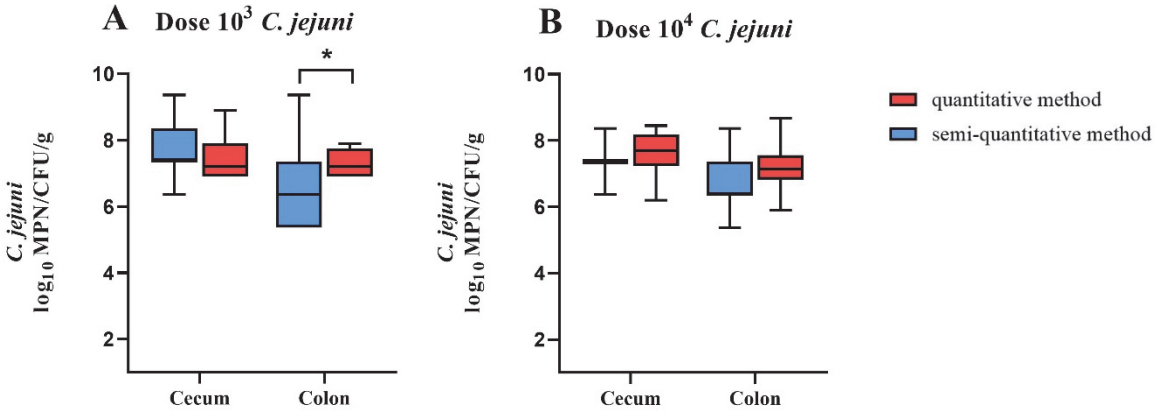

Figure 3: Results of both preliminary trials after necropsy in intestinal contents obtained by the semi-quantitative or quantitative method. **(A)** Mean $\log_{10}$ MPN/CFU of *C. jejuni* per gram in intestinal content of broilers challenged with 10^3 CFU of *C. jejuni*. **(B)** Mean $\log_{10}$ MPN/CFU of *C. jejuni* per gram in intestinal content of broilers challenged with 10^4 CFU of *C. jejuni*. Blue boxes represent results obtained using the semi-quantitative method while red boxes represent results obtained using the quantitative method. The box plots show the 5th and 95th percentiles**. *** (p = 0.043)**.**

4.2. Alternative breed (Ranger Gold)

Colonization with *C. jejuni* can in principle be influenced by many factors, including breed. The objective of this trial was to determine the possible influence of an alternative breed with lower daily weight gain in combination with a reduced stocking density on *Campylobacter* colonization. For this purpose, the slow-growing breed Ranger Gold was used. In addition, the stocking density was reduced to 25 kg/m². This specific breed is a combination of a brown feathered Ranger female that provides excellent reproduction and a gold male that provides exceptional performance in terms of robustness, feed conversion and weight gains.

The results of the cloacal swabs are shown in **Figure 4**. In comparison to the control group, *C. jejuni* numbers in cloacal swabs taken from Ranger Gold broilers differed significantly (p < 0.0001) at 8-, 11-, 15- and 18 dpi. The highest reduction of *C. jejuni* counts in cloacal swabs of Ranger Gold broilers (*Md* = 4.36 $\log_{10}$ MPN) was demonstrated in comparison to the control group (*Md* = 6.36 $\log_{10}$ MPN) at 15 dpi. These results correspond to a log reduction of 2 log (p < 0.0001; r = 0.7). Similar results were observed at eight, 11 and 18 dpi. Ranger Gold broilers showed at each sampling point lower bacterial counts (*Md* = 4.36 $\log_{10}$ MPN) compared to the control group (*Md* = 5.36, 5.36 and 5.86 $\log_{10}$ MPN), corresponding to a log reduction of < 1.5 $\log_{10}$ MPN/cloacal swabs (at 8 dpi p < 0.0001; r = 0.57, at 11 dpi p < 0.0001; r = 0.74 and at 18 dpi p < 0.0001; r = 0.67), respectively. At 25 and 31 dpi, we observed a significant increase in the overall *C. jejuni* load (*Md* = 6.36 and 5.36 $\log_{10}$ MPN) of Ranger Gold broilers, however, it is not possible to compare counts with the control group as in this group cloacal swabs were not taken due to the extended rearing period of Ranger Gold broiler chickens. At the end of the trial after necropsy Ranger Gold broilers did not show significantly decreased cecal *C. jejuni* colonization (p = 0.86) but demonstrated significantly reduced *C. jejuni* numbers in the colon (*Md* = 6.36 $\log_{10}$ MPN/g) in comparison to the control group (*Md* = 7.36 $\log_{10}$ MPN/g), corresponding to a log reduction of 1 log MPN/g (p = 0.001; r = 0.41) (**Figure 5**).

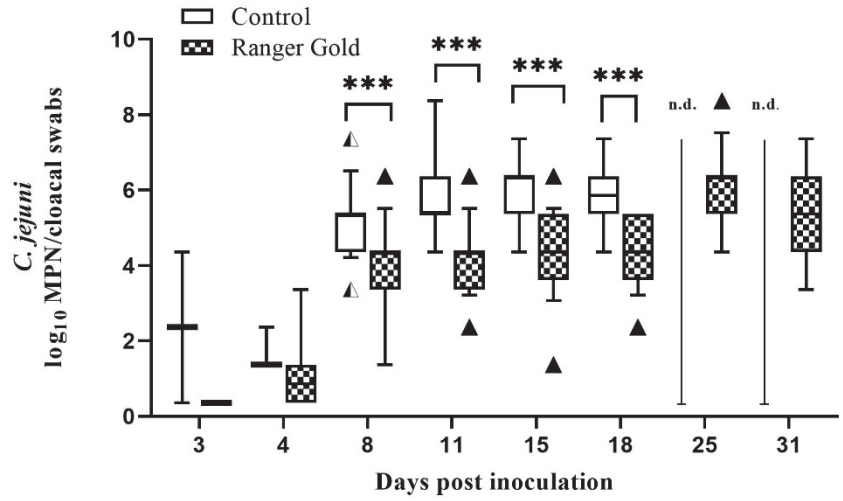

Figure 4: *C. jejuni* colonization of the control and alternative breed group during rearing obtained by semi-quantitative analysis by semi-quantitative analysis. *C. jejuni* counts in $\log_{10}$ MPN in cloacal swabs at distinct time points after oral inoculation of the seeders on day 10. White boxes represent the control group (Ross 308 broilers challenged with *C. jejuni*); boxes with a black-white checkered pattern represent the alternative breed group (Ranger Gold broilers challenged with *C. jejuni)*. The data presented were obtained from 36 sentinels per group. The box plots show the 5th and 95th percentiles (whiskers) and outliers (shown as black-white triangles for the control group and black triangles for the alternative breed group). Medians (bold line) and significance levels (p values) determined by the Mann Whitney *U*-test are indicated. Time points showing a significant reduction in *Campylobacter* counts compared to the control group are marked with three asterisks: *** (p < 0.0001). n.d. not determined.

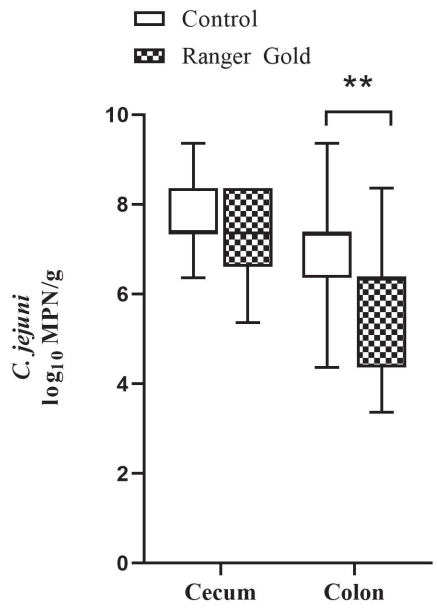

Figure 5: *C. jejuni* colonization in intestinal contents of the control and alternative breed group obtained by semi-quantitative analysis. *C. jejuni* counts in $\log_{10}$ MPN of 36 sentinels per group and per gram in intestinal content upon necropsy (23 dpi). White boxes represent the control group (Ross 308 broilers challenged with *C. jejuni*); boxes with a black-white checkered pattern represent the alternative breed group (Ranger Gold broilers challenged with *C. jejuni)*. The box plots show the 5th and 95th percentiles (whiskers) and outliers (shown as black-white triangles for the control group and black triangles for the alternative breed group). Medians (bold line) and significance levels (*p* values) determined by the Mann Whitney *U*-test are indicated Bars marked by an asterisk differ significantly: ** ($p < 0.001$).

4.3. Bacteriophages

Finally, the effect of phages provided by the TiHo on *Campylobacter* colonization was investigated in the same experimental setup (seeder bird model) as described above. Therefore, a combination of group II (LVL1/1) and group III (NCTC 12673) phages was used. Both phages have already proven successful in previous *in vitro* studies due to their specificity and ability to lyse the infectious strain. The cocktail was administered continuously via drinking water over a 24-hour period at a final concentration of 10^7 plaque forming units (PFU)/ml for three consecutive days before necropsy. For this purpose, the cocktail was diluted at a 1:10 ratio in the drinking water and prepared once a day on each of the 29th, 30th, and 31st days of life.

The results of the cloacal swabs are shown in **Figure 6**. In comparison to the control group, *C. jejuni* counts in cloacal swabs of broilers treated with phages differed significantly ($p < 0.0001$) before (19 dpi; $r = 0.43$) and during phage application (20 dpi; $r = 0.63$ and 21 dpi; $r = 0.61$). Even at 8 dpi, the *C. jejuni* counts of phages-treated broilers were lower (*Md* = 4.86 $\log_{10}$ MPN) in comparison to the control group (*Md* = 5.36 $\log_{10}$ MPN). However, this difference did not yet significantly differ ($p = 0.05$). Phage application reduced *C. jejuni* counts by 1 log in cloacal swabs from *Md* = 5.36 $\log_{10}$ MPN at 19 dpi to *Md* = 4.36 $\log_{10}$ MPN at 20, 21, 22 and

23 dpi. In the control group, however, *C. jejuni* counts were consistently equal at each sampling time examined (19, 20 and 21 dpi, *Md* = 5.36 $\log_{10}$ MPN). At 22 and 23 dpi, no cloacal swabs were taken in the control group. At the end of the trial after necropsy phages failed to significantly decrease *C. jejuni* colonization in the cecum (p = 0.05) (*Md* = 6.86 $\log_{10}$ MPN/g vs. *Md* = 7.36 $\log_{10}$ MPN/g) but significantly reduced *C. jejuni* numbers in the colon ($p < 0.0001$; r = 0.52) of phage treated broiler chickens (*Md* = 5.36 $\log_{10}$ MPN/g vs. *Md* = 7.36 $\log_{10}$ MPN/g). The log reduction in the colon was 2 $\log_{10}$ MPN/g (**Figure 7**).

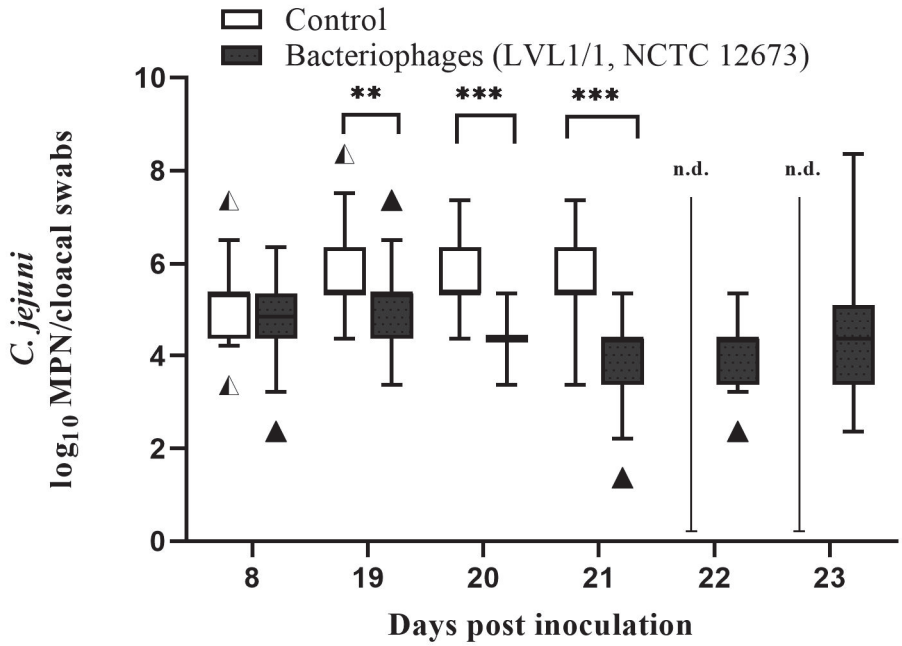

Figure 6: *C. jejuni* colonization of the control and phages group during rearing obtained by semi-quantitative analysis. *C. jejuni* counts in $\log_{10}$ MPN in cloacal swabs at distinct time points after oral inoculation of the seeders on day 10. White boxes represent the control group (broilers challenged with *C. jejuni* and not treated with phages); dark gray dotted boxes represent broilers challenged with *C. jejuni* and treated with phages via drinking water 21, 22 and 23 dpi. The data presented were obtained from 36 sentinels per group. The box plots show the 5th and 95th percentiles (whiskers) and outliers (shown as black-white triangles for the control group and black triangles for the group receiving phages). Medians (bold line) and significance levels (p values) determined by the Mann Whitney *U*-test are indicated. Time points showing a significant reduction in *Campylobacter* counts compared to the control group are marked with asterisks: ** ($p < 0.001$), *** ($p < 0.0001$). n.d. not determined

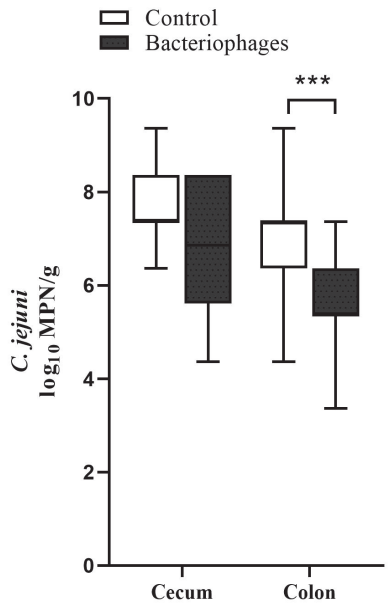

Figure 7: *C. jejuni* colonization in intestinal contents of the control group and the group receiving phages obtained by semi-quantitative analysis. *C. jejuni* counts in $\log_{10}$ MPN of 36 sentinels per group and per gram in intestinal content upon necropsy (23 dpi). White boxes represent the control group (broilers challenged with *C. jejuni* and not treated with phages); black boxes represent broilers challenged with *C. jejuni* and treated with phages via drinking water. The box plots show the 5th and 95th percentiles (whiskers) and outliers (shown as black-white triangles for the control group and black triangles for the group receiving phages). Medians (bold line) and significance levels (*p* values) determined by the Mann Whitney *U*-test are indicated. Time points showing a significant reduction in *Campylobacter* counts compared to the control group are marked with asterisks: *** ($p < 0.001$).

5. Discussion

Despite tremendous effort, effective and easily applicable alternative intervention strategies to control *Campylobacter* prevalence and colonization on broiler farms are still inadequate worldwide (Hakeem and Lu 2020). Attempts to counteract *Campylobacter* colonization in broiler flocks by following strict hygiene and biosecurity measures have been shown to be potentially effective (Gibbens et al. 2001; Hermans, Pasmans, et al. 2011; Newell et al. 2011; Georgiev et al. 2017) but may only be partly effective or not always sufficient on their own as broiler chickens are at constant risk of *Campylobacter* contamination (Sahin et al. 2003; Wagenaar et al. 2008; Hermans, Van Deun, Messens, et al. 2011). This deficiency stresses the need for specific further investigations (Soro et al. 2020; Abd El-Hack et al. 2021). To successfully combat the bacterium at the farm level, it is not only necessary to prevent and impede its introduction into poultry flocks, its transmission, and its subsequent colonization but also to reduce and minimize its overall prevalence in poultry flocks (Abd El-Hack et al. 2021). The present study addresses various non-biosecurity based intervention measures and their efficacy to reduce *C. jejuni* colonization in broiler chickens of slaughter age. In this context, possible factors that might have influenced the efficacy of the intervention measures evaluated in this study and thus hindered the reduction of the *Campylobacter* load will be discussed in the following. Furthermore, additional benefits of two non-biosecurity based intervention measures (carvacrol and organic acids) as a mitigation measure against *Campylobacter* contamination will be outlined and, as an extension of the current research approach, the possible combination of intervention measures with synergistic potential to reduce *C. jejuni* will be discussed. Finally, the implementation of non-biosecurity based intervention measures in commercial broiler production will be assessed.

5.1. *In vivo* efficacy of non-biosecurity based intervention measures in reducing *C. jejuni* colonization in broiler chickens

The objective of this study was to evaluate the efficacy of (i) carvacrol, (ii) a complex CE culture (Aviguard®), (iii) a mixture of different organic acids (sorbic acid, benzoic acid, propionic acid), (iv) an alternative slow-growing broiler breed (Ranger Gold) in a combination with a reduced stocking density of 25 km/m² and (v) phages, in reducing *C. jejuni* colonization in broiler chickens in an *in vivo* broiler chicken model (seeder bird model). To provide an overview of the respective results of each trial (of the individually applied measures), the previously published results (from three publications) are presented along with yet unpublished results. The respective graphical representation of the results is referred to by the figure number of the original publication (as applicable).

5.1.1. Carvacrol (publication I)

Carvacrol (a primary constituent of plant essential oils) and its antimicrobial activity against *Campylobacter* have been the subject of many different *in vitro* and *in vivo* studies (Friedman et al. 2002; Grilli et al. 2013; O'Bryan et al. 2015). In particular, given its antimicrobial properties, carvacrol is a promising alternative to conventional antimicrobials (Micciche et al. 2019). In this trial, dietary supplementation of carvacrol in a concentration of 120 mg/kg feed four days post-hatch until the end of the trial (during an entire fattening period) significantly reduced *Campylobacter* counts ($p \leq 0.02$) in cloacal swabs (publication I; Figure 1A) taken eight, 11, 15 and 18 dpi. Nevertheless, carvacrol feed supplementation failed to reduce *Campylobacter* cecal colonization (publication I, Figure 1B). In comparison to the control group, *C. jejuni* counts in the ceca showed no significant difference ($p > 0.05$). However, we observed significantly reduced *C. jejuni* numbers in the colon of carvacrol-treated animals ($p < 0.0001$) in comparison to the control group. Colon counts were reduced by 1 $\log_{10}$ MPN/g. In accordance, Kelly et al. (Kelly et al. 2017) found similar *Campylobacter* cecal counts in broiler chickens after dietary carvacrol supplementation. Based on our results, we hypothesize, that the selected dosage of 120 mg/kg feed was successful in decreasing *C. jejuni* colonization during animal starter and grower periods but not at the end of the trial (time of necropsy). This is supported by the fact that Arsi et al. (Arsi et al. 2014) observed significantly reduced *C. jejuni* cecal counts in 10-day-old broiler chickens after having administered 1% carvacrol in feed. Similar results were also observed in mice, where the application of carvacrol lowered *C. jejuni* loads and alleviated symptoms in a clinical murine model for human campylobacteriosis (Mousavi et al. 2020). The authors administered carvacrol at a considerably higher concentration of 500 mg/L to abiotic IL-$10^{-/-}$ mice via the drinking water as a preventive measure and observed that mice from the carvacrol cohort harbored two log orders of magnitude lower pathogen loads in their intestines and also showed significantly reduced disease symptoms.

Nevertheless, the reason for the failure of carvacrol to reduce *C. jejuni* cecal colonization at the end of our trial in our setting remains unclear. As set out previously (see section 1.5.1), essential oils (such as carvacrol) have a strong *in vitro* bactericidal activity against *Campylobacter*. In terms of *in vivo* studies, however, their antimicrobial effect is still insufficient and not consistent with *in vitro* studies (Gracia et al. 2016). One explanation might be that, if unformulated carvacrol is applied as a feed additive, its antibacterial efficacy on *Campylobacter* cecal colonization may be diminished since carvacrol can be rapidly absorbed in the upper GIT (Meunier et al. 2006; Michiels et al. 2008; Wang et al. 2009; Du et al. 2015). Likewise, carvacrol has poor chemical-physical properties such as poor water solubility which complicates its therapeutic use (Naghdi Badi et al. 2017; Marinelli et al. 2019). For this reason,

new research technologies have been designed to increase and enhance the *in vivo* solubility and stability of carvacrol through the use of specific drug delivery systems (prodrug approach) (Marinelli et al. 2019) that delay its release (Eusepi et al. 2020) so that it can reach the target area in the GIT, i.e., the ceca of broiler chickens (Allaoua et al. 2018), without compromising its antibacterial activity.

Another possible reason for the limited efficacy in this trial might be that the dose of carvacrol used was too low to achieve a significant *Campylobacter* cecal load reduction in broiler chickens at slaughter age. According to Arsi et al. (Arsi et al. 2014), it is necessary to add carvacrol to the feed in a dose of 1%, which is significantly higher than the dose used in this trial. However, in terms of application in commercial poultry farms, such a high dose is uneconomical. Since already dietary carvacrol in the amount of 200 mg/kg significantly reduces feed intake and weight gain of broilers (Lee et al. 2003) it can only be used at relatively low concentrations. Furthermore, carvacrol is currently only approved in a maximum dose of 120–160 mg/g feed additive (equaling 10.4–16.8 mg/kg feed) for broiler fattening in Germany and in the EU (see Commission Implementing Regulation (EU) 2020/996). In any case and contrary to the assessment of Arsi et al. (Arsi et al. 2014), the administration of increased carvacrol doses does not appear to be accompanied by an increased reduction of *Campylobacter* cecal loads (Allaoua et al. 2018).

The limited efficacy of carvacrol in this trial could, in general, also be based on specific attributes of essential oils. Essential oils are volatile, their stability and bioactivity can be compromised by temperature, light, metals, water and oxygen availability in production systems (Turek and Stintzing 2013). Their high reactivity is another obstacle to their direct application in feeds, as reactivity with the feed matrix may affect their activities.

In general, it is postulated that high protein diets (as supplied in poultry production) protect pathogenic bacteria (Aureli et al. 1992; Pandit and Shelef 1994; Tassou et al. 1995; Burt 2004) and lower the biological effects of essential oils (Śliwiński et al. 2002). Recent findings indicate that dietary composition (Zeng et al. 2015) and physical structure (Burt 2004) may limit the antibacterial activity of essential oils. Furthermore, poorly mixed feed with unevenly distributed carvacrol may cause individual chickens within the flock to consume different amounts of carvacrol. However, we were very strict about proper storage (premixed feed was stored in airtight containers) and homogeneous distribution of carvacrol in the feed, which should have minimized the aforementioned factors. Finally, the possible emergence of bacteria resistant to carvacrol should also be considered when evaluating its reduced efficacy. Such resistance to carvacrol was previously demonstrated for gram-positive *Listeria monocytogenes* and gram-negative bacteria like *Pseudomonas aeruginosa* and *Escherichia coli* (Ait-Ouazzou et al. 2013;

Pesingi et al. 2019). In general, however, resistance to components of essential oils rarely occurs because they contain many different molecules that have different modes of action (Becerril et al. 2012).

5.1.2. Complex CE culture (publication II)

A balanced and stable microbiome composition is critical for the nutrient metabolism, the intestinal mucosal barrier and the immune functions of broiler chickens (Richards, Fothergill, et al. 2019; Wu et al. 2021). In turn, an unstable microbiome is susceptible to naturally occurring microbiome alterations resulting from myriad confounding factors such as environmental influences (e.g., seasonal and geographical climate changes) host factors (e.g., genetics and gut development), increase of broiler age (Oakley et al. 2014), medication, housing, *Campylobacter* colonization (especially the time of infection) (Patuzzi et al. 2021) or feed (Borda-Molina et al. 2018; Takeshita et al. 2021), which may induce changes in microbiota abundance and diversity and thus are associated with the colonization of pathogens in the chicken gut (Kers et al. 2018; Diaz Carrasco et al. 2019; Takeshita et al. 2021). In this context, the administration of complex CE cultures in broiler production has proven effective in promoting the early development of a stable microbiota community, thereby counteracting susceptibility of broiler chickens to such naturally occurring microbiome alterations and ultimately reducing colonization with pathogenic bacteria (Richards, Fothergill, et al. 2019).

In this trial, CE culture administration (using a practical approach via spray and drinking water) yielded a consistent and statistically significant reduction of *C. jejuni* counts in cloacal swabs 8, 11, 16 and 18 dpi (the maximum observed log reduction was 2 $\log_{10}$ MPN) (publication II, Figure 1A). Moreover, at the end of the trial after necropsy, we determined a significantly decreased *C. jejuni* cecal load in 33-day-old broilers. In comparison to the control group, the cecum of broilers receiving the CE culture showed significantly reduced *Campylobacter* counts (reduction of 1 $\log_{10}$ MPN/g). Likewise, colon counts were significantly lower (reduction of about 2 $\log_{10}$ MPN/g) (publication II, Figure 1B). Our observations are in line with several other studies which provided circumstantial evidence for the protective role of CE cultures against *Campylobacter* colonization (Aho et al. 1992; Schoeni and Wong 1994; Hakkinen and Schneitz 1999; Schneitz and Hakkinen 2016). We assume that an early administration of the complex CE culture to newly hatched chickens in combination with a second treatment via the drinking water had a stabilizing effect on microbiota composition and abundance while also reducing susceptibility to the aforementioned confounding factors.

Although our observations are promising, earlier attempts using CE cultures of different compositions revealed a varying potential for the reduction of *Campylobacter* colonization (Aho et al. 1992; Schoeni and Wong 1994; Mead et al. 1996; Mead 2000; Wagenaar et al. 2006;

Schneitz and Hakkinen 2016; Ty et al. 2022). Furthermore, experimental data regarding the effect of the specific CE product used in this trial on *C. jejuni* colonization and infection of the vertebrate host are sparse. So far, only two studies have been published while one had been conducted with broiler chickens (Ty et al. 2022) and the other one with mice (Heimesaat, Weschka, et al. 2021). While the results obtained by Ty et al. (Ty et al. 2022) reflect the success found in this trial, three consecutive oral CE culture applications to microbiota-depleted IL-10$^{-/-}$mice after infection with *C. jejuni* strain 81-176 did not affect their gastrointestinal *C. jejuni* colonization (Heimesaat, Weschka, et al. 2021). Remarkably, however, in the same study, the treatment resulted in improved clinical outcome, less distinct pro-inflammatory immune responses and attenuated apoptotic cell responses in infected large intestines during acute campylobacteriosis of mice.

While the relevance of the study by Heimesaat et al. (Heimesaat, Weschka, et al. 2021) conducted with mice is difficult to evaluate in the current context, the remarkable discrepancies between studies conducted with broiler chickens are likely due to differences in rearing conditions, methods of administration or challenge, the timing of administration, CE culture compositions (few bacterial strains or abundance of different bacteria), donor material, bird strain, stress, and rearing length (Pivnick 1982; Mead 2000). Similarly, incorrect storage (e.g., oxygen exposure) may affect CE culture efficacy (Wagner 2006). Moreover, it should be considered that the composition of CE cultures may vary considerably between different batches. Consequently, the efficacy of the CE culture may be compromised if the number of bacterial strains that contribute significantly to CE activity is reduced (Wagner 2006). It would thus be beneficial to determine whether differences in CE culture composition actually have an impact on anti-*Campylobacter* activity.

Furthermore, even though it has not been addressed in this trial, it is reasonable to assume that one of the most decisive factors affecting the efficacy of CE cultures is the broiler diet. Dietary components that escape host digestion and absorption serve as substrates for intestinal bacterial growth and significantly determine microbiota composition (Pan and Yu 2014) as well as *Campylobacter* establishment. Thus, diets that do not promote or support the growth of bacteria from CE cultures may prevent their establishment and therefore their protective effect against pathogenic bacteria. For example, it has been shown recently that lower crude protein content and a specific amino acid composition of the diet reduced the mucin (the main constituent of the intestinal mucus layer) released in the digestive tract of broilers (Visscher et al. 2018). Apparently, there is a correlation between crude protein content in the ratio and the synthesis of mucins (Ravindran et al. 2008). It is well established that *C. jejuni* preferentially colonizes the mucus layer and the presence of mucin appears to be crucial for its growth and survival (Van Deun, Pasmans, et al. 2008), which in turn would

explain the difficult establishment of *C. jejuni* when it lacks certain amino acids (serine, aspartic acid, glutamic acid, and proline) important for its metabolism (Guccione et al. 2008; Visscher et al. 2018). Therefore, it is not surprising that diets, that on the one hand, do not promote or support the growth of bacteria from CE cultures and on the other hand, are rich in protein, may favor *C. jejuni* colonization because the bacteria can claim certain resources essential nutrients (essential amino acids) for its metabolism (Baker and Han 1994) and attachment sites without any competition. However, these are only assumptions so far because ration and microbiome analyses have unfortunately not been part of this trial. Thus, we are not able to illustrate potential differences in microbiome composition after CE culture treatment. These should be the subject of further investigations in the future.

Regarding public health, it must be considered that bacteria in CE products may be undefined, microbial resistant and include virulence genes that pose a risk to human customers (Wagner 2006), which is why they have not yet been approved for poultry fattening in Germany and the EU. Indeed, approval requires a full characterization of their contents under some legislation (e.g., under the European Regulation on additives for use in animal nutrition (Regulation (EC) No 1831/2003)). Nevertheless, CE products may significantly contribute to the control of *Campylobacter* as a foodborne pathogen if these hurdles are addressed and overcome in the future.

5.1.3. Organic acids (publication III)

Due to the ban on the use of antibiotics as growth promoters in the EU in 2006, poultry farms are increasingly exposed to the risk of disease occurrence (Dittoe et al. 2018). In this context, acidic compounds consisting of organic acids are under discussion as a possible control strategy against *Campylobacter* since they represent a relatively low-cost alternative to antibiotics and can be easily administered via feed or drinking water (Mani-López et al. 2012; Meunier et al. 2016). In this trial, a mixture consisting of four different organic acids (benzoic-, propionic-, sorbic- and acetic acid) was applied daily via the drinking water (in a dilution of 1:30) during the entire growth period and its efficacy to reduce fecal shedding of *C. jejuni* and intestinal *C. jejuni* colonization was determined. The addition of organic acids to the drinking water adjusted the drinking water to pH 6.0. During the entire rearing period, we observed a significantly reduced fecal shedding of the group receiving organic acids (lower *Campylobacter* counts in cloacal swabs) (publication III, Figure 1A). However, at the end of the trial *Campylobacter* colonization in cecal and colon contents did not differ significantly ($p > 0.05$) in comparison to the control group (publication III, Figure 1B). Previous *in vivo* studies also yielded inconsistent results regarding the antibacterial effects of organic acids as feed supplements (de los Santos et al. 2008; Van Deun, Haesebrouck, et al. 2008; Skånseng et al.

2010; Solís de los Santos et al. 2010; Hermans et al. 2012). The reason for this discrepancy remains unclear. The failure to reduce intestinal *C. jejuni* carriage could be attributed to the inability of organic acids to affect the lower part of the GIT, the ability of bacteria (*C. jejuni)* to develop resistance to organic acids as well as their impeding effect on beneficial host bacteria (like *Lactobacillus* spp.) (Dittoe et al. 2018).

Indeed, a large part of their bacterial action is associated with reaching sufficiently effective concentrations in the various compartments of the GIT (Dittoe et al. 2018). The concentration of organic acids necessary to develop effective antibacterial properties in the intestinal tract may depend on several factors. For example, the pH of the GIT rises almost to pH 7 as the intestine progresses (Wolin 1974; Miller and Sulavik 1996) which is unfavorable for acid efficacy. The weakly acidic nature of organic acids requires preferably an acidic pH value as they are more abundant and most effective in their undissociated form. In this state, organic acids are able to diffuse to the cytoplasmic membrane of pathogens (Davidson et al. 2012) and thus exert their antimicrobial activity. In this context it should be noted that water acidification with high acid concentrations might affect the flavor of the drinking water, therefore decreasing the palatability (Walsh et al. 2007; Haughton et al. 2013) and reducing the chickens' water assumption. In particular, pH values below 5.0 are assumed to make water unpalatable (Tabler et al. 2013; Jacobs et al. 2020). However, to circumvent this issue, we acidified the drinking water to a pH value of 6.0 which should have avoided an alienated water taste.

The lack of reduction in the cecum could also simply be explained by the inability of organic acids to reach their destination in the first place (Ricke 2003). It should be taken into account that the direct effects of organic acids on pathogenic bacteria are often limited to the crop and gizzard (Hume et al. 1993; Thompson and Hinton 1997) and proximal small intestine (Goodarzi Boroojeni et al. 2014). Given that organic acids exert their effects primarily in the crop, it is evident that with the oral inoculation of 10^4 *C. jejuni* in this trial, some *C. jejuni* may still manage to successfully pass through the crop, escape the action of the acids and thus colonize the ceca of broilers.

In addition, the specific efficacy of certain organic acids is highly dependent on intraluminal acid concentrations produced by lactic acid bacterial populations in the GIT (Papatsiros et al. 2013). Previous studies have demonstrated that feeding organic acids in poultry diets reduced both, the amount of lactic acid and *Lactobacilli* present in the GIT (Impey and Mead 1989; Thompson and Hinton 1997). For this reason, their potentially deleterious effect on beneficial bacteria should not be underestimated (Dittoe et al. 2018). Although microbiome studies were not part of this trial, we still cannot rule out the possibility that *Lactobacillus* populations were

also reduced. If so, this may have favored *Campylobacter* colonization since the bacteria were unrivaled in terms of niche occupation.

As previously mentioned (see section 1.5.1.), resistance to organic acids may occur as a response to a stressful environment. Likewise, it has been reported that bacteria are able to adapt to acid stress by induction of an acid tolerance response. This response allows them to reduce their internal pH to a tolerable level that ensures continued survival and growth, as well as protection against organic acids (Ricke 2003). Thus, resistance or adaptation might be a possible explanation for the missing efficacy of *Campylobacter* cecal colonization in this trial. It is quite conceivable that the prolonged and continuous exposure to organic acids during the experiment caused the *C. jejuni* strain to develop an increased tolerance to organic acids. Indeed, this assumption is consistent with a study by Ren et al. (Ren et al. 2021), which showed that malic acid reduced *Campylobacter* cecal carriage when added to the drinking water for five consecutive days prior to slaughter, as opposed to when added over a total period of three weeks. As a result, treatment with organic acids might have led to the onset of organic-acid-tolerant bacterial populations. Nevertheless, susceptibility testing of collected re-isolates during the experiment did not indicate the presence of *Campylobacter* populations tolerant to organic acids (re-isolates MIC values equal to those of the initially inoculated *C. jejuni* strain BfR-CA-14430).

5.1.4. Alternative breed (Ranger Gold) and reduced stocking density (unpublished)

Exploiting genetic variation takes advantage of the lower susceptibility and higher evolutionary potential of certain hosts. Interestingly, the existence of heritable resistance to *C. jejuni* colonization has already been established in several chicken lines (Li et al. 2010; Li et al. 2011; Li et al. 2012; Bailey et al. 2018). In this trial, we aimed to determine whether the use of an alternative slow-growing breed (Ranger Gold) and a reduced stocking density of 25 kg/m² may have an impact on *C. jejuni* colonization in broiler chickens. Although we observed significantly reduced *C. jejuni* counts in cloacal swabs up to 18 dpi, at the end of the trial we observed a significant increase in the *C. jejuni* load which was consistent with high *Campylobacter* loads in the cecum. Surprisingly, we observed significantly reduced *C. jejuni* numbers in the colon. Nevertheless, the increase in colonization associated with high *C. jejuni* loads in the cecum suggests that this measure is unlikely to be an effective control strategy against *Campylobacter* in poultry farms.

We believe that the temporarily lower *C. jejuni* counts in the cecum were simply due to the lower daily gains that led to the observed growth and weight differences between the Ranger Gold and the control group. While differences in the susceptibility of chicken breeds for *Campylobacte*r colonization have been observed (Stern et al. 1990; Boyd et al. 2005), such

differences in colonization also occur between individual broiler chickens of the same flock, housed under identical conditions (Wagenaar et al. 2008). It has already been shown that *C. jejuni* colonization varies widely within a poultry flock (average load varies between 5 to 6 log CFU/g) (Battersby et al. 2017). Accordingly, Gormley et al. (Gormley et al. 2014) observed no significant difference in cecal load between birds of different genotypes both in mixed- and single-genotype pens. Likewise, Chinotan et al. (Chintoan-Uta et al. 2020) observed no major differences in microbial populations and resistance or susceptibility to colonization was not conferred by transferring gut bacteria between different inbred chicken lines. Similarly, Humphrey et al. (Humphrey et al. 2014) found no significant impact of broiler breed on *C. jejuni* levels. In contrast, Georgiev et al. (Georgiev et al. 2017) found that specific breeds are less likely than others to be highly colonized with *Campylobacter*. Nonetheless, it appears, that the potential for genetic control over the degree of *Campylobacter* colonization among commercial broilers is likely to be limited (Bailey et al. 2018).

5.1.5. Bacteriophages (unpublished)

The potential of phages to mitigate *Campylobacter* colonization in chickens has been shown in several trials (Loc Carrillo et al. 2005; Wagenaar et al. 2005; El-Shibiny et al. 2009; Carvalho et al. 2010; Fischer et al. 2013; Kittler et al. 2013; Chinivasagam et al. 2020; D'Angelantonio et al. 2021). In this trial, we applied a combination of group II (LVL1/1) and group III (NCTC 12673) phages via the drinking water. Phage treatment on three consecutive days before necropsy (29–31 days of age) significantly reduced *C. jejuni* counts in cloacal swabs ($p < 0.0001$). At the end of the trial, however, the treatment failed to decrease *C. jejuni* colonization in the cecum but significantly ($p < 0.0001$) reduced *C. jejuni* numbers in the colon (reduction of 2 $\log_{10}$ MPN/g). Likewise, Kittler et al. (Kittler et al. 2013) observed significantly reduced *Campylobacter* cecal counts in one trial (> 3 $\log_{10}$ units) but the same reduction was not achieved in two other replicates. Hammerl et al. (Hammerl et al. 2014) did not yield a significant reduction in the cecum after having applied two groups III phages (CP14, CP 81) either.

One conceivable factor limiting the efficacy of the phage therapy could have been a loss of stability (Malik et al. 2017) of the phage cocktail in the drinking water. When phages are administered via drinking water, it is necessary to guarantee that the applied phage titer remains stable for a prolonged period to ensure sufficient uptake by the animals and consequently sufficient concentration in the cecum (Sanjay and Seema 2012; Chinivasagam et al. 2020; Alomari et al. 2021). In this trial, however, evaluation of phage stability in the drinking water immediately after the addition and after 24 hours yielded no significant concentration decrease. Therefore, it is rather unlikely that stability loss was the reason for not achieving a significant *Campylobacter* reduction in the cecum.

Another possible factor for the limited efficacy of the phage therapy could have been the failure to achieve a sufficiently high titer of phages in the cecum (Chinivasagam et al. 2020) which can be caused, in particular, by the phages lacking robustness against the GIT environment where they encounter low pH values (Leverentz et al. 2001). In this trial, however, both phages were found in fecal, cecal and colon samples (NCTC 12673 ≈ mean 4.0–5.0 log_{10} PFU/ml; LVL1/1 ≈ mean 3.0–5.0 log_{10} PFU/ml) (unpublished data), which supports successful phage persistence and replication in the GIT. Moreover, both phages achieved equally stable concentrations in the cecum (mean 4.9 log_{10} PFU/ml) (unpublished data). Authors of other studies observed similar or even lower titers of phages (ranging from log_{10} 3.2 to log_{10} 6.5 PFU/g cecal contents) which resulted in a successful reduction of *C. jejuni* colonization of broiler chickens (Loc Carrillo et al. 2005; Wagenaar et al. 2005; El-Shibiny et al. 2009; Carvalho et al. 2010; Fischer et al. 2013). Thus, it can be reasonably ruled out that the limited efficacy of the phage therapy was caused by low phage concentrations in the cecum.

A more likely reason for the limited results of the phage therapy in this trial could have been the way of administration of the phages. In particular, dose and timing are critical to the success of phage therapy in reducing *Campylobacter* loads in chickens (Levin and Bull 1996; Payne and Jansen 2001; Weld et al. 2004). The most effective dose for phage therapy was indicated to be 7 log_{10} PFU. Deviating doses (> 9 log_{10} PFU or < 5 log_{10} PFU) generally appear to be less effective (Loc Carrillo et al. 2005). Particularly at higher doses, this is likely due to phage aggregation and nonspecific association with digesta or non-host bacteria (Rabinovitch et al. 2003; Connerton et al. 2011). Indeed, phages seem to require a host density threshold, so called “phage proliferation threshold” (Wiggins and Alexander 1985; Payne and Jansen 2001), to proliferate sufficiently and to target or control pathogenic bacteria (Connerton et al. 2011). Accordingly, in this trial, the phage cocktail was continuously administered via the drinking water at a final concentration of 7 log_{10} PFU/ml although El-Shibiny et al. (El-Shibiny et al. 2009) found that only high doses of phages (10^9 PFU) reduced *C. jejuni* and *C. coli* cecal counts. In accordance with the assumption that phage administration towards the end of rearing appears to achieve the greatest success (Loc Carrillo et al. 2005; El-Shibiny et al. 2009), phage treatment in this trial was carried out on three consecutive days before necropsy. Nevertheless, it might be possible that such repeated phage administration was a reason for the limited success. In fact, an extended administration has been shown to be detrimental as *Campylobacter* populations commence to recuperate (Richards, Connerton, et al. 2019).

Finally, the most likely factor responsible for the failure of the phages to reduce *Campylobacter* populations in the cecum of broiler chickens in this trial is the development of resistance. The emergence of resistance of *C. jejuni* post-phage treatment occurs upon passage through the broiler gut and was reported to occur in 1–14% of the isolates (Loc Carrillo et al. 2005; El-

Shibiny et al. 2009; Fischer et al. 2013; Hammerl et al. 2014; Richards, Connerton, et al. 2019). Indeed, we identified *C. jejuni* isolates collected post-phage treatment to show emerging resistance to both phage classes. Interestingly, observed resistance rates to phage NCTC 12673 were greater (23.6%) than to phage LVL1/1 (2.8%) (unpublished data). Thus, it is reasonable to assume, that emerge of resistant bacteria to both phages limited their efficacy to reduce *C. jejuni* colonization in the chicken ceca. Nevertheless, as assumed by Weld et al. (Weld et al. 2004), physiological differences between phage/bacterial growth *in vitro* and growth *in vivo*, phage-bacteria specificity, endo- and exotoxins in phage lysates, phage contamination, and phage-specific host immune response may also explain the seemingly unpredictable results of phage administration *in vivo*.

5.2. Additional benefits of using carvacrol and organic acids

To ensure a targeted reduction of the *Campylobacter* load, established intervention measures should preferably exert their effects in the cecum since it is the primary site of *C. jejuni* colonization. Nevertheless, reduced colonization of other intestinal segments (such as the colon) is also expected to lower the overall *Campylobacter* load of broiler carcasses during processing. Accordingly, even though the examined non-biosecurity based intervention measures were not observed to lower *C. jejuni* loads in the cecum (with the exception of the CE culture), the results of these measures are still relevant to poultry practice. Given the complex nature of transmission, these measures may leverage their effects outside the host and still provide a vast contribution in targeting and controlling the bacterium. In this respect, they can be exploited as additional treatments to raise the threshold for colonization via oral exposure (Wales et al. 2019). Preventive measures such as organic acids or carvacrol may be valuable in lowering *Campylobacter* survival outside the host, thereby eliminating feed and water as potential environmental sources. Although *Campylobacter* is generally not present in fresh feed and water (Humphrey et al. 1993; Gregory et al. 1997; van de Giessen et al. 1998), both can become contaminated from other sources, such as broiler feces (Gregory et al. 1997), rendering them potential infectious vehicles for horizontal *Campylobacter* transmission within the broiler houses (Sahin et al. 2003; Schroeder et al. 2014). Similarly, litter is supposed to become a potential source of infection (Sahin et al. 2015) if it is accumulated with *Campylobacter* contaminated broiler feces. As broiler chickens have permanent access to feed and water and are prone to extensive coprophagy (Wagenaar et al. 2008) but also equally ingest litter from the floor (Svihus et al. 2009) they are at ubiquitous risk of being colonized by *Campylobacter* stemming from the environment.

To minimize this risk, drinking water acidification through the use of organic acids aims to lower the pH value in the environment thus rendering it hostile for *Campylobacter* (pH values < 4.5

are detrimental for *Campylobacter*) with the overall survivability of *Campylobacter* being considerably diminished (Jansen et al. 2014). This likely not only delays and reduces the risk of introduction and partly prevents transmission (van Bunnik et al. 2012), but also reduces the likelihood of a colonization of the entire broiler flock (Allain et al. 2014; Jansen et al. 2014). Accordingly, the use of organic acids can provide a convenient way to significantly improve drinking water sanitation and sanitization (Wales et al. 2019).

Recently, Wagle et al. (Wagle et al. 2020) found that carvacrol is a potential antimicrobial compound that suppresses biofilms formed by *Campylobacter*, thereby eliminating them as a potential environmental reservoir. Consequently, by lowering the ability of *Campylobacter* to integrate into mixed-species biofilms (Joshua et al. 2006; Reuter et al. 2010; Brown et al. 2014), the use of carvacrol provides great potential for another preventive approach that can effectively limit the transmission of this pathogen and reduce its overall incidence (Elgamoudi and Korolik 2021).

5.3. Synergistic interaction of individual measures

In summary, each of the examined non-biosecurity based intervention measures demonstrated promising potential. Nonetheless, they may not be sufficient to prevent *C. jejuni* transmission and colonization at the farm level when applied individually. Therefore, applying multiple measures at the same time might prove more effective. The combination of selected individual measures may overcome their limited reducing effect in the cecum by broadening the range of antimicrobial activity since essential oils, organic acids, phages and CE cultures have different mechanisms of action and thus a combination may have synergistic effects, i.e., an effect that is greater than the sum of their individual effects (Hakeem and Lu 2020).

5.3.1. Combination of organic acids and carvacrol

Research has demonstrated that both organic acids and essential oils have the capability to improve broiler performance, body weight gain and reduce pathogenic bacteria (Denli et al. 2004; Cabuk et al. 2006; Johny et al. 2010; Samanta et al. 2010; Adil et al. 2011; Banday et al. 2015; Youssef et al. 2021). Although the complete mechanism of action of organic acids and essential oils is not yet fully understood, it is clear that both exhibit a pathway of action that can be considered to be similar: while organic acids are able to directly reduce the pH in the poultry GIT (Samanta et al. 2008; Panda et al. 2009), carvacrol promotes the growth and establishment of probiotic microflora (e.g., *Lactobacillus*) and also its SCFA production (Tiihonen et al. 2010; Du et al. 2015; Mohammadpour et al. 2015; Hashemipour et al. 2016; Nobakht et al. 2016; Kelly et al. 2017; Yin et al. 2017; Yang et al. 2019; Irawan et al. 2020). Consequently, organic acids as well as essential oils improve gut health as they directly affect epithelial cells by providing them with SCFA as an energy source for growth (Kasubuchi et al.

2015; Gadde et al. 2017). Likewise, organic acids and carvacrol interfere with pathogenic bacteria by either penetrating their surrounding membrane or disrupting and disturbing physiological membrane function (Lambert et al. 2001; Biggs and Parsons 2008). Furthermore, both carvacrol and organic acids are known to increase and enhance nutrient digestibility by elevating protein retention (Amad et al. 2011; Gadde et al. 2017). By affecting the protein metabolism, carvacrol and organic acids together could contribute to reduce *Campylobacter* loads in poultry as *C. jejuni* is known to acquire amino acids for its oxidative energy metabolism while growing within the poultry' ceca (Stahl et al. 2012). Accordingly, enhanced ileal absorption of amino acids may restrict a major nutrient source for *Campylobacter* catabolism (Velayudhan et al. 2004). Moreover, organic acids have been shown to stimulate the growth of antagonistic probiotic microflora (Baffoni et al. 2012; Baffoni et al. 2017), leading to a shift in the intestinal microbiota towards more homogenous and distinct populations and increased *Lactobacillus* colonization of the chicken ileum (Nava et al. 2009; Weber et al. 2012) which in turn may inhibit *C. jejuni* establishment as its colonization has been shown to be associated with lower abundances of *Lactobacillus* (Kaakoush et al. 2014; Connerton et al. 2018; Dittoe et al. 2018; Sakaridis et al. 2018; Patuzzi et al. 2021; Takeshita et al. 2021).

Further, it is conceivable that organic acids lead to a synergistic interaction by lowering the pH in the GIT (Yang et al. 2019) and creating a hostile environment for *Campylobacter* as it has been shown that the antibacterial effect of carvacrol is enhanced in an acidic environment (Rivas et al. 2010; Ait-Ouazzou et al. 2011; Nostro et al. 2012). Especially at lower pH values, carvacrol appears to be less dissociated and therefore much more hydrophobic. As a result, carvacrol increasingly attaches to hydrophobic regions of membrane proteins. Accordingly, better distribution into the lipid phase of the bacterial membrane increases the antibacterial activity of carvacrol (Marchese et al. 2018). Also, organic acids are thought to have bacteriostatic and bactericidal properties as they can suppress metabolic functions of pathogens at the cellular level which are essential for their survival (Russell 1992; Ricke 2003). In addition to the antimicrobial effect, it is relevant to note that organic acids and carvacrol seem to be particularly active in different parts of the GIT: while organic acids show positive effects in crop and gizzard, carvacrol appears to act rather in the distal GIT (Langhout 2000; Basmacioğlu-Malayoğlu et al. 2016). Consequently, a homogeneous distribution of both substances throughout the GIT could not only hinder the survival of *Campylobacter* during passage through different parts of the GIT but also ultimately its colonization in the cecum. During its passage, *Campylobacter* encounters acids and bile acids (Wagle et al. 2020). Indeed, a previous *in vitro* study illustrated that carvacrol increased the susceptibility of *C. jejuni* to acid and bile and reduced its ability to detect and respond to cell population density

by gene regulation (quorum sensing) due to decreased signal production of the molecule autoinducer-2 activity (Wagle et al. 2020).

5.3.2. Combination of bacteriophages and CE cultures

Recent findings indicate that both CE cultures and phages are beneficial for intestinal health by modulating the gut microbiome in chickens (Wagner 2006; Clavijo and Flórez 2018; Upadhaya et al. 2021). While the use of CE cultures allows the quick establishment of a competitive microbiota after hatching, thereby potentially influencing and altering the composition of the GIT microbiota (Wagner 2006), phages particularly affect specific pathogenic microbial populations and promote the proliferation of beneficial microbiota (Clavijo and Flórez 2018). Since the attachment of *Campylobacter* to the intestinal mucus layer can be considered an essential part of its pathogenic strategy, it stands to reason that primarily those bacteria competing for the same niche can successfully displace *Campylobacter* (Lee et al. 1986; Alemka et al. 2012). In competition with *Campylobacter* for chicken intestinal cecal mucus, *Lactobacillus* (a major component of commercial CE cultures) once again has proven to be an advantageous antagonist, successfully inhibiting the adhesion of *C. jejuni in vitro* (Alemka et al. 2012; Ganan et al. 2013). The ability of *Lactobacilli* to reduce *Campylobacter* adhesion to the mucus layer may in turn contribute to the enhanced ability of phages (when applied in a timely manner) to target and infect *C. jejuni* cells as they are no longer surrounded by protective mucus. Such synergistic effect may further be amplified by the ability of phages to increase *Lactobacillus* populations (Upadhaya et al. 2021). This conjecture is consistent with a study illustrating that a combination of phages along with a CE product is significantly more effective in reducing *Salmonella* carriage in chickens than either treatment on its own (Toro et al. 2005). Accordingly, it is reasonable to consider that the combined use of phages and CE cultures might also control *Campylobacter* colonization: first, through beneficial antagonistic bacteria contained in the CE culture, which not only compete with *Campylobacter* for niches and nutrient sources but also impede its colonization and establishment within the mucus layer of the cecal crypts by stabilizing the microbiota and, second, by phages through their specific infection and lysis of *Campylobacter* cells which is enhanced by the mechanism of action of the CE culture.

5.4. Prospects and implementation in broiler production

Campylobacter mitigation at the farm level still faces many hurdles in commercial poultry production. Due to its ubiquitous nature, *Campylobacter* frequently occurs as a member of the poultry gastrointestinal microbial community, which challenges the optimization of intervention strategies (Olson et al. 2021). Besides, the establishment and implementation of specific intervention measures at the farm level entail the fulfillment of certain requirements:

(i) compliance with specific regulations, (ii) efficacy, (iii) consumer acceptance, (iv) reasonable costs, (v) avoidance of adverse effects on feed uptake and broiler performance, (vi) harmlessness to chicken and human health and (vii) the absence of sensory properties that affect the final product (Meunier et al. 2016; Soro et al. 2020). In addition, universal implementation is difficult to realize on a global level because poultry production in different parts of the world varies significantly in terms of the type of operation (indoor or outdoor), the equipment used for feed and water supply, the type of litter used (new or reused between groups, different litter material), the microclimate, the type of ventilation and finally the breed of chicken used. These differences affect the efficacy of certain intervention measures against *Campylobacter* and may determine which measures are ultimately preferable to achieve the greatest risk reduction (Facciolà et al. 2017).

Furthermore, a major limitation is the transferability of studies conducted under experimental conditions to commercial farms. Indeed, experimental broiler chicken models cannot fully mimic actual conditions on commercial farms. In contrast, it is difficult to control experimental conditions on farms because complex environmental factors such as biosecurity, housing type and climate can affect the composition of the microbiota in the chicken gut (Kers et al. 2018; Takeshita et al. 2021). Moreover, the costs and benefits of intervention measures remain difficult to estimate in terms of large-scale feasibility and the practical viability appears to vary widely across broiler industries and countries (Wagenaar et al. 2008). Oftentimes, as in the case of CE cultures and phages, the enforcement of specific legislations or harmonized EU regulatory frameworks is required (Use et al. 2017). Specifically, uniform application of measures across all EU Member States is difficult and the lack of effective tools, especially those aimed at reducing colonization of the poultry intestine, could limit the reduction of the risk of campylobacteriosis in humans (Meunier et al. 2016). Moreover, as mentioned earlier (see section 5.1.2) CE cultures may be considered a potential source of pathogens (Mead 2000) and may harbor transferable antimicrobial drug resistance or virulence genes (Wagner 2006) that could pose a risk to human health. In turn, the application of organic acids and carvacrol is cheap and quite simple to implement at the farm level, as they are usually considered safe and are already listed as authorized feed additives in the EU under Regulation (EC) No 1831/2003. Unfortunately, there are still limits to their administration at the farm level since the required doses often exceed the legally permissible amount or are no longer accepted by the animals due to flavor alterations (Franz et al. 2010). Encapsulation methods have been suggested to improve *in vivo* efficiencies by protecting the active compound from degradation before it reaches its place of action. However, these new approaches and particular formulations of active compounds considerably increase the overall treatment costs (Meunier et al. 2016). With regard to the long-term feasibility of certain measures the use of

organic acids, in particular, must be considered as they may damage water pipes (corrosion) or promote the formation of *Campylobacter* biofilms which in turn can lead to the formation of a new environmental reservoir (Jansen et al. 2014). Long-term use of organic acids may also promote the development of bacterial adaptation and resistance. However, development of resistance has been reported as a rather rare event (Suresh et al. 2018). An obstacle to the direct application of essential oils such as carvacrol and their incorporation into food and feed is that their stability and bioactivity can be affected by temperature, light, metals, and the availability of water and oxygen in production systems (Turek and Stintzing 2013). Further, the reactivity of essential oils with the feed matrix may influence their activities. For example, a study indicated lower biological effects of phytogenic feed additives present in fibrous diets (Beauchemin and McGinn 2006) or high protein diets (Śliwiński et al. 2002). Equally problematic is the acceptance by the animals. It cannot be ruled that unpredictable effects occur, like antagonistic and synergistic effects, possible interactions of essential oils and aroma substances with other substances (Stevanović et al. 2018).

Regarding the administration of phages at the farm level prior to slaughter, the occurrence and the emergence of phage-resistant *Campylobacter* post-phage treatment (1–14% of the isolates), consumer safety as well as production costs should be thoroughly considered (Jäckel et al. 2019; Richards, Connerton, et al. 2019; Żbikowska et al. 2020). However, safety concerns should not be a major drawback as phages are highly specific and can infect only a limited number of host bacteria. Even oral consumption of high amounts is expected to be completely harmless to humans (Hermans, Van Deun, Messens, et al. 2011). Only in rare cases, phages may interact with host immune systems and result in a harmful but reversible immune response (Alisky et al. 1998; Kutateladze and Adamia 2010) as a result of improper phage purification (Skurnik et al. 2007). Against this background, phages as components of commercial products are currently already approved for the elimination of pathogens from food products of animal origin in the USA, Canada, Israel, Australia and some European countries (France, Sweden, Switzerland and the Netherlands). At the same time, the demand and number of approvals of phage preparations as substances generally recognized as safe continue to increase worldwide (Dec et al. 2020). Nevertheless, the efficacy of phage administration is hampered by the practical inability to estimate the predominant spectrum of *C. jejuni* field strains in poultry flocks until the initial onset (usually between 14 and 21 days of age). The preparation of large amounts of phages against different *Campylobacter* field strains is ruled out because the material costs associated with manufacturing costs cannot be justified. Consequently, it is only practical to administer a phage cocktail that has previously been shown to be effective against the majority of field strains (Loc-Carrillo and Abedon 2011). For large-scale applications on broiler farms, it may be advantageous to establish rapid detection

methods to provide an earlier indication of predominant background *Campylobacter* strains in the respective broiler houses. Similarly, the environmental impact of phage release and consumer acceptance of phage-treated food needs to be determined (Meunier et al. 2016).

6. Conclusion

The outline of this study was to determine whether various non-biosecurity based intervention measures have the potential to reduce *Campylobacter* spp. colonization in broiler production. In this study, we demonstrated that each of the measures evaluated (carvacrol, organic acids, a complex CE culture, different broiler breed (Ranger Gold) in combination with a reduced stocking density and phages) has the potential to reduce *C. jejuni* colonization in broiler chickens during rearing. Similarly, all measures, except the administration of organic acids, successfully decreased *C. jejuni* colonization in the colon which is known to contribute to fecal contamination of broiler carcasses during slaughter. However, only one of the measures evaluated, the complex CE culture, proved to be effective in reducing *C. jejuni* colonization in the cecum as well.

In conclusion, the results of this study strongly suggest that a reduction of *C. jejuni* colonization in broiler chickens can be achieved by non-biosecurity based intervention measures, in particular the use of a complex CE culture seems highly promising. Even though organic acids and carvacrol were not shown to decrease the *C. jejuni* load in the cecum of broiler chickens, they may be valuable in lowering *C. jejuni* survival outside the host, thereby eliminating feed and water as potential environmental sources. Nevertheless, given that the efficacy of all measures evaluated herein may be vulnerable to many different factors, reducing *Campylobacter* remains challenging. Likewise, successful establishment and implementation, the costs and benefits of non-biosecurity based intervention measures remain difficult to estimate in terms of large-scale feasibility and the practical viability appears to vary widely across broiler industries and countries. Besides, strategies to mitigate *Campylobacter* colonization in poultry should consider the complex nature of its transmission and may require both, preventive and therapeutical approaches since *Campylobacter* rapidly colonizes entire broiler flocks after its introduction and broiler chickens then carry high numbers of *C. jejuni* in their intestinal tract.

Thus, there is still an urgent need for reliable and practical intervention measures in primary production and further research in all areas is necessary and relevant. Most importantly, large-scale field trials need to be conducted to investigate the practical effect of distinct control strategies in a commercial poultry production setting. Due to the ubiquitous presence of *C. jejuni* in the environment, successful elimination of this pathogen will most likely be achieved through the implementation of stringent biosecurity measures in conjunction with a strategic combination of existing and novel control strategies or by using multiple approaches that target different stages of the poultry chain. In particular, to combat *C. jejuni* colonization at multiple

different levels, a combination of organic acids and carvacrol as well as CE cultures and phages may be a promising treatment option that should be further examined in the future.

7. Summary

Non-biosecurity based intervention measures against *Campylobacter* spp. in broiler production

Campylobacter is a commensal microorganism that generally colonizes the ceca of avian species and is detected in high prevalence in poultry farms. Once colonized with *C. jejuni*, chickens may remain *C. jejuni* carriers and excretors until slaughter, which inevitably increases the potential for carcass contamination during processing, in turn allowing transmission of the pathogen to humans. While *Campylobacter* carriage in broilers is mostly asymptomatic, human infection via contaminated meat causes abdominal pain, fever and acute enteritis and occasionally serious late sequelae like the Guillain-Barré syndrome, reactive arthritis and the Miller-Fisher syndrome. The objective of the present study was to establish a broiler chicken colonization model which targets natural infection and keeps conditions close to commercial poultry production. This model was subsequently used to determine the efficacy of different non-biosecurity based intervention measures to reduce *C. jejuni* shedding and colonization in broiler chickens.

First, we conducted two preliminary studies in which we aimed at determining the lowest inoculation dose necessary to successfully colonize all 20 broiler chickens (of breed Ross 308) after 2 days. For this purpose, we used a dose consisting of 10^3 and 10^4 CFU of a *C. jejuni* strain. Results revealed that the dose consisting of 10^4 CFU/500µl fulfilled the desired requirement and was therefore used as an inoculation dose for further experiments.

Second, we aimed at examining the effect of different non-biosecurity based intervention measures to reduce *C. jejuni* colonization in broiler chickens using the established seeder bird model. The study consisted of several experimental groups, each with a specific intervention measure. One group served as a control group while the other group received one of the following treatments: an essential oil (carvacrol), a complex CE culture (Aviguard®), a blend of different organic acids or a combination of two phages. Another group did not receive any treatment but was conducted with an alternative slow-growing breed (Ranger Gold) in combination with a reduced stocking density (25 kg/m^2). Each of the measures evaluated showed evident potential to reduce *C. jejuni* colonization and shedding in broiler chickens during rearing (growing period). However, only one of the measures evaluated, the complex CE culture, proved to be effective in reducing *C. jejuni* colonization in the cecum. In contrast, colonization of the colon with *C. jejuni* was significantly reduced by all evaluated measures, except for organic acids.

In summary, the complex CE culture can be considered most valuable for the control of *Campylobacter* in poultry farms because it led to a significant *C. jejuni* load reduction in the cecum. Even if the other measures did not lead to a reduction in the cecum, they still showed notable potential. Therefore, it cannot be ruled out that further optimizations and new technologies will improve their applicability. Further research, especially large-scale field trials, is needed to investigate the practical effect of distinct control strategies in a commercial poultry production setting.

8. Zusammenfassung

Nicht biosicherheitsbasierte Interventionsmaßnahmen gegen *Campylobacter* spp. in der Broilerproduktion

Campylobacter sind kommensale Mikroorganismen, die häufig den Blinddarm vieler Geflügelarten kolonisieren und somit vor allem in Geflügelbetrieben häufig in hohen Prävalenzen nachgewiesen werden. Ist eine Kolonisierung erfolgt, bleiben die Masthühner über die gesamte Mastperiode mit *Campylobacter* (*C.*) *jejuni* kolonisiert, sodass sie bis zur Schlachtung Träger und Ausscheider von *C. jejuni* sind. Während des Schlachtprozesses und der Verarbeitung kann es sodann zu einer Kontamination der Geflügelschlachtkörper kommen, wodurch wiederum eine Übertragung des Erregers auf den Menschen ermöglicht wird. Während die Kolonisierung mit *Campylobacter* bei Masthähnchen meist asymptomatisch verläuft, kann eine *Campylobacter*-Infektion beim Menschen infolge des Verzehrs von kontaminierten Fleischprodukten Symptome wie Bauchschmerzen, Fieber und akuter Enteritis sowie gelegentlich schwerwiegende Spätfolgen wie das Guillain-Barré-Syndrom, reaktive Arthritis und das Miller-Fisher-Syndrom hervorrufen. Ziel der vorliegenden Studie war es, zunächst ein experimentelles Kolonisierungsmodell für Hühner zu entwickeln, welches die natürliche *Campylobacter*-Infektion von Masthühnern möglichst realitätsnah abbildet. Anschließend wurde dieses Modell verwendet, um die Wirksamkeit verschiedener nicht biosicherheitsbasierter Interventionsmaßnahmen zur Verringerung der Kolonisierung von Masthühnern mit *C. jejuni* zu ermitteln.

In einem ersten Schritt wurden zwei Vorstudien durchgeführt, um die niedrigste Inokulationsdosis zu ermitteln, die 2 Tage nach oraler Inokulation zur stabilen Kolonisierung aller inokulierten Masthühner der Rasse Ross 308 führt. Zu diesem Zweck wurde ein gut charakterisierter *C. jejuni* Stamm verwendet und je eine Dosis von 10^3 und 10^4 koloniebildenden Einheiten (KbE) verabreicht. Die Dosis von 10^4 KbE/500µl erfüllte die gewünschten Anforderungen und wurde daher als Inokulationsdosis für die weiteren Versuche verwendet.

In einem zweiten Schritt wurde sodann die Wirkung verschiedener nicht biosicherheitsbasierter Interventionsmaßnahmen zur Verringerung der *C. jejuni* Kolonisierung von Masthühnern untersucht. Die Studie umfasste mehrere Versuchsgruppen, mit jeweils einer spezifischen Interventionsmaßnahme. Eine Gruppe diente jeweils als Kontrollgruppe, während die andere Gruppe eine der folgenden Behandlungen erhielt: ein ätherisches Öl (Carvacrol), eine komplexe Competitive-Exclusion-Kultur (Aviguard®), eine Mischung aus verschiedenen organischen Säuren oder eine Kombination aus zwei Bakteriophagen. Eine

weitere Gruppe erhielt keine Behandlung, sondern wurde mit einer alternativen, langsam wachsenden Rasse (Ranger Gold) in Kombination mit einer deutlich reduzierten Besatzdichte (25 kg/m^2) gehalten. Jede Maßnahme zeigte ein deutliches Potenzial zur Verringerung der Kolonisierung von Masthähnchen mit *C. jejuni* während der Aufzucht (Wachstumsperiode). Allerdings erwies sich nur eine Maßnahme, die komplexe Competitive-Exclusion-Kultur, als wirksam gegen die *C. jejuni*-Kolonisierung im Blinddarm. Im Gegensatz dazu wurde die Besiedlung des Dickdarms von Masthühnern mit *C. jejuni* durch alle bewerteten Maßnahmen, mit Ausnahme der Applikation von organischen Säuren, deutlich reduziert.

Im Ergebnis erwies sich die Applikation der komplexen Competitive-Exclusion-Kultur als der vielversprechendste Ansatz zur Kontrolle von *Campylobacter*, da diese zu einer signifikanten Verringerung der *C. jejuni*-Belastung im Blinddarm der Masthühner führte. Auch wenn durch die anderen Maßnahmen keine Reduktion im Blinddarm zu erzielen war, zeigten diese dennoch auch ein Potenzial, die Kolonisierung von Masthühnern mit *C. jejuni* sowie die Ausscheidung von *C. jejuni* zu reduzieren. Folglich ist nicht auszuschließen, dass weitere Optimierungen oder der Einsatz neuer Technologien deren praktischen Nutzen verbessern könnten. Weitere Studien, insbesondere groß angelegte Feldversuche sind erforderlich, um die praktische Anwendbarkeit der untersuchten Maßnahmen in einer kommerziellen Geflügelproduktion zu beurteilen.

9. References

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IV. List of publications

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Szott, V.; Reichelt, B.; Friese, A.; Roesler, U. (2019): **Impact evaluation of non-biosecurity based measures on *Campylobacter* spp. prevalence in a seeder-bird model**. **CHRO 2019 Belfast,** Belfast, 08.-12.09.2019.

Szott, V.; Reichelt B, Friese, A.; Roesler, U. (2019): **Impact evaluation of non-biosecurity based measures on *Campylobacter* spp. prevalence in a seeder-bird model**. XIX International Congress of ISAH 2019, Breslau, 08.-12.09.2019.

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colonization in broiler chickens. Tagung der DVG-Fachgruppe Bakteriologie und Mykologie, online, 14.-16.06.2021.

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Szott, V; Reichelt, B.; Daehre, K.; Roesler, U. (2018): **Establishing a seeder bird model to evaluate the effectiveness of non-biosecurity based measures on *Campylobacter* prevalence in broiler flocks**. International Symposium on Zoonoses Research**,** Berlin, 17.10.2018.

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VII. Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

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