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# *In vivo* efficacy of carvacrol on *Campylobacter jejuni* prevalence in broiler chickens during an entire fattening period

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## 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<sub>2</sub>O<sub>2</sub>) 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<sup>2</sup> 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<sup>4</sup> 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



wavelength of 600 nm ( $OD_{600}$ ) in order to obtain an inoculum containing  $1 \times 10^6$  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 were 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

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 <sup>a</sup>	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

<sup>a</sup>Megajoules of metabolizable energy.

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).

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.

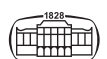
On day 33 of age (average weight 2.0 kg) all 36 sentinels per group were euthanized using ZKS poultry pliers (Corstechnology, Neerstedt, Netherlands) after confirming deep anesthesia. The animals were dissected and intestinal content (cecum and colon) was collected for *C. jejuni* enumeration and determination. 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

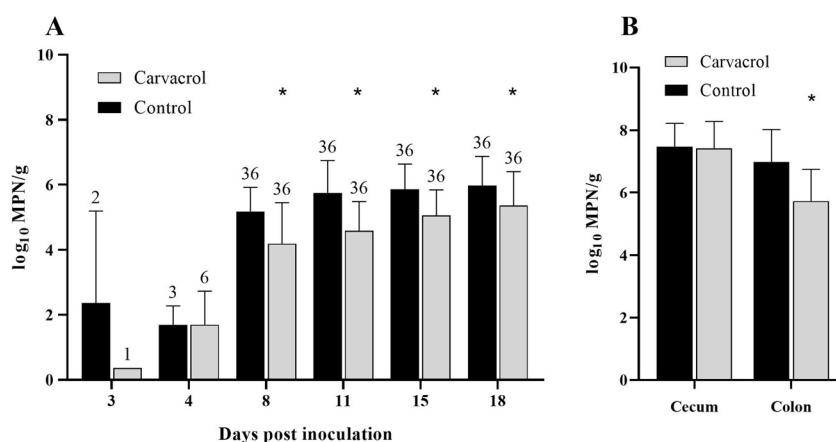


Fig. 1. (A) Mean  $\log_{10}$  most probable number (MPN) of *C. jejuni* per gram in cloacal swabs at each point in time after inoculation. (B) Mean  $\log_{10}$  MPN of *C. jejuni* per gram in intestinal content. Black bars represent the control group (broilers challenged with *C. jejuni* and not treated 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.

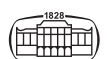
The results of this *in vivo* study demonstrate that a supplementation with 120 mg/kg feed of carvacrol is able to reduce *C. jejuni* load in cloacal swabs significantly at a 0.6 log<sub>10</sub> minimum. As McLendon et al. [49] enlightened in a recent study, the use of cloacal swabs is a reliable method 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<sup>7</sup> 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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