Is allicin able to reduce *Campylobacter jejuni* colonization in broilers when added to drinking water?

J. Robyn,* G. Rasschaert,*[1] D. Hermans,† F. Pasmans,† and M. Heyndrickx*†

*Institute for Agricultural and Fisheries Research (ILVO), Technology and Food Science Unit, Brusselsesteenweg 370, Melle, Belgium; and †Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, Merelbeke, Belgium

ABSTRACT Reducing *Campylobacter* shedding on the farm could result in a reduction of the number of human campylobacteriosis cases. In this study, we first investigated if allicin, allyl disulfide, and garlic oil extract were able to either prevent *C. jejuni* growth or kill *C. jejuni* in vitro. Allyl disulfide and garlic oil extract reduced *C. jejuni* numbers in vitro below a detectable level at a concentration of 50 mg/kg (no lower concentrations were tested), whereas allicin reduced *C. jejuni* numbers below a detectable level at a concentration as low as 7.5 mg/kg. In further experiments we screened for the anti-*C. jejuni* activity of allicin in a fermentation system closely mimicking the broiler cecal environment using cecal microbiota and mucus isolated from *C. jejuni*-free broilers. During these fermentation experiments, allicin reduced *C. jejuni* numbers below a detectable level after 24 h at a concentration of 50 mg/kg. In contrast, 25 mg/kg of allicin killed *C. jejuni* in the first 28 h of incubation, but anti-*C. jejuni* activity was lost after 48 h of incubation, probably due to the presence of mucin in the growth medium. This had been confirmed in fermentation experiments in the presence of broiler cecal mucus. Based on these results, we performed an in vivo experiment to assess the prevention or reduction of cecal *C. jejuni* colonization in broiler chickens when allicin was added to drinking water. We demonstrated that allicin in drinking water did not have a statistically significant effect on cecal *C. jejuni* colonization in broilers. It was assumed, based on in vitro experiments, that the activity of allicin was thwarted by the presence of mucin-containing mucus. Despite promising in vitro results, allicin was not capable of statistically influencing *C. jejuni* colonization in a broiler flock, although a trend toward lower cecal *C. jejuni* numbers in allicin-treated broilers was observed.

Key words: *Campylobacter jejuni*, allicin, in vivo, broiler, drinking water

2013 Poultry Science 92:1408–1418
http://dx.doi.org/10.3382/ps.2012-02863

INTRODUCTION

Since 2005, *Campylobacter* infections caused by thermotolerant *Campylobacter* species [more specifically *Campylobacter jejuni* and *Campylobacter coli* (Tauxe, 2002)] have been the leading cause of human bacterial gastroenteritis in many developed countries (Ailes et al., 2008; EFSA, 2010a, 2011). Because the broiler cecum can be colonized to a high degree by *C. jejuni*, broiler chickens can serve as a potential reservoir for *Campylobacter* strains pathogenic to humans (Altekruse et al., 1999; Fields and Swerdlow, 1999; Friis et al., 2010). Intestinal colonization with *Campylobacter* during rearing can lead to contamination of carcasses during processing (Herman et al., 2003; Rasschaert et al., 2006; Rosenquist et al., 2006). Contamination can arise during defeathering and evisceration due to contaminated feces leaking from the cloaca or a ruptured cecum (Berrang et al., 2001; Smith et al., 2007). Worldwide, the prevalence of *Campylobacter* contamination of poultry carcasses is reported to be 60 to 80% on average (Suzuki and Yamamoto, 2009; EFSA, 2010b). Broiler chicken meat contaminated with *C. jejuni* is believed to be responsible for up to 40% of human campylobacteriosis cases (EFSA, 2010c) in the European Union.

According to a Belgian risk assessment (Messens et al., 2007), if a 1, 2, or 3 log reduction of the *Campylobacter* contamination on carcasses would be achieved, the incidence of human campylobacteriosis cases in Belgium would be reduced by 48, 85, and 96%, respectively.

On-farm intervention measures could result in possible reduction of contamination levels of the carcasses of these animals. Because the intestine of living poultry is the only amplification site for *Campylobacter* through-

Received October 29, 2012.
Accepted January 24, 2013.
[1]Corresponding author: Geertrui.rasschaert@ilvo.vlaanderen.be

out the entire food chain, one could aim to either prevent *Campylobacter* introduction and transmission in poultry flocks or to reduce intestinal *Campylobacter* counts in colonized animals. Reducing the cecal *Campylobacter* load in poultry during primary production can be expected to significantly reduce the incidence of human campylobacteriosis (Lin, 2009).

Medicinal use of *Allium sativum* (garlic) has been widespread throughout human history. Crushed garlic has long been known to display antibacterial properties (Hann and Koch, 1996; Thomson and Ali, 2003). Various garlic preparations have been shown to exhibit a wide spectrum of antibacterial activity against gram-negative bacteria, including *Campylobacter jejuni*, as well as gram-positive bacteria (Cowan, 1999; De Wet et al., 1999; Friedman et al., 2002; Cutler et al., 2009; Fujisawa et al., 2009; Perry et al., 2009; Lu et al., 2011). This effect is primarily attributed to sulfur-containing organic compounds (Harris et al., 2001; Kyung and Lee, 2001; Sivam, 2001; Lu et al., 2011; Rose et al., 2005) such as allicin (Ankri and Mirelman, 1999; Miron et al., 2000; Kyung, 2012), ajoene (Naganawa et al., 1996), and various diallyl sulfides (O'Gara et al., 2000; Tsao et al., 2007; Lu et al., 2011). The active molecule, allicin, a thiosulfinate, is formed during lysis of alliin by alliinase (Lawson and Hughes, 1992; Rabinkov et al., 1994), an enzyme located in a garlic clove compartment separated from its substrate alliin (Ellmore and Feldberg, 1994). Allicin contains an oxidized disulfide bond [–S(O)–S–], which generally reacts with the thiol groups of cellular proteins and enzymes. By targeting these enzymes (Focke et al., 1990; Rabinkov et al., 1998; Miron et al., 2000; Waag et al., 2010) or cellular processes (Feldberg et al., 1988; Ghannoum, 1988), allicine inhibits the growth of microorganisms. For example, allicin was capable of partially inhibiting DNA and protein synthesis in *Salmonella enterica* serovar Typhimurium and had an immediate effect on RNA synthesis (Feldberg et al., 1988). More research is needed into the possibility of supplementing plant-derived antimicrobial compounds to the feed or drinking water to combat cecal *Campylobacter* colonization in poultry.

The first aim of this study was to investigate if allicin was able to prevent or reduce colonization of *C. jejuni* in a fermentation system mimicking the broiler cecal environment. The second aim was to determine the ability of allicin to prevent or reduce cecal *C. jejuni* colonization in vivo.

MATERIALS AND METHODS

Bacterial Strains and Culture Media

Campylobacter jejuni strains MB 4185 (KC 40) and MB 3834 (KC 96.1), collected from broiler houses and their surroundings (Herman et al., 2003) and NCTC 11168 (= LMG 8842, BCCM/LMG, Ghent University, Ghent, Belgium; Véron and Chatelain, 1973) were grown on *Campylobacter* Blood-Free Selective Agar base plates (CCDA, CM0739, Oxoid, Basingstoke, UK) + CCDA selective supplement (SR0155, Oxoid; modified charcoal cefoperazone deoxycholate agar, **mCCDA**). The strains were incubated at 41.5°C for approximately 24 h under a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) in a Forma Series II 3110 Water-Jacketed CO_2 incubator (Thermo Scientific, Waltham, MA; all microaerobic incubations were done in this incubator, unless otherwise mentioned). *Campylobacter jejuni* bacteria were enumerated by preparing 10-fold dilutions in Ringer's solution (BR0052G, Oxoid), which were spread on mCCDA and incubated microaerobically at 41.5°C for 24 to 48 h.

Determination of Concentrations of Garlic-Derived Products Active Against C. jejuni

The concentrations of garlic-derived products active against *C. jejuni* were determined at pH 6.5 using HCl or NaOH to obtain the desired pH values. Allicin (as part of Allimax, a very concentrated garlic preparation for animals, Allicin Animal Care Int., Vaassen, the Netherlands), alliin (74264, Sigma, St. Louis, MO), garlic oil extract (W530316, Sigma), and allyl disulfide (A35801, Sigma) were added to nutrient broth No. 2 (**NB2**, CM0067, Oxoid) + 0.05% (wt/vol) mucin from porcine stomach type II (M1778, Sigma) + 0.05% (wt/vol) mucin from porcine stomach type III (M2378, Sigma), to a final concentration of 500, 250, 125, 62, 31, 15, and 7.5 mg/kg for allicin; 100 and 50 mg/kg for garlic oil extract and allyl disulfide, and 50 mg/kg for alliin. All garlic-derived products were filter sterilized before addition. Overnight cultures of the *C. jejuni* strains (NCTC 11168, MB 4185, and MB 3834) to be tested were grown on mCCDA, suspended in Ringer's solution, and inoculated in the growth medium described above at an initial concentration of approximately 10^3 cfu/mL.

The action of allicin on *C. jejuni* strains NCTC 11168, MB 4185, and MB 3834 was studied in the presence and absence of *Campylobacter* growth supplement (SR0232E, Oxoid) in the growth medium described above. The influence of the alliine, allyl disulfide, and garlic oil extract was only tested against *C. jejuni* MB 4185 in growth medium described above without supplements. Incubation was performed for 48 h at 41.5°C under microaerobic conditions. Ten-fold dilutions of samples taken at 0, 24, and 48 h were plated out on mCCDA and incubated for 24 to 48 h under microaerobic conditions at 41.5°C for enumeration of *C. jejuni*.

Growth Conditions of Batch Cultures

Selected allicin concentrations (125, 100, 50, 25, and 10 mg/kg) exhibiting in vitro *C. jejuni* inhibition using the above-mentioned methods were tested in batch

culture experiments under controlled circumstances reproducing the pH (6.5), temperature (41.5°C), and atmospheric conditions (5% O_2, 10% CO_2, 85% N_2) that favor *C. jejuni* growth in the broiler cecum. *Campylobacter jejuni* MB 4185 was grown overnight on mCCDA under microaerobic conditions at 41.5°C and subsequently diluted in Ringers solution to a concentration of 10^5 to 10^8 cfu/mL, depending on the final concentration needed in the growth medium. The New Brunswick Scientific BioFlo110 fermentor vessels (New Brunswick Scientific, Enfield, CT) containing 500 mL of NB2 + 0.05% (wt/vol) mucin from porcine stomach type II + 0.05% (wt/vol) and mucin from porcine stomach type III were sterilized in a Fedegari FVA3/A1 (Fedegari Autoclavi SpA, Albuzzano, Italy) autoclaved for 10 min at 121°C. The sterile fermentor vessels were inoculated with the *C. jejuni* MB 4185 strain to a final concentration of 10^5 to 10^6 cfu/mL for experiments with cecal background flora or to a final concentration of approximately 10^3 cfu/mL for experiments without cecal background flora (see below). One vessel (control) only contained the *C. jejuni* strain in 500 mL of the incubation medium described above. The second vessel (experimental) contained the *C. jejuni* strain in a total volume of 500 mL consisting of the incubation medium to which different volumes of 500 mg/kg of sterile allicin were added before incubation up to the desired final concentration.

Incubation in the fermentor vessels was performed at 41.5°C and pH was kept stable at 6.5 using 5 *M* NaOH. The atmosphere was kept microaerobic by blowing a gas mixture of 5% O_2, 10% CO_2, and 85% N_2 (Air Liquide, Paris, France) directly into the growth medium.

Batch Fermentation Experimental Designs

The aim of the first experimental design was to investigate the therapeutic effect of allicin. In that experiment, various allicin concentrations with previously shown anti-*Campylobacter* activity (125, 50, 25, and 10 mg/kg) were added at the same time as the initial *C. jejuni* MB 4185 inoculation. The aim of the second experimental design was to examine the protective effect of allicin. In that experiment, the lowest bactericidal allicin concentration identified during the first experimental design was added 24 h before the *C. jejuni* MB 4185 strain.

To simulate broiler cecal conditions more closely, both experimental designs were repeated with the lowest allicin concentration that showed complete bactericidal features in the presence of cecal microbiota. One milliliter of a 10^{-3} dilution of broiler cecal microbiota was added to the growth medium at the start of each fermentation experiment. The cecal microbiota were obtained from cecal droppings collected from *C. jejuni*-negative broiler chickens and kept at −20°C in Trypton Soy Broth (TSB, CM0129, Oxoid) with 15% glycerol (vol/vol; 8.18709.1000, Merck, Darmstadt, Germany). During fermentation experiments, samples were aseptically taken at 0 (time of *C. jejuni* inoculation), 3, 24, 26, 28, and 48 h. All fermentation experiments were carried out in duplicate, except for the protective experimental design with 50 mg/kg of allicin, which was only tested once. Enumeration of *C. jejuni* was carried out as described above.

Influence of Mucus from Broiler Ceca on Allicin Activity

To determine whether sulfur-containing nonbacterial components residing in the cecal broiler environment affect the anti-*Campylobacter* activity of 50 mg/kg of allicin, we examined the bactericidal effect of allicin in the presence of mucus derived from the ceca of *Campylobacter*-free broilers (Hermans et al., 2010). In this experiment, *C. jejuni* MB 4185 was inoculated at an initial concentration of approximately 10^4 cfu/mL in 10 mL of NB2 + 0.05% (wt/vol) mucin from porcine stomach type II + 0.05% (wt/vol) mucin from porcine stomach type III containing either mucus (control), 50 mg/kg of allicin, or both mucus and 50 mg/kg of allicin. Mucus was tested at 0.2, 0.02, and 0.002 mg of protein/mL. Samples were aseptically taken at 0, 3, 24, 26, 28, and 48 h of incubation. Enumeration of *C. jejuni* was carried out as described above.

Effect of pH and Bile Salts on Allicin Activity

To test the conservation of the anti-*C. jejuni* activity of allicin during passage through the broiler gastrointestinal tract, we performed an in vitro experiment mimicking conditions of the gastrointestinal tract of broiler chickens. To do so, 25 mL of sterilized NB2 + 0.05% (wt/vol) mucin from porcine stomach type II + 0.05% (wt/vol) mucin from porcine stomach type III was dispensed in eight 50-mL centrifuge tubes (430829, Corning Inc., Corning, NY). Two centrifuge tubes were used as positive control and contained only the growth medium described above. Two tubes contained a final concentration of 50 mg/kg of filter-sterilized allicin in the growth medium. Two tubes contained a final concentration of 50 mg/kg of filter-sterilized allicin and 0.5% of filter-sterilized bile salts (B3301, Sigma) in the growth medium. The last 2 tubes, which contained 50 mg/kg of sterile allicin, were adjusted to pH = 3 (stomach pH) using HCl and incubated at 41.5°C for 3 h. Subsequently, the pH was adjusted to approximately 6.5 (broiler intestinal pH) using NaOH and 0.5% (wt/vol) of filter-sterilized bile salts was added. All tubes were subsequently inoculated with 10^4 cfu of *C. jejuni* MB 4185 and microaerobically incubated at 41.5°C for 48 h. Samples were taken at 6, 24, and 48 h. Enumeration of *C. jejuni* was carried out as described above.

Experimental Birds

Day-of-hatch Ross broiler chickens of both sexes from a local farm were raised in 2 groups in the experimental

facility of the Faculty of Veterinary Medicine of Ghent University for 14 d. Directly after inoculation with *C. jejuni* on d 14, broilers were housed separately in the experimental facility of the Faculty of Veterinary Medicine of Ghent University. Broilers were provided with feed and water ad libitum. Husbandry, euthanasia methods, experimental procedures, and biosafety precautions were approved by the Ethical Committee of the Ghent University Faculty of Veterinary Medicine, Ghent, Belgium (EC no. 2011/127). Chicks were proven to be *Campylobacter*-free before beginning the experiment.

In Vivo Trial with Allicin Added to Drinking Water

An in vivo experiment was carried out to determine the effect of allicin in drinking water on *Campylobacter* susceptibility of broilers and cecal *Campylobacter* survival. Day-of-hatch broiler chicks (n = 69) were randomly divided into 2 groups ($n_1 = 36$, $n_2 = 33$) and were housed separately. Birds of the first group (control) were given standard drinking water. Chicks of the second group (experimental) were given drinking water supplemented with allicin at a final concentration of 25 mg/kg. This concentration was determined to be tolerated by the chicks (unpublished results). At the age of 14 d, all chicks of both groups were orally inoculated with approximately 5.0×10^2 to 1.0×10^3 cfu/mL of the highly colonizing *C. jejuni* strain MB 4185. Subsequently chicks were transferred to separate housing (Hermans et al., 2012). Forty-eight hours later, all chicks were euthanized by injection of T61 (Intervet, Brussels, Belgium) in the wing vein (500 μL per chick), and the ceca and their contents were collected for *C. jejuni* enumeration (see below).

Cecal C. jejuni Enumeration

The ceca (including the contents) were cut into small fragments, weighed, and diluted 1:9 (wt/vol) in NB2 with Preston *Campylobacter* selective supplement and *Campylobacter* growth supplement for *C. jejuni* enumeration. After homogenization, a 10-fold dilution series was made in Ringer's solution. One hundred milliliters of each dilution was spread onto mCCDA plates. After 24 to 48 h of incubation at 41.5°C under microaerobic conditions, characteristic colonies were counted.

Statistical Analysis

Experimental data were analyzed using Statistica (StatSoft, Tulsa, OK) software. The significance level was set at 0.05. *Campylobacter jejuni* counts were log-transformed before statistical analysis. A one-way ANOVA was carried out to compare the means of the log-transformed counts in chicken cecal contents of all groups (treated and control groups) of the seeder model. Significant differences were assessed by Tukey post hoc tests.

RESULTS

Determining Active Concentrations of Garlic-Derived Products Against C. jejuni

As evidenced in a first experiment (results not shown), allicin was capable of killing 3 *C. jejuni* strains (MB 4185, MB 3834, and NCTC 11168) at relatively low concentrations. After 24 h of microaerobic incubation in the presence of allicin at concentrations ranging from 500 to 7.5 mg/kg, no living *C. jejuni* cells could be detected, whereas in the control experiment the MB 4185, MB 3834, and NRRL B-11168 *C. jejuni* strains grew to 6×10^5, 2.3×10^6, and 2×10^5 cfu/mL, respectively. This bactericidal influence is thwarted by adding *Campylobacter* growth supplement to the growth medium (results not shown): after 24 h in the presence of allicin concentrations up to 62 mg/kg, all 3 *C. jejuni* strains were capable of reaching numbers comparable with control numbers (approximately 10^7 cfu/mL). This indicates an increase in the minimum bactericidal concentration of allicin against *C. jejuni* from <7.5 mg/kg to a concentration ranging between 62 to 125 mg/kg.

Figure 1 shows that garlic oil as well as allyl sulfide are bactericidal against *C. jejuni* MB 4185 at 100 and 50 mg/kg; no *C. jejuni* could be found after 24 h or 48 h of microaerobic incubation in 2 repetitions. In the presence of alliine (50 mg/kg), *C. jejuni* MB 4185 was able to grow to numbers comparable with or higher than those growing in normal growth medium.

Batch Fermentation Experimental Designs

In a therapeutic experimental design, 50 and 125 mg/kg of allicin reduced *C. jejuni* MB 4185 numbers below detectable levels after 3 and 24 h, respectively (Figure 2A,B). The mean $\log_{10}$ value of *C. jejuni* numbers in the control vessel lacking allicin had increased approximately 2 to 5 $\log_{10}$ after 3 h (50 mg/kg experiment, Figure 2B) and 24 h (125 mg/kg experiment, Figure 2A) of incubation, respectively. When 10 mg/kg of allicin was added to the growth medium, no influence on *C. jejuni* MB 4185 growth was observed compared with the control (Figure 2D).

Growth of *C. jejuni* MB 4185 was impaired for the first 28 h of incubation in the presence of 25 mg/kg of allicin (Figure 2C); the mean $\log_{10}$ value of *C. jejuni* numbers in the control vessel lacking allicin was 4 to 6 $\log_{10}$ higher than the mean $\log_{10}$ value of *C. jejuni* numbers in the vessel containing 25 mg/kg of allicin. More specifically, after 3 h of incubation, the mean $\log_{10}$ value of *C. jejuni* numbers in the control fermentor vessel rose from 3.22 to 4.98 $\log_{10}$ cfu/mL, whereas that in the allicin-containing vessel dropped from 3.22

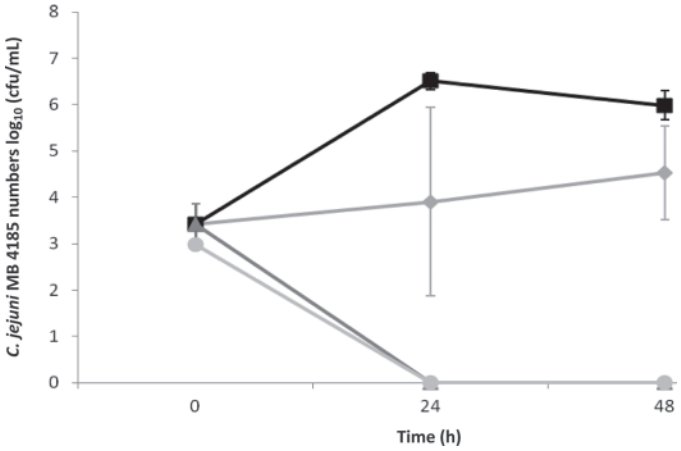

Figure 1. Determination of minimum bactericidal concentration of alliine, allyl disulfide, and garlic oil against *Campylobacter jejuni* MB 4185. Garlic oil and allyl sulfide lower *C. jejuni* MB 4185 numbers below detectable levels at 100 and 50 mg/kg concentrations. Experiments were carried out at 41.5°C and under a microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2). ◆: $\log_{10}$ (cfu/mL) of *C. jejuni* MB 4185 under noninhibitory growth circumstances (positive control). ■: $\log_{10}$ (cfu/mL) of *C. jejuni* MB 4185 in growth medium with 50 mg/kg of alliine. ●: $\log_{10}$ (cfu/mL) of *C. jejuni* MB 4185 in growth medium with 50 mg/kg garlic oil or 50 mg/kg of allyl disulfide. ▲: $\log_{10}$ (cfu/mL) of *C. jejuni* MB 4185 in growth medium with 100 mg/kg of garlic oil or 100 mg/kg of allyl disulfide (values represented by ▲ coincide with values represented by ●).

$\log_{10}$ cfu/mL at time of inoculation to 1.25 $\log_{10}$ cfu/mL. Additionally, whereas the mean $\log_{10}$ value of *C. jejuni* numbers in the control fermentor vessel remained stable around 6.63 $\log_{10}$ cfu/mL over the course of 24 to 28 h of incubation, the mean $\log_{10}$ value of *C. jejuni* numbers in the 25 mg/kg allicin-containing fermentor vessel was still low, although it rose from 1.23 to approximately 3.00 $\log_{10}$ cfu/mL during those 4 h of incubation. The mean $\log_{10}$ value of *C. jejuni* numbers in both fermentor vessels reached approximately 6.5 $\log_{10}$ cfu/mL after 48 h of incubation.

Figure 3 shows that 50 mg/kg of allicin is capable of exerting its bactericidal influence in an experimental design testing for the protective effect of allicin against *C. jejuni* MB 4185 (addition of 50 mg/kg of allicin to the growth medium 24 h before *C. jejuni* incubation). No live *C. jejuni* MB 4185 cells were found after 24 to 48 h of incubation under these conditions.

In the presence of broiler cecal background flora, allicin at a concentration of 50 mg/kg was able to suppress *C. jejuni* growth for at least 28 h of incubation in both the therapeutic experimental design (Figure 4A; allicin addition at the same time as *C. jejuni* MB 4185 incubation) and the protective experimental design (Figure 4B; allicin addition 24 h before *C. jejuni* MB 4185 incubation). When screening for a therapeutic effect of 50 mg/kg of allicin, no live *C. jejuni* colonies could

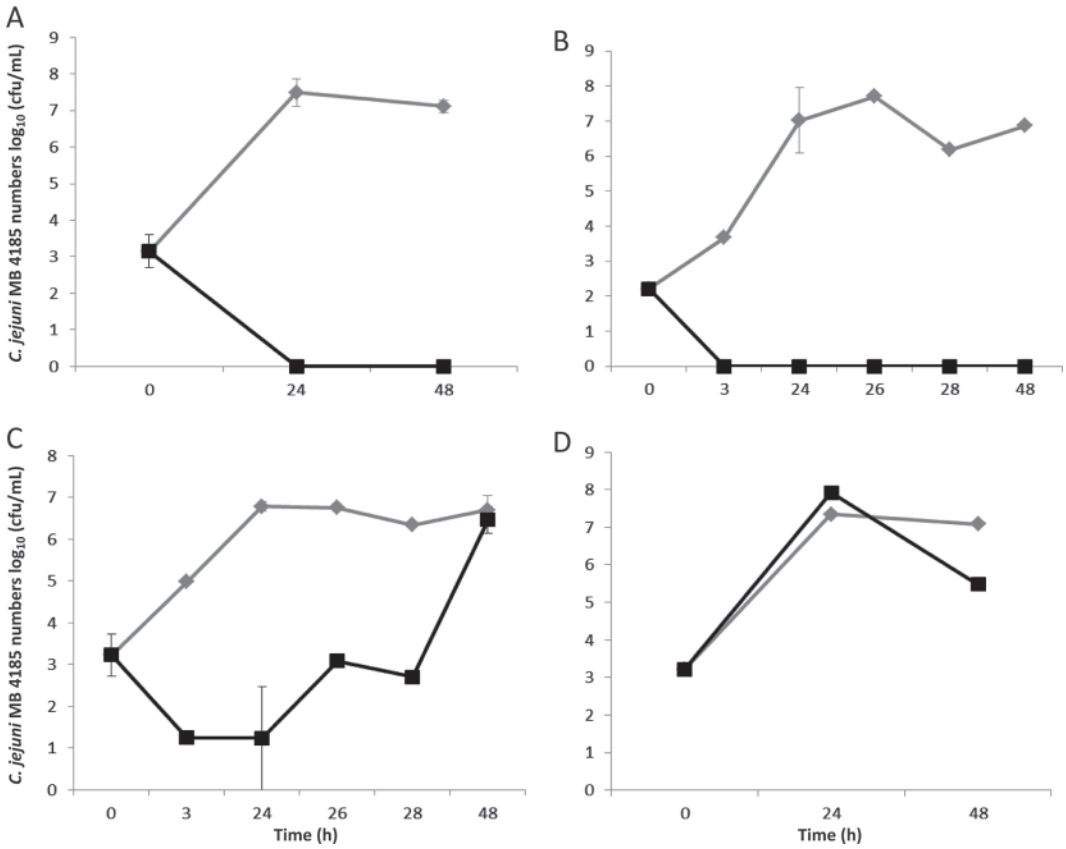

Figure 2. Therapeutic influence of A) 125 mg/kg, B) 50 mg/kg, C) 25 mg/kg, and D) 10 mg/kg of allicin on *Campylobacter jejuni* MB 4185 numbers. Experiments were carried out under simulated broiler cecal conditions: 41.5°C, pH = 6.5, and microaerobic atmosphere. This experimental design screens for a therapeutic effect of allicin. ◆: $\log_{10}$ (cfu/mL) of *C. jejuni* MB 4185 in growth medium under noninhibitory growth circumstances (positive control). ■: $\log_{10}$ (cfu/mL) of *C. jejuni* MB 4185 in growth medium with allicin.

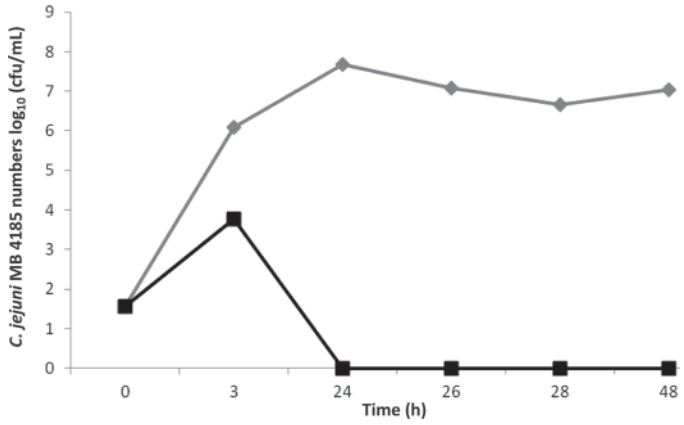

Figure 3. Protective influence of 50 mg/kg of allicin on *Campylobacter jejuni* MB 4185 numbers when added 24 h before *C. jejuni* MB 4185 incubation. Experiments were carried out under simulated broiler cecal conditions: 41.5°C, pH = 6.5, and microaerobic atmosphere. ◆: $\log_{10}$ (cfu/mL) of *C. jejuni* MB 4185 in growth medium under noninhibitory growth circumstances (positive control). ■: $\log_{10}$ (cfu/mL) of *C. jejuni* MB in growth medium with 50 mg/kg of allicin.

be detected during the first 28 h of incubation. After 48 h of incubation, a loss of anti-*C. jejuni* activity was observed, as the mean $\log_{10}$ value of *C. jejuni* numbers in the vessel containing 50 mg/kg of allicin (3.13 ± 3.13 $\log_{10}$ cfu/mL) had increased and was not statistically different ($P > 0.05$) from the mean $\log_{10}$ value of *C. jejuni* numbers in the control vessel (no allicin present; 6.77 ± 0.19 $\log_{10}$ cfu/mL; Figure 4A).

In the first 28 h of screening for a protective effect of 50 mg/kg of allicin toward *C. jejuni* MB 4185, the mean $\log_{10}$ value of *C. jejuni* numbers in the control vessel (approximately 6.7 $\log_{10}$ cfu/mL) was more than 4 log higher than the mean $\log_{10}$ value of *C. jejuni* numbers in the allicin-containing fermentor vessel (1.9 $\log_{10}$ cfu/mL), except for samples taken after 3 h of *C. jejuni* incubation, which were not statistically different ($P > 0.05$). After 48 h of *C. jejuni* incubation, this difference decreased over the next 20 h of incubation until it was no longer statistically different ($P > 0.05$), indicating a loss of anti-*C. jejuni* activity. The mean $\log_{10}$ value of *C. jejuni* numbers in the 50 mg/kg allicin-containing vessel amounted to 3.85 ± 3.85 $\log_{10}$ cfu/mL after 48 h of incubation, whereas that in the control vessel remained fairly stable (6.90 ± 0.05 $\log_{10}$ cfu/mL; Figure 4B).

Influence of Mucus from Broiler Ceca on Allicin Activity

The addition of 0.002 or 0.02 mg/mL of broiler cecal mucus to the growth medium had no influence on the bactericidal activity of 50 mg/kg of allicin (Figure 5A, B).

Only when 0.2 mg/mL of broiler cecal mucus was added to the growth medium was the anti-*C. jejuni* influence of 50 mg/kg of allicin lost (Figure 5C). In both regular growth medium (positive control) and in growth medium containing 50 mg/kg of allicin and 0.2 mg/mL mucus, *C. jejuni* MB 4185 was able to grow to 6.7 and 7.0 $\log_{10}$ cfu/mL, respectively, after 24 h of incubation. After 48 h, *C. jejuni* MB 4185 numbers decreased more rapidly in growth medium containing 50 mg/kg of allicin and 0.2 mg/mL mucus than in the regular growth medium (5.0 $\log_{10}$ cfu/mL as opposed to 6.2 $\log_{10}$ cfu/mL).

Effect of pH and Bile Salts on Allicin Activity

No effect of the acidic treatment (pH 3) during 3 h or the presence of 0.5% bile salts was detected on the anti-*C. jejuni* activity of 50 mg/kg of allicin, whether allicin

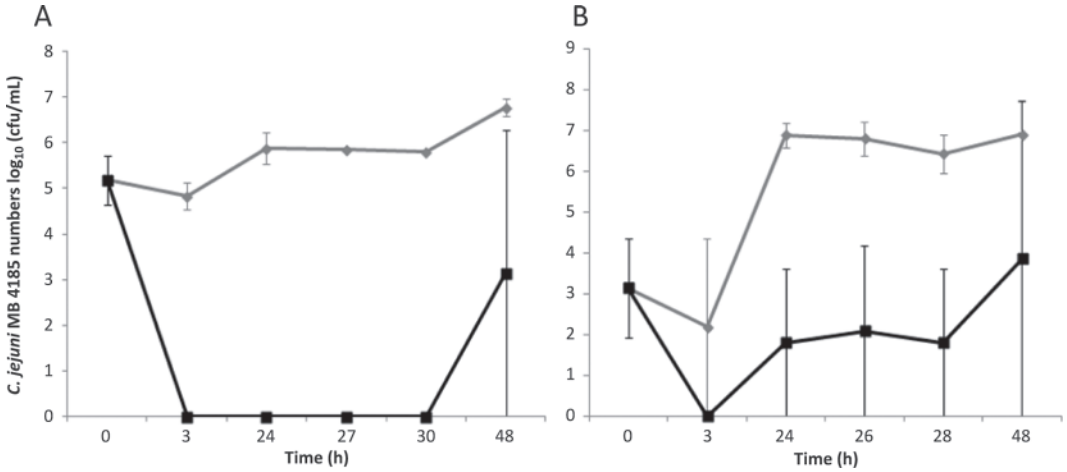

Figure 4. Influence of 50 mg/kg of allicin on *Campylobacter jejuni* MB 4185 numbers in the presence of broiler cecal background flora. A) A total of 50 mg/kg of allicin inoculated at the same time as *C. jejuni* MB 4185 incubation in growth medium containing background flora isolated from *Campylobacter*-free broiler ceca; B) 50 mg/kg of allicin inoculated 24 h before *C. jejuni* MB 4185 incubation in growth medium containing background flora isolated from *Campylobacter*-free broiler ceca. Experiments were carried out under simulated broiler cecal conditions: 41.5°C, pH = 6.5, and microaerobic atmosphere. ◆: $\log_{10}$ (cfu/mL) of *C. jejuni* MB 4185 in growth medium under noninhibitory growth circumstances (positive control) and added broiler cecal background flora. ■: $\log_{10}$ (cfu/mL) of *C. jejuni* MB in growth medium with allicin and broiler cecal background flora.

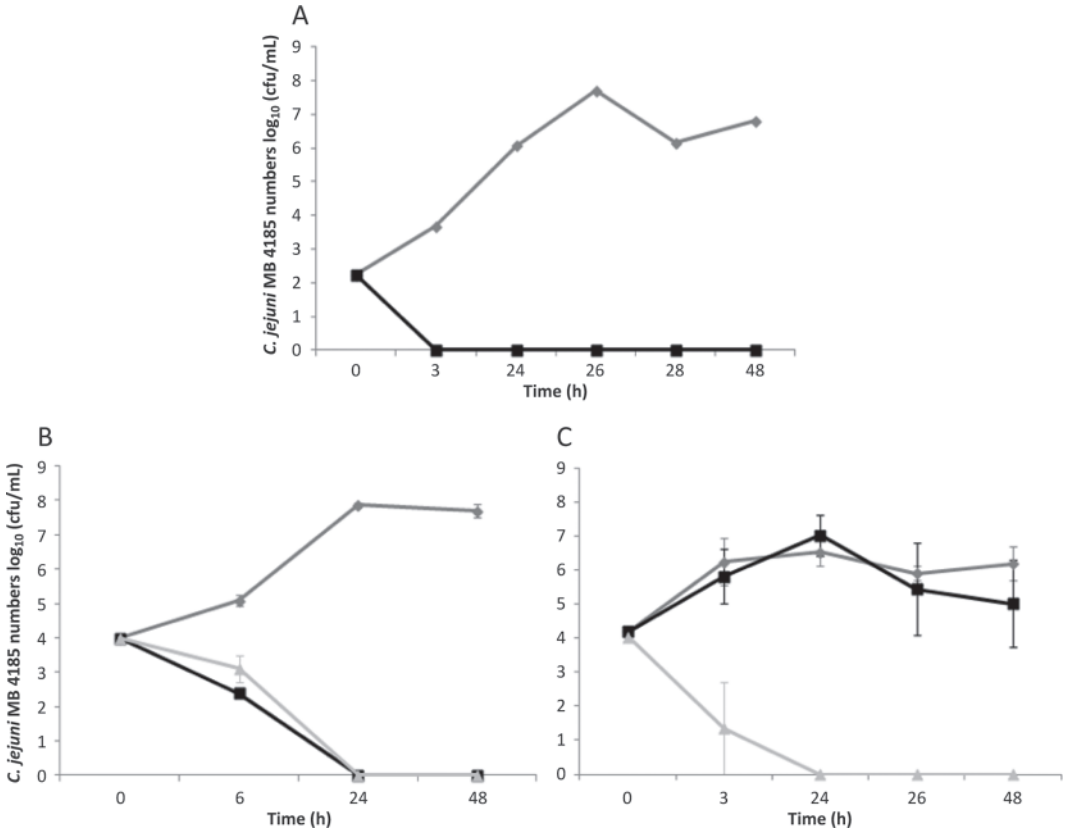

Figure 5. Influence of 50 mg/kg of allicin on *Campylobacter jejuni* MB 4185 numbers in the presence of cecal mucus from *Campylobacter*-free broilers. A) A total of 50 mg/kg of allicin inoculated at the same time as *C. jejuni* MB 4185 incubation in growth medium containing 0.002 mg/mL mucus; B) 50 mg/kg of allicin inoculated at the same time as *C. jejuni* MB 4185 incubation in growth medium containing 0.02 mg/mL mucus; C) 50 mg/kg of allicin inoculated at the same time as *C. jejuni* MB 4185 incubation in growth medium containing 0.2 mg/mL mucus. Experiments were carried out under closely simulated broiler cecal conditions in Falcon tubes: 41.5°C and microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2). ◆: $\log_{10}$ (cfu/mL) of *C. jejuni* MB 4185 in growth medium under noninhibitory growth circumstances (positive control). ■: $\log_{10}$ (cfu/mL) of *C. jejuni* MB in growth medium with 50 mg/kg of allicin and mucus (0.2; 0.02 and 0.002 mg/mL). ▲: $\log_{10}$ (cfu/mL) of *C. jejuni* MB 4185 in growth medium with 50 mg/kg of allicin. In 5A, ▲ and ■ gave the same result; thus, only ■ was displayed.

was exposed to a combination of acidic environment and 0.5% bile salts or exposed solely to 0.5% bile salts. Live *C. jejuni* cells could not be detected after 24 h of incubation in growth medium containing 0.5% bile salts + 50 mg/kg of allicin nor in growth medium to which 50 mg/kg of allicin and 0.5% bile salts was added after being kept at a pH of 3 for 3 h. *Campylobacter jejuni* MB 4185 grew to 3.3×10^7 cfu/mL in growth medium containing only 0.5% bile salts (positive control; results not shown).

In Vivo Trial with Allicin in Drinking Water

The mean $\log_{10}$ value ± SE of the number of *C. jejuni* colonies found in the ceca of broilers raised on standard drinking water or on 25 mg/kg of allicin-containing drinking water amounted to 6.01 ± 0.25 and to 5.38 ± 0.33, respectively. Although there was no statistically significant difference ($P = 0.130$) in mean cecal *C. jejuni* numbers between broilers raised on standard drinking water or on allicin-containing drinking water, there was an observable trend for ($P = 0.130$) lower cecal *C. jejuni* numbers in broilers raised on allicin-containing drinking water (Figure 6). All broilers in both treatment groups were found to be colonized. Influence of allicin on other cecal microbiota was not assessed.

There was no significant difference ($P > 0.05$) in the mean weight of broilers raised on standard (362.03 ± 42.03 g) or allicin-containing drinking water (354.97 ± 46.92 g).

DISCUSSION

Despite declining numbers of registered campylobacteriosis cases in some parts of the world in recent years, the disease remains problematic in developed countries (EFSA, 2011). Despite increasing evidence that poultry is the number 1 contributor to campylobacteriosis in humans (Altekruse et al., 1999), no effective strategy to clear *Campylobacter* from broiler flocks has yet been developed (Hermans et al., 2011).

In the current study, we investigated if allicin was able to inhibit *C. jejuni* growth in in vitro experiments mimicking the broiler chicken cecal environment and if it was able to (1) reduce susceptibility of broilers to *C. jejuni* and (2) reduce cecal *C. jejuni* colonization in an in vivo experiment in broilers. To our knowledge, this is the first study of anti-*Campylobacter* activity of allicin

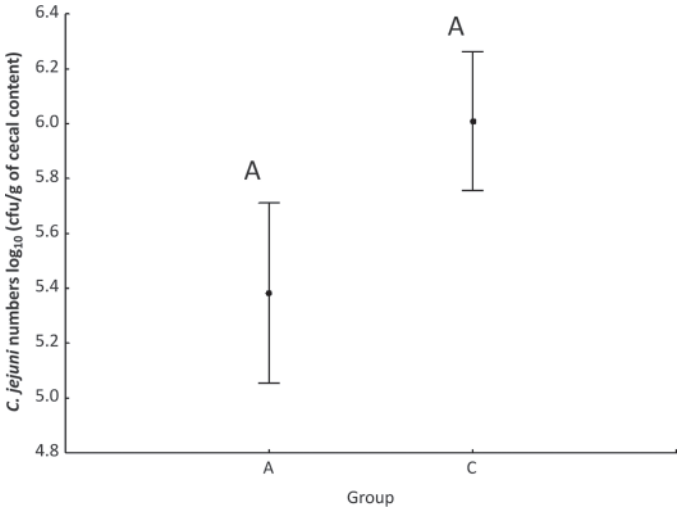

Figure 6. In vivo trial with 25 mg/kg of allicin in drinking water of broiler chickens. Broilers were separated into 2 groups. At 14 d of age, broiler chickens were orally inoculated with 5.0×10^2 to 1×10^3 cfu of *Campylobacter jejuni* MB 4185 and housed individually. On d 16, all broilers were euthanized and cecal *C. jejuni* MB 4185 numbers were determined. C: Mean cecal *C. jejuni* numbers of broilers provided with standard drinking water. A: Mean cecal *C. jejuni* numbers of broilers provided with drinking water containing 25 mg/kg of allicin. Bars denote ± SE. Common letters (A) on each curve designate nonsignificant differences ($P = 0.130$).

in an in vivo experiment in broiler chickens. We also tested the in vitro anti-*Campylobacter* activity of other allicin-related chemical components (allyl disulfide, diallyl disulfide, and diallyl trisulfide).

Activity of alliin, the allicin precursor (Rabinkov et al., 1994), was tested because it is more stable than allicin and thus would be easier to use as a drinking water/feed supplement. It was demonstrated that alliin had no inhibitory activity toward *C. jejuni.* Alliin is always present in garlic cloves; thus, one can presume that this chemical compound either does not have the same activity as allicin or does not exhibit activity by the same mechanisms as allicin. If that were the case, thiol-containing enzymes or tissue of the garlic clove itself could be affected. This also explains why the substrate alliin and the enzyme alliinase, which produces allicin from alliin, are located in different compartments of the garlic clove (Ellmore and Feldberg, 1994; Koch and Lawson, 1996).

We also tested various allicin degradation products, i.e., allyl disulfide and garlic oil extract (consisting of allyl disulfide, diallyl disulfide, and diallyl trisulfide) for anti-*Campylobacter* activity. These compounds can normally be spontaneously formed from allicin (Brodnitz et al., 1971), in water or in a basic environment (Block, 1985). Their activity was tested because these sulfur-containing compounds will probably be formed as degradation products in the drinking water, feed, or in the gastrointestinal tract of broilers when used as additives during primary production. We found that both allyl disulfide and garlic oil extract (a mixture of diallyl sulfide, diallyl disulfide, and diallyl trisulfide) were capable of lowering *C. jejuni* numbers below a detectable level at a concentration of 50 mg/kg in in vitro experiments.

In preliminary in vitro experiments, we demonstrated a disadvantageous effect of a *Campylobacter* growth supplement on the anti-*Campylobacter* activity of allicin, especially at lower concentrations (≤62 mg/kg). An explanation for the influence of *Campylobacter* growth supplement (which contains metabisulphite, pyruvate, and sulfate) was not experimentally investigated. We presumed that either the growth supplement interacts directly with *Campylobacter* colonies to protect them from allicin influence or metabisulphite, being an ambident nucleophile, performs a S_N2 nucleophilic substitution on allicin. This might result in a split of the S-allyl bond in the R-S(O)S-allyl compound (R also being an allyl group in the case of allicin).

We performed in vitro fermentation experiments under controlled temperature, pH, and atmosphere and in the presence of broiler cecal mucus and background flora to further elucidate the ability of allicin to inhibit *C. jejuni* growth under conditions closely simulating the broiler ceca. We could see an in vitro influence of both the presence of cecal background flora and the presence of mucins (of broiler and nonbroiler origin) on the anti-*Campylobacter* activity of different allicin concentrations.

Although the *C. jejuni* inhibiting activity of 25 mg/kg of allicin in the in vitro fermentation experiments disappeared between 28 to 48 h of incubation, broilers were provided with drinking water containing 25 mg/kg of allicin during the in vivo experiment. Because broilers drink more often than once every 28 to 48 h, we assumed that they ingested enough allicin to maintain the inhibitory effect of allicin. It was also confirmed that passage through an acidic environment and contact with bile salts, which occur during passage through a general animal gastrointestinal tract, had no influence on the anti-*C. jejuni* activity of allicin.

As the results of the in vivo experiment showed, broilers provided with allicin-containing drinking water did not have significantly lower cecal *C. jejuni* numbers than broilers in the control groups, although a trend toward lower cecal *C. jejuni* numbers could be observed (almost 1 $\log_{10}$). The discrepancies between the in vivo and in vitro results might be partly explained by the interaction of allicin with other intestinal/cecal or upstream bacteria as demonstrated in the in vitro fermentation experiments or by the presence of mucus in the intestinal and cecal environment of broilers.

The influence of mucins on allicin activity can be explained by the presence of cysteine rich amino- and carboxy-terminal regions in mucins, which generally help to establish disulfide linkages within and between mucin monomers (Albrecht and Bernkop-Schnürch, 2007). The antimicrobial activity of thiosulfinates such as allicin is cancelled by compounds containing a -SH moiety, such as cysteine, coenzyme A, and glutathione, but overall not by non-SH compounds (Fujisawa et al., 2009). Generally, most microbial cells do not have,

or have only small amounts of, glutathione (Smirnova and Oktyabrsky, 2005) and other -SH-containing compounds. They thus lack the ability to reactivate the essential SH-containing enzymes that are thiolated by allicin. Bacterial cells are therefore more sensitive to allicin than mammalian cells (Smirnova and Oktyabrsky, 2005). As the mucus layer covers the entire chicken intestinal tract, it must play a role in influencing the activity of allicin. Hermans et al. (2010) and Van Deun et al. (2008a) have already proven that chicken intestinal mucus protects *C. jejuni* from the antibacterial activity of capric acid and the sodium salt of butyric acid, respectively. Furthermore, because *C. jejuni* is preferentially attracted to mucus-filled crypts due to the chemotaxis of *C. jejuni* toward mucins (Beery et al., 1988), *C. jejuni* colonies are located in an environment that is disadvantageous for allicin activity. The exact concentration of mucus present in the cecum is not defined. The amount of cysteine, coenzyme A, and glutathione present in the cecum, mucus, or entire gastrointestinal tract is also not defined. This makes it difficult to predict the total loss of active allicin from the moment it is ingested until it reaches the cecum. *Campylobacter jejuni* colonies might also avoid extensive contact with allicin by undergoing several cycles of adherence, invasion, and escape from the epithelial cell layer in the cecum, followed by fast replication in mucus and reinvasion of the cell layer (Van Deun et al., 2008b).

Using in vitro fermentation experiments, we also demonstrated that the presence of broiler cecal background flora affected allicin activity. Using the protective in vitro fermentation design, we furthermore demonstrated how this influence grew along with increasing incubation time. As incubation time increases, more interaction becomes possible between allicin and non-*C. jejuni* cecal background flora. As Smirnova and Oktyabrsky (2005) described, once an allicin molecule reacts with a bacterial component, it is no longer capable of reacting with other components because it becomes irreversibly bound. This implies that interaction with non-*C. jejuni* cecal background flora reduces the amount of allicin available for interaction with *C. jejuni*. We could not determine if allicin indeed reacted with other cecal microbiota because we did not research the influence of allicin on the cecal microbiota during the in vivo experiment. Several studies, however, have stated that allicin or garlic-derived components are capable of influencing the survival of a wide range of bacterial species, some of which will probably be present in the broiler cecum (Ross et al., 2001; Ruddock et al., 2005; Cai et al., 2008; Cutler et al., 2009; Fujisawa et al., 2009; Khodavandi et al., 2010).

A possible way to counteract the loss of activity due to –SH-containing compounds might be by coating allicin to make sure allicin undergoes no deleterious effects due to interaction with other bacteria or –SH-containing compounds before reaching the cecum. This does not solve the problem of intestinal mucus, however. This might be remediated by the combined use of 1) coated allicin and 2) probiotics as prophylactics in feed. Ganan et al. (2012) recently stated that the human probiotic strains *Lactobacillus rhamnosus* GG, *Propionibacterium freudenreichii* spp. *shermanii* JS, and a starter culture *Lactoccoccus lactis* spp. *lactis* strain were able to adhere well to chicken intestinal mucus. This adhesion reduced the binding of *Campylobacter* spp. to mucus when the aforementioned strains colonized the mucus before the pathogen. This might increase the availability of *Campylobacter* spp. strains to the bactericidal effects of allicin.

In conclusion, our results show strong inhibiting properties of allicin and its degradation products toward *C. jejuni* in vitro, as previously reported (Lu et al., 2011, 2012). However, under the test conditions applied, 25 mg/kg of allicin, when given preventively in drinking water, was not able to decrease cecal *C. jejuni* counts in broilers or protect broilers from *C. jejuni* colonization. This might be due to the localization of *C. jejuni* species in close contact with intestinal mucus in cecal crypt cells and the inactivation of allicin by cysteine groups of mucin or other gastrointestinal bacteria. Our proposals for further research include studying the in vivo activity of different diallyl sulfides and their interaction with mucins, coupled with the measures described in this paper.

ACKNOWLEDGMENTS

This work was financed by a grant of the Federal Public Service of Health, Food Chain Safety and Environment (Brussels, Belgium): project RT08/8-Campoul. We thank Lien Vande Maele, Marc Verlinden, Jungang Liang, and Shaoji Li of the Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, for help during the in vivo experiment. We thank Miriam Levenson of the Institute for Agricultural and Fisheries Research (ILVO) for proofreading the manuscript.

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