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1 **Medium chain fatty acid feed supplementation reduces the probability of *Campylobacter jejuni***
2 **colonization in broilers**

3

4

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20 **Abstract**

21

22 *Campylobacteriosis* in humans is associated with handling and consumption of contaminated broiler
23 meat. Reduction of the number of *Campylobacter*-colonized broiler flocks could potentially be
24 realized by decreasing their susceptibility for colonization. The aim of this study was to determine the
25 effect of feed supplementation with a mixture of medium chain fatty acids (C₈-C₁₂) on susceptibility of
26 broilers for *Campylobacter* colonization, feed conversion and body weight gain. Two experiments
27 were carried out with individually housed commercial broilers. The birds were fed with medium chain
28 fatty acids supplemented feed (n=227), or received feed without supplement (n=87). The birds were
29 inoculated with a dose of *Campylobacter jejuni* varying between log₁₀ 1.19 - 5.47 CFU. During 14
30 days after inoculation, cecal or fecal samples were collected, in which the presence of *C. jejuni* was
31 determined by bacterial culture. Beta-binomial dose response modeling of the colonization status at 14
32 days post-inoculation was performed to estimate the *C. jejuni* dose necessary to colonize 50% of
33 inoculated broilers, which was estimated to be 200 times higher in broilers fed with supplemented feed
34 (log₁₀ 4.8 CFU) than in control broilers (log₁₀ 2.5 CFU). Feed conversion was not affected by feed
35 supplementation, while body weight gain was 49 g higher in broilers fed with supplemented feed.
36 These findings indicate that susceptibility of broilers for *Campylobacter* colonization is decreased by
37 supplementation with medium chain fatty acids, and that feed supplemented with this mixture may be
38 a promising tool for the reduction of *Campylobacter* colonization in commercial broiler flocks.

39

40 **Keywords:** *Campylobacter*, broiler, feed supplementation, fatty acid, dose response

41

42

43 Introduction

44

45 Meat products from broilers colonized with *Campylobacter jejuni* are considered an important source
46 of human campylobacteriosis (Friedman et al., 2004), and reduction of human exposure is an
47 important goal of public health programs (EFSA, 2005). One way to achieve this is to prevent
48 intestinal colonization of broilers with *Campylobacter* spp., either by prevention of exposure or by
49 reducing the susceptibility for colonization (Wagenaar et al., 2006). Because highly effective
50 biosecurity measures to prevent exposure might be difficult to implement, reduction of susceptibility
51 of broilers for colonization, here defined as the probability of colonization upon exposure, may be an
52 alternative way to reduce the number of colonized flocks.

53 One way to reduce the susceptibility is to change the micro-environment in the gastro-
54 intestinal tract in such a way that ingested *Campylobacter* bacteria are inactivated or unable to reach
55 the lower intestines. It has been shown that short and medium chain fatty acids (SCFA and MCFA,
56 respectively) have *in vitro* anti-*Campylobacter* activity that is additional to the *Campylobacter*
57 inactivating effect of an acidified micro-environment (Chaveerach et al., 2002; Thormar et al., 2006).
58 *In vivo*, feed supplemented with high level SCFA results in a reduced chance of colonization in
59 broilers, but also in reduced body weight gain (BWG), which is economically undesirable (Heres et
60 al., 2004). Lower fatty acid concentrations might be necessary to prevent BWG loss, preferably
61 combined with improved effectivity at higher pH, to extend the anti-*Campylobacter* activity
62 throughout a larger part of the gastro-intestinal tract.

63 Whereas SCFA activity ceases at $\text{pH} \geq 5.5$ (Chaveerach et al., 2002), *in vitro* studies showed
64 that 1-Monoglycerid of Capric acid, a MCFA, has anti-*Campylobacter* activity in feed mixed with a
65 buffer at pH 5.5 and in feed mixed with tap water at pH 7.0 (Thormar et al., 2006). Consequently,
66 MCFA might inactivate *Campylobacter* cells in the crop (pH 4.5) and the intestines, where the pH is
67 approximately 5.8-6.0 (Chang and Chen, 2000; Farner, 1942). A low concentration mixture of MCFA
68 could therefore be a promising tool to decrease the susceptibility without negative effects on weight
69 gain or feed conversion.

70 The aim of this study was to determine whether an acidified feed containing 1% MCFA was
71 able to reduce the susceptibility of broilers for colonization with *Campylobacter jejuni*. Additionally,
72 the effects of supplemented feed on BWG and feed conversion rate (FCR) were determined.

73

74

75 **Material and methods**

76

77 *Experimental Design*

78 Two experiments were carried out subsequently. For each experiment hatching eggs, originating from
79 one commercial broiler breeder flock (Ross 308) were purchased at 17 days of incubation. Chicks
80 were hatched at the experimental facilities of the Faculty of Veterinary Medicine of Utrecht
81 University. After hatching broilers were randomly assigned to control feed (CF) or supplemented feed
82 (SF), which they received throughout the experiments. CF was an antibiotic and anti-coccidia drug
83 free, mashed diet. SF was the same diet, with 1% soybean-oil substituted by 1% LodestarTM C8-10
84 (Loders Crokiaan, Wormerveer, the Netherlands), which is produced by fractional distillation of palm
85 kernel oil free fatty acids, and typically consists of 56% C₁₀, 30% C₈, 10% C₁₂, <3% C₆, and <3%
86 other lipids. The composition and calculated chemical analyses of both diets is shown in table 1.

87 In experiment 1 (exp. 1), a group of 150 day-old chicks was provided CF, based on random
88 selection of the birds, and a group of 47 chicks was provided SF. In experiment 2 (exp. 2), chicks were
89 placed in multiple groups: 192 day-old chicks were randomly divided into 32 groups of 6 chicks each,
90 22 groups receiving CF (132 chicks) and 10 groups SF (60 chicks). Birds were housed in a well-
91 controlled facility, on litter floors. Groups were separated by walls. Water and feed was available *ad*
92 *libitum*, and from 7 days of age a daily dark period of 6 hours was applied.

93 At 14 days of age, by random selection 114 of the initially 150 CF and 42 of 47 SF broilers
94 (exp. 1), and 113 of 132 CF and 43 of 60 SF broilers (exp. 2) were weighed and housed individually in
95 wired cages of 40x40 cm, with closed, littered floors, which were situated in four identical
96 compartments. In exp. 1, broilers in both treatment groups were randomly divided over the cages in all
97 compartments. In exp. 2, broilers per treatment group were evenly divided over the compartments, and

98 within each compartment, broilers were randomly assigned to cages. The sides and back of each cage
99 were covered with plastic sheets. The distance between the cages was 40 cm at least.

100

101 *Inoculation*

102 One dose of 10^9 CFU of *C. jejuni* C356, originating from a broiler flock (Jacobs-Reitsma et al., 1995)
103 and stored in glycerol at -80°C , was orally administered to 3 five-day-old broilers, which were housed
104 with 3 non-inoculated contact broilers. Three days post-inoculation (PI) *C. jejuni* was isolated from the
105 ceca of one contact broiler. This chicken-passaged strain, here referred to as *C. jejuni* C356P (C356P),
106 was stored in glycerol at -80°C and used in experiments 1 and 2.

107 Before inoculation, C356P was freshly cultured in Heart Infusion Broth (micro-aerobically,
108 37°C , overnight) and diluted in saline to obtain the intended inoculation doses. Based on power
109 calculations, using results from a pilot study (data not shown), and presuming an additive effect on the
110 inoculation dose required to colonized 50% of the birds (CD_{50}) of approx. \log_{10} 1.5 CFU, SF broilers
111 were inoculated with higher doses C356P than CF broilers (table 2). In each compartment, non-
112 inoculated broilers were housed in 4 randomly selected cages (sentinel) to detect *Campylobacter*
113 transmission. Broilers, except sentinels, were orally inoculated with 0.25 ml of the C356P inoculation
114 suspensions at 14 days of age (exp. 1; n=140) or 18 days of age (exp. 2; n=139; one broiler died one
115 day prior to inoculation). The inoculation doses were randomly divided over the compartments. To
116 limit variation between individuals, all broilers were feed-deprived for 11 hours prior to inoculation,
117 and provided feed directly afterwards. The concentration of *Campylobacter* in the administered
118 inocula was determined by plating on modified charcoal cefoperazone deoxycholate agar (mCCDA)
119 (Biotrading Benelux B.V., Mijdrecht, the Netherlands).

120

121 *Sampling and Testing*

122 Six (exp. 1) or seven days (exp. 2) prior to inoculation, broilers were tested for the presence of
123 *Campylobacter* by culture of a fecal dropping on mCCDA. All broilers tested negative. After
124 inoculation, birds were sampled at 4, 8, and 11 days post inoculation (PI) by swabbing fresh cecal

125 droppings if present, or otherwise a swab of a fresh fecal dropping. If neither could be obtained, a
126 swab from cloacal content was taken. In addition, at 1, 2, 3, 7, and 9 days PI 1 sentinel per
127 compartment and 1 to 5 inoculated broilers per inoculation dose were placed in cardboard boxes with
128 wire floors for 4 hours, to collect cloacal swabs and fresh cecal droppings (or fecal droppings if not
129 present). Different material was sampled with the intention to compare the sensitivity of each of these
130 sampling methods. Because positivity was limited to one of two sample types only incidentally,
131 sensitivity of different sample mediums was considered equal. At the end of the trial (14 days PI),
132 cecal contents were sampled after euthanasia by cervical dislocation.

133 The person sampling the broilers was blinded to dose groups and could not distinguish
134 sentinels from inoculated broilers. Long-sleeved plastic gloves were changed for each broiler to avoid
135 cross-contamination. Samples were collected with sterile swabs and transported to the laboratory in
136 modified Amies transport medium without charcoal (Biotrading, Mijdrecht, the Netherlands) within 6
137 hours. Samples were streaked on mCCDA plates and incubated micro-aerobically at 42°C, and
138 examined for the presence of *Campylobacter*-suspect colonies after 24 and 48 hours. Microscopic
139 examination of morphology and motility was used as confirmation. Broilers were considered
140 *Campylobacter*-colonized and were excluded from further sampling when at least one sample tested
141 positive. Ethical aspects of the experiments were judged and approved by the animal ethical
142 committee of Utrecht University.

143 To assess the effect of MCFA feed supplementation on technical performance, BWG and feed
144 intake during the period of individual housing (>14 days of age) were recorded in exp. 2.

145

146 *Statistical Analyses*

147 The effect of MCFA feed supplementation on susceptibility was assessed by fitting a Beta-binomial
148 dose response model (Teunis and Havelaar, 2000). According to this model, inoculation with dose D
149 results in a probability of colonization $\text{Pr}_{\text{inoc}}(D)$:

$$150 \quad \text{Pr}_{\text{inoc}}(D) = 1 - \left(1 + \frac{D}{\beta}\right)^{-\alpha}$$

151 The underlying assumption of the model is that each bacterium can independently establish
 152 colonization, but that hosts may differ in their susceptibility (Teunis and Havelaar, 2000). The two
 153 parameters (instead of single probabilities for each dose) provide opportunities to assess the dose
 154 response relation over a wide range of exposure doses, and to compare treatments tested with different
 155 ranges of inoculation doses. Furthermore, the dose-response model could be used to calculate a CD_{50} :
 156 the colonization dose resulting in a 50% probability of infection.

157 Because sentinels were detected positive after day 4 PI in exp. 2 (table 2), colonization as a
 158 result of transmission could not be excluded for the inoculated birds either. Therefore, in the main
 159 analysis (using data up to day 14 PI) we corrected for transmission by estimating a transmission
 160 probability Pr_{tr} , different for each room and treatment group. Consequently $Pr_+(D)$, the probability of
 161 being colonized, was equal to

$$162 \quad Pr_+(D) = 1 - (1 - Pr_{tr})^{1 - Pr_{inoc}}(D)$$

163 Additionally, a more simple analysis was performed using the colonization status up to day 4
 164 PI, without the correction for transmission. In the day-4 analysis, $Pr_+(D)$ was equal to $Pr_{inoc}(D)$.

165 For both analyses (day-4 and day-14), four different models were fitted to see if there were
 166 group and treatment effects: the first with separate dose response relations for each of the 4 treatment
 167 groups (CF_1 vs. CF_2 vs. SF_1 vs. SF_2), the second with combined CF groups (CF_{12}) vs. combined SF
 168 groups (SF_{12}), the third with combined exp. 1 groups vs. exp. 2 groups, and the fourth with all groups
 169 combined. All parameters were estimated by maximum likelihood. The corrected Akaike Information
 170 Criterium (AIC_c) (Hurvich and Tsai, 1989) was used to decide which model explained the data best
 171 and whether different dose response relations should be adopted for (combined) treatment groups.

172 Prior to inoculation, some groups of broilers got diarrhea at 1 week of age. To study the
 173 possible confounding effect of diarrhea on susceptibility, data of exp. 2 were analyzed as described
 174 above, in 4 groups, with diarrhea status instead of experiment. AIC_c was used to assess if the
 175 occurrence of this symptom was associated with an increased or decreased susceptibility to
 176 *Campylobacter* colonization.

177 Linear regression analyses (SPSS 15.0.1.) were performed to assess if feed treatment affected
 178 BWG and FCR in exp. 2. Sixty-nine broilers, which were detected *Campylobacter*-colonized before or
 179 at 4 days PI, were weighed and sexed at 28 days of age, while the remaining 84 broilers (19 colonized
 180 and 65 non colonized) were weighed and sexed at 32 days of age. To correct for this, age and final
 181 colonization status were included as dependent variables, next to the variables sex and feed treatment,
 182 resulting in the equations

$$183 \text{ BWG}_i = \beta_0 + \beta_1 \text{Feed}_{i1} + \beta_2 \text{Sex}_{i2} + \beta_3 \text{Age}_{i3} + \beta_4 \text{Col}_{i4} + \varepsilon_i,$$

184 and,

$$185 \text{ FCR}_j = \beta_0 + \beta_1 \text{Feed}_{j1} + \beta_2 \text{Sex}_{j2} + \beta_3 \text{Age}_{j3} + \beta_4 \text{Col}_{j4} + \varepsilon_j.$$

186

187

188 **Results**

189

190 *Colonization*

191 In exp. 1, 35 of 46 colonized birds, and in exp. 2, 72 of 89 colonized birds were detected
 192 *Campylobacter*-positive in the first 4 days PI.(table 2). In exp. 1, the sentinels remained negative, but
 193 in exp. 2, 4 of 16 sentinels were detected *Campylobacter*-positive, at days 7, 8, 8 and 14 days PI. Four
 194 birds in exp. 1 and one bird in exp. 2 died after day 4 PI (table 2).

195

196 *Dose Response: Effect on Susceptibility*

197 The best fitting day-14 model (lowest AIC_c) included separate dose response relation for CF₁₂
 198 and SF₁₂, indicating equal relations in both experiments. Dose response relations of CF₁₂ and SF₁₂
 199 show parallel sigmoid shapes (figure 1). CD₅₀ for CF₁₂ was log₁₀ 2.5 CFU (95% CI: 2.2-2.8) and CD₅₀
 200 for SF₁₂ was log₁₀ 4.8 CFU (95% CI: 4.4-5.2) (figure 1). The AIC_c of alternative models were at least
 201 3.95 higher.

202 The best day-4 model included separate dose response relations for all four treatment groups
 203 (CF₁, CF₂, SF₁, and SF₂). CD₅₀ for CF₁ and CF₂ were log₁₀ 2.8 CFU (95% CI: 2.6-3.3) and 2.1 CFU

204 (95% CI: 1.8-2.3), respectively. CD_{50} for SF₁ and SF₂ were \log_{10} 8.5 CFU (95% CI: 4.8 - ∞) and 4.7
205 CFU (95% CI: 4.2-5.2), respectively. The AIC_c of alternative models were at least 12.2 higher.

206 In exp. 2, diarrhea occurred around 1 week of age, with similar frequencies in both treatment
207 groups (14/22 CF groups and 5/10 SF groups; Fisher exact test: $P=0.70$), suggesting that diarrhea was
208 not feed related. In a separate analysis of data of exp. 2 inclusion of a variable describing whether a
209 broiler originated from a group with diarrhea did not result in a better fit, with AIC_c being approx. 4
210 points higher in both the day-4 and day-14 models.

211

212 *Effect on technical performance*

213 The broiler that died during the exp. 2 (table 2) and a broiler that was lame during the last few days of
214 the experiment were excluded from this analysis. BWG was estimated 49 ± 24 g higher in SF broilers
215 compared to CF broilers ($p=0.044$) when correcting for the effect of sex ($p<0.001$), age ($p<0.001$), and
216 final colonization status ($p=0.398$). FCR was estimated 0.061 ± 0.034 lower in broilers with were
217 provided supplemented feed ($p=0.075$) when correcting for sex ($p=0.179$), age ($p=0.183$), and final
218 colonization status ($p=0.735$).

219

220

221 **Discussion**

222

223 In two experiments the effect of MCFA feed supplementation on susceptibility and technical
224 performance of broilers was studied. The effect on susceptibility was assessed by determining the
225 relation between the *C. jejuni* inoculation dose and the subsequent occurrence of colonization,
226 resulting in dose response curves of SF broilers shifted to the right compared to CF broilers, indicating
227 that SF broilers required a higher inoculation dose to become colonized than CF broilers. The effect of
228 MCFA feed supplementation could also have been assessed by comparing the percentage of colonized
229 broilers exposed to equal inoculation doses (\log_{10} 2.19 CFU in exp. 1, and \log_{10} 2.47 and 4.37 CFU in
230 exp. 2) at 14 days PI (table 2). Although this would also have illustrated the susceptibility reducing
231 effect of the treatment (Fisher exact test: all $p<0.05$), we would not have been able to predict the

232 probability of colonization for other inoculation doses, nor would we have been able to correct for
233 transmission.

234 We used the Beta-binomial dose response model (Teunis and Havelaar, 2000) to estimate dose
235 response curves and CD_{50} s. Besides the fact that this analysis resulted in less parameters to be
236 estimated than separate analyses for different doses, and in the possibility to use different doses for
237 different treatments, it also turned out useful to correct for transmission. We used the colonization
238 status at 14 days PI to estimate the dose response relations, but when the *Campylobacter* colonization
239 status at 4 days PI was used in the alternative (day-4) model, similar estimates were obtained. This
240 similarity suggests that most broilers colonized after 4 days PI were colonized by transmission. Based
241 on the results in this study, challenge experiments with *Campylobacter* might not necessarily have to
242 last longer than 4 days to estimate the dose response relation properly.

243 Analysis of technical performance showed that BWG was increased in SF broilers, while FCR
244 was not affected. The effect on BWG might have been caused by antimicrobial effects of the fatty
245 acids, as feed supplementation with Capric Acid and Lauric acid, two MCFAs, has been shown to
246 decrease the concentration of *Clostridium perfringens* in jejunum and ileum of *C. perfringens*
247 challenged broilers (Jansman et al., 2006). Although feed supplementation with antimicrobial agents
248 has the potential to improve feed efficiency (Dibner and Richards, 2005; Jansman et al., 2006), in this
249 study no significant effect on FCR was observed.

250

251

252 **Conclusion**

253

254 The number of *C. jejuni* bacteria required to colonize 50% of inoculated broilers was estimated 200
255 times higher in broilers fed with supplemented feed than in control broilers. Although the working
256 mechanism of supplemented feed remains to be elucidated, this effect on susceptibility is a promising
257 finding for the implementation of MCFA feed supplementation as an intervention for reduction of
258 susceptibility in broilers. As the *Campylobacter* exposure dose that broilers experience in the field is

259 unknown, field trials are necessary to determine to what extent MCFA supplementation reduces
260 *Campylobacter* colonization in the field.

261

262

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264

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- 296
- 297
- 298

299 **Table 1.** Ingredient composition and calculated analysis of the diets

Item	Amount (%)	
Ingredients	Control Feed	Supplemented Feed
Wheat	36.53	36.53
Soybean meal	21.20	21.20
Corn	20.00	20.00
Peas	15.00	15.00
Soybean oil	3.95	2.95
Calcium carbonate	1.44	1.44
MCFA ^b	0.00	1.00
Monocalcium phosphate	0.57	0.57
Lysine 65%	0.35	0.35
Sodium chloride	0.26	0.26
Methionine	0.25	0.25
Premix	0.25	0.25
Sodium bicarbonate	0.10	0.10
Threonine	0.04	0.04
Choline chloride	0.03	0.03
Endoxylanase	0.02	0.02
Phytase	0.01	0.01
Calculated analysis		
CP	19.60	19.60
ME _n (kcal/kg) [*]	2790	2790
C8 [†]	0.00	0.30
C10 ^b	0.00	0.56
C12 ^b	0.00	0.10

300

* Energetic value of MCFA and soybean oil was assumed to be equal

† 1% LodestarTM C8-10

301 **Table 2.** Colonization status of broilers (exp. 1 and exp. 2).

302 Rows represent different feed treatment groups (CF = control feed; SF = supplemented feed) and *C. jejuni* C356P inoculation

303 doses (\log_{10} CFU/broiler). Nominators in cells express the number of broilers detected *Campylobacter*-positive, and

304 denominators express the number of broilers sampled throughout 4 or 14 days post-inoculation (PI).

305

Exp. 1				Exp. 2			
treatment	Dose	Time (days PI)		treatment	Dose	Time (days PI)	
group	C356P	4	14	group	C356P	4	14
CF	-	0/16	0/16	CF	-	0/16	4/16
CF	1.19	0/33	1/32 ^a	CF	1.47	8/32	13/32
	2.19	9/33	12/32 ^b		2.47	21/33	25/33
	3.19	20/32	21/31 ^a		3.47	30/32	31/32
SF	2.19	1/21	2/21	SF	2.47	0/10	2/10
	4.49	5/21	9/20 ^a		3.47	2/11	2/11
					4.47	2/10	3/9 ^a
					5.47	9/11	9/11

306 ^a A broiler died after being tested negative at 4 dpi.

307 ^b A broiler died after being tested positive at 4 dpi.

308

309 **Figure 1.** Dose response curves for the final model of the day-14 analysis
310 The relation between inoculation dose en the probability of colonization occurring as a result of inoculation, $Pr_{inoc}(D)$, up to
311 14 days PI, for combined experiments (1 and 2). Best fitted curves, with 95% confidence bounds, represent relations for
312 control feed (CF₁₂) and MCFA supplemented feed (SF₁₂) fed broilers. Dots represent raw data as observed in the
313 experiments, not corrected for the occurrence of transmission.
314 << Figure 1.tif >>
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