EFFECT OF DIETARY MEDIUM-CHAIN FATTY ACIDS ON *CAMPYLOBACTER JEJUNI* IN BROILER CHICKENS*

P. Hovorková[1,2], E. Skřivanová[1,2], E. Kudrnová[1], M. Marounek[1,2]

[1]Institute of Animal Science, Prague-Uhříněves, Czech Republic
[2]Czech University of Life Sciences, Department of Microbiology, Nutrition and Diabetics, Prague, Czech Republic

The inhibitory properties of a commercial product Fortibac® containing medium-chain fatty acids on *Campylobacter jejuni* were determined. The product is a mixture of $C_{6:0}$-$C_{14:0}$ fatty acids. After testing the antibacterial properties towards *C. jejuni* in *in vitro* conditions, an experimental infection on broiler chickens was performed to confirm the results. The product was admixed with feed (final concentrations 0, 0.25, and 0.5%) and broiler chickens were artificially infected with *C. jejuni* VFU 612. The chickens were infected on day 16 of age, while the aforementioned feed mixtures were used during the entire fattening period (days 0–35). After the infection, the dynamics of *C. jejuni* shedding was evaluated among treated groups and the control. Reduction of the number of campylobacters by the product with medium-chain fatty acids was not confirmed *in vivo*. It is assumed that the final amount of potentially active fatty acids in the digestive tract was not sufficient. The product, however, had a clear beneficial impact on mortality of infected chickens.

inhibition; campylobacteriosis; poultry; pathogen

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INTRODUCTION

Bacterial diseases of poultry have still been a topical problem. The presence of bacterial pathogens in commercial poultry farms increases expenses of farmers and often threatens the health of humans. The annual incidence of *Campylobacter* spp. in developed European countries is estimated at 4.4–9.3 per 1000 population (WHO, 2012). Within the EU, 214 268 confirmed human cases of campylobacteriosis were reported in 2012 (EFSA, 2014). Pathogenic bacteria of the *Campylobacter* genus represent the main risk of zoonosis. Their occurrence is frequently related to poultry meat (Batz et al., 2011). Campylobacteriosis is usually treated by fluorochinolin antibiotics (e.g. ciprofloxacin), or by macrolide antibiotics (e.g. erythromycin) (Gupta et al., 2004).

There are critical studies focused on the use of antibiotics, both for treatment and for prevention of bacterial infections. The reason for this criticism is the increasing resistance of bacteria to antibiotics. This phenomenon has contributed to the restriction on the use of in-feed antibiotics in 2006 (EU Directive No. 1831/2003/CE). Unfortunately, the in-feed antibiotics ban may increase proliferation of pathogenic bacteria, contamination of animal products, and consequently the safety of the food chain may be threatened. Thus, there is a pressing need for new non-antibiotic antibacterial agents in order to protect food safety and animal health. Organic acids represent a suitable alternative to antibiotics. Medium-chain fatty acids (MCFA) containing 8–12 carbon atoms are non-toxic compounds with a promising antibacterial activity. The greatest potential for the reduction of Gram-negative bacteria have caprylic and capric acids (C_8 and C_{10}, respectively). Lauric acid (C_{12}) is very efficient in reduction of Gram-positive bacteria (Lieberman et al., 2006; Desbois, Smith, 2010). Antibacterial

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effects of MCFA have been observed both in *in vitro* experiments, and in infected animals.

The aim of the present study was to evaluate the effect of a commercial product containing MCFA on counts of *Campylobacter jejuni*, both in *in vitro* and *in vivo* experiments.

MATERIAL AND METHODS

Bacterial strain

C. jejuni strain VFU 612 was a gift of Prof. Steinhauserová (University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic).

Determination of fatty acid composition

The fatty acid composition of Fortibac® (Delacon Biotechnik, Šumperk, Czech Republic) was determined by gas chromatography (GC/FID) in the Institute of Animal Science, Prague-Uhříněves, Czech Republic. Alkaline trans-methylation of extracted fatty acids was carried out according to standard ISO 5509 (1994). For gas chromatography analysis of methyl esters, a HP 6890 Gas Chromatograph (Agilent Technologies, Inc., Santa Clara, USA) with a programmed 60 m DB-23 capillary column (J&W Scientific, Folsom, USA) has been applied. Fatty acids were identified on the basis of retention times compared to the retention times of FAME Mix 37 standards (Sigma-Aldrich, Prague, CZ).

In vitro experiment

A broth microdilution method was used to determine the *in vitro* activity of the product (Fortibac®) on *C. jejuni*, following the methodology described in Hecht et al. (1999). *C. jejuni* VFU 612 was inoculated into microtitration wells of a 96-well plate containing a twofold dilution series of Fortibac® (0.156–10 g/l) in selective growth broth Nutrient Broth No. 2, Campylobacter growth supplement, and Preston Campylobacter selective supplement (Oxoid, Basingstroke, UK). All concentrations of the product were tested in triplicate, in three independent experiments. The bacterial inoculum was standardized to achieve a final concentration of 6 $\log_{10}$ CFU/ml, using McFarland scale. As an antibiotic control, tetracycline was used (Sigma-Aldrich). The inoculated plates were incubated at 37°C for 48 h in microaerophilic atmosphere (CampyGen, Oxoid, UK). The absorbance (405 nm) was measured using Infinite® 200 instrument (Tecan Group Ltd., Männedorf, CH). The minimum inhibitory concentration (MIC) of Fortibac® was defined as the mean of concentrations resulting in growth reduction greater than 80%, as compared to the growth control.

In vivo experiment

The experiment was performed under the supervision of the Ethical Committee of the Institute of Animal Science (Prague-Uhříněves, Czech Republic) and the Central Commission for Animal Welfare of the Ministry of Agriculture of the Czech Republic.

One-day cockerels Ross 308 ($n = 60$) (XAVERgen, a.s., Říčany, Czech Republic) were randomly divided into four groups of fifteen animals (positive control, negative control, group FB0.25, and group FB0.50), and housed in four floor pens. Room temperature was 32°C in the first week, 30°C in the second week, and 27°C for the rest of experiment. The chickens were kept in the floor pens for the first two weeks of life. At fourteen days of age, 12 cockerels from each group were chosen and housed in individual cages. Two days after moving to the individual cages (day 16 of age), all cockerels except one group (negative control) were infected *per os* with *C. jejuni* VFU 612 (0.5 ml of culture containing 10^8 CFU/ml).

Animals of both control groups were fed *ad libitum* with a wheat-corn based granulated diet, free of antimicrobials (Biopharm, Pohoří-Chotouň, Czech Republic), containing dry matter, crude protein, and crude fat at 883, 218, and 61 g/kg, respectively; nitrogen-corrected apparent metabolizable energy was 12.59 MJ/kg. The chickens from the groups FB0.25 and FB0.50 were fed the same diet, except of the supplementation with Fortibac®, as follows: FB0.25 group received the feed supplemented with 0.25% of Fortibac®, whereas FB0.5 group received 0.50% of Fortibac® supplementation in feed. To check the fatty acid profile of feed mixtures, the contents of fatty acids were determined by gas chromatography of fatty acids methylesters after the extraction of lipids and alkaline trans-methylation of fatty acids (Folch et al., 1957). These feed mixtures were fed for the whole period of broiler fattening. Fortibac® was also analyzed for its fatty acids profile, using the same analytical method.

From day 14 of age, the excrements of 5 broilers from each group (selected at random) were collected for microbiological analyses, in two-day intervals (days 14, 16, 18, 20, 22, 24, 26, and 28 days of age). To analyze the numbers of excreted campylobacters, 1 g of excreta was diluted with sterile saline, serially diluted (ten-fold dilutions), and 100 µl of each dilution were streaked on selective agar plates (Campylobacter Agar Base, Campylobacter Growth Supplement, Preston Campylobacter Selective Supplement, Laked Horse Blood, all purchased from Oxoid, UK). Plates were incubated in triplicate at 37°C for 48 h under N_2–CO_2–O_2 atmosphere (85:10:5). Colonies grown on the selective agar plates were further confirmed by Gram-staining and morphology. 48

Chickens were individually weighed once a week, mortality was checked daily. If any mortality occurred during the experiment, cadavers were immediately

Table 1. Concentration of MCFA[a] in feeds of broilers.

Acid	Feed mixture		
	Control[b]	FB 0.25[c]	FB 0.50[d]
Caproic ($C_{6:0}$)	75	75	85
Caprylic ($C_{8:0}$)	60	440	885
Capric ($C_{10:0}$)	75	425	905
Lauric ($C_{12:0}$)	100	125	160

[a] Average of two analyses, both in triplicate (mg/kg)

[b] No Fortibac®was added

[c] Fortibac® at 0.25 % was added

[d] Fortibac® at 0.50 % was added

examined in the State Veterinary Institute (Prague-Lysolaje, Czech Republic). Since day 14 of age, feed consumption was also recorded on a daily basis. On day 35 of age, chickens were euthanized with inhalation of Isofluranum (Torrex Chiesi CZ Ltd., Prague, Czech Republic), followed by cervical dislocation. After the euthanasia, chickens were dissected and samples of caecum contents, liver, and spleen were taken for the bacteriological analysis (performed as described above).

Statistical analysis

The data were statistically analyzed using one-way analysis of variance. Differences between treatment means in terms of *Campylobacter* spp. shedding were tested by the Scheffé's test. The differences were considered significant at $P < 0.05$. Data on mortality of chickens were analyzed by means of Fisher's exact test. The STATISTICA software (Version 10) was used.

RESULTS

The MIC of Fortibac® determined by the broth microdilution method was 0.625 g/l. As was determined by gas chromatography, Fortibac® in 100 g contains 2.84 mg (0.0028%) of caproic acid, 2905 mg (2.9%) of caprylic acid, 3334 mg (3.3%) of capric acid, and 30 mg (0.03%) of lauric acid.

The concentration of MCFA in feed of the experimental chickens is shown in Table 1. Based on the analysis, the product contains a mixture of saturated C_6–C_{14} fatty acids, with a predominance of caprylic (C_8) and capric (C_{10}) acids.

The effect of a dietary mixture of fatty acids on *Campylobacter* spp. shedding in experimental chickens is shown in Table 2. Two days after infection (day 18 of age), *Campylobacter* spp. were detected in the excreta of all infected chickens (3.84–4.95 log10 CFU/g). There were no statistically significant differences in counts of campylobacters among the infected groups. Also in the following days of the microbiological analysis, no statistically significant differences among the infected groups were detected and no trends were observed. Furthermore, no effect of the additive on daily feed intake and daily weight gain was observed (data not shown). Average daily feed intake was 80.2 g, average daily weight gain was 46.5 g.

Table 3 presents results of the bacteriological analysis of cadavers. In the positive and negative control groups, five chickens (42%) and two chickens (17%) died, respectively. There was no mortality in both treatment groups.

DISCUSSION

C. jejuni is prominently associated with poultry, and the contaminated poultry meat is responsible for a significant percentage of intestinal infection diseases in humans. In the present study, the Fortibac® product containing MCFA was tested in *in vitro* experiments in order to determine its minimum inhibitory concentration against *C. jejuni*. In the subsequent experiment, the effect of feed supplemented with Fortibac® at 0.25 and 0.50% on mortality of chickens, and shedding of campylobacters in excreta of infected chickens, was investigated. The MIC of Fortibac® determined by the broth microdilution method was 0.625 g/l. As was determined by gas chromatography, Fortibac® contains caprylic and capric acid at 2.9 and 3.3%, respectively, which is consistent with data presented by the manufacturer.

Our results on the inhibition of *Campylobacter* spp. by MCFA (*in vitro*) are in agreement with results of other authors. Grilli et al. (2013) informed that the MIC of caprylic acid was 0.9%, Molatová et al. (2011) demonstrated the antibacterial action of

Table 2. Counts of campylobacters in control and treated chickens

Age of chicks (days)	16	18	20	22	24	26	28	30
Positive control	< 2*	3.91[a] ± 0.67	4.97[a] ± 0.66	6.54[a] ± 0.86	6.95[a] ± 0.47	6.88[a] ± 0.32	6.33[a] ± 0.63	6.34[a] ± 0.49
Negative control	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2
FB 0.25	< 2	4.95[a] ± 0.52	5.05[a] ± 0.78	6.56[a] ± 0.33	6.87[a]	6.94[a] ± 0.43	6.55[a] ± 0.64	6.42[a] ± 0.25
FB 0.5	< 2	3.84[a] ± 0.40	5.00[a] ± 0.61	6.97[a] ± 0.43	6.53[a]	6.65[a] ± 0.29	6.13[a] ± 0.42	6.55[a] ± 0.41

[a] Values in the same column with the same superscript are not significantly different at P≥0.05

*Values bellow the detection limit (2 Log10 CFU/g) were not included in the statistical analysis

Table 3. Results of bacteriological analysis of cadavers

	Mortality (%)	Bacteriological analysis	
		Intestine	Organs
Positive control	42[b]	*Kleibsella pneumoniae* *Campylobacter jejuni,* *Enterobacter cloacae*	*Campylobacter jejuni,* *Proteus sp.,* *Enterococci*
Negative control	17[ab]	*Escherichia coli,* *Clostridium perfringens*	*Escherichia coli*
FB 0.25	0[a]	-	-
FB 0.5	0[a]	-	-

[a, b] values with the same index within column are not statistically different

caprylic and capric acid mixture (1 : 1) at 0.25%. Our feed mixture supplemented with Fortibac® at 0.25% contained caprylic and capric acid at 0.044 and 0.0425%, respectively. If Fortibac® was added at 0.50%, the corresponding concentrations of caprylic and capric acid were 0.0885 and 0.0905%.

In our experiment, Fortibac® did not influence counts of campylobacters in excreta of infected chickens. Similar results were reported by Hermans et al. (2012). The dosing of Fortibac®, however, was rather low, while maybe higher doses of this preparation are necessary to obtain conclusive results. On the other hand, no mortality of chickens in groups fed Fortibac®-supplemented feed was observed. Two chickens (i.e. 17%) died in the negative control group (non-infected chickens fed the basal diet). Five chickens (42%) died in the positive control (infected chickens fed the basal diet). The difference in mortality of both control groups was statistically significant. Several chickens died due to mixed infections. Thus, we assume that Fortibac® is efficient against other enteropathogenic bacteria, which colonize chicken intestine. Fortibac® may be more efficient against Gram-positive bacteria because its principal components are caprylic and capric acid.

Depending on the solubility, fatty acids can be added into drinking water. Hermans et al. (2012) observed the effect of caproic, caprylic, capric, and lauric acids added as emulsions into drinking water on counts of campylobacters in the digestive tract of broiler chickens. Fatty acids did not reduce counts of campylobacters, however, the susceptibility of chickens to infection was decreased. The encapsulation of MCFA enhanced their efficacy, as shown by Molatová et al. (2011). The coated MCFA bypass the stomach, and exert antibacterial activity in lower parts of the digestive tract.

CONCLUSION

The study did not confirm the inhibitory activity of Fortibac® added to the feed of broilers against *C. jejuni*, presumably due to its low dosing. The mortality of chickens fed this preparation, however, was significantly reduced.

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Corresponding Author:

doc. MVDr. Eva S k ř i v a n o v á , Ph.D., Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Microbiology, Nutrition and Dietetics, Kamýcká 129, 165 21 Prague 6-Suchdol, Czech Republic, phone: +420 224 382 678, e-mail: skrivanovae @af.czu.cz