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Kim van Deun, Freddy Haesebrouck, Filip van Immerseel, Richard Ducatelle, Frank Pasmans. Short chain fatty acids and lactate as feed additives to control *Campylobacter jejuni* infections in broilers.. Avian Pathology, 2008, 37 (04), pp.379-383. 10.1080/03079450802216603 . hal-00540120

**HAL Id: hal-00540120**

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Submitted on 26 Nov 2010

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**Short chain fatty acids and lactate as feed additives to control *Campylobacter jejuni* infections in broilers.**

Journal:	<i>Avian Pathology</i>
Manuscript ID:	CAVP-2008-0010.R2
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	30-Mar-2008
Complete List of Authors:	Van Deun, Kim; Ghent University, Department of Pathology, bacteriology and poultry diseases Haesebrouck, Freddy; Ghent University, Pathology, Bacteriology and Avian Diseases Van Immerseel, Filip; Ghent University, Pathology, Bacteriology and Avian Diseases Ducatelle, Richard; Ghent University, Pathology, Bacteriology and Avian Diseases Pasmans, Frank; Ghent University, Pathology, Bacteriology and Avian Diseases
Keywords:	Campylobacter jejuni, poultry, butyrate, short chain fatty acids

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Cavp-2008-0010

Short chain fatty acids and L-lactate as feed additives to control *Campylobacter jejuni* infections in broilers.

**Kim Van Deun\***, Freddy Haesebrouck, Filip Van Immerseel, Richard Ducatelle, Frank Pasmans

*Department of Pathology, Bacteriology and Avian Diseases, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium.*

**Short title:** Colonization of broilers by *C. jejuni*

Corresponding author:

Tel: ++32 (0) 9 264 74 31; fax: ++32 (0) 9 264 74 94.

Email: [kim.vandeun@ugent.be](mailto:kim.vandeun@ugent.be)

*Received: 21 January 2008*

Cavp-2008-0010

Short chain fatty acids and L-lactate as feed additives to control *Campylobacter jejuni* infections in broilers.

**Kim Van Deun\*, Freddy Haesebrouck, Filip Van Immerseel, Richard Ducatelle, Frank Pasmans**

### Abstract

The usefulness of butyrate, acetate, propionate and L-lactate for the control of *C. jejuni* infections in broilers was assessed. For this purpose, the effect of these acids on the growth of *C. jejuni* in broth and intestinal mucus was determined as well as their influence on the invasiveness of *C. jejuni* in intestinal epithelial cells. From these *in vitro* obtained results, one acid was retained for use as feed additive in an *in vivo* trial.

Butyrate was the most successful of the short chain fatty acids (SCFA), with 12.5 mM being bactericidal for *C. jejuni* at pH 6.0. Propionate and acetate had a bacteriostatic effect at 50 mM. None of the SCFA had a bactericidal effect at pH 7.5 at a maximum concentration of 50 mM. Mucus increased the minimum bactericidal concentration of butyrate, but not the bacteriostatic concentrations of propionate or acetate. When *C. jejuni* was incubated in growth sub-inhibitory concentrations of butyrate, acetate or propionate or 25 mM L-lactate, no alteration in the invasive capabilities of *C. jejuni* in Caco-2 cells was observed. The addition of butyrate coated micro-beads to the feed was unsuccessful to reduce *C. jejuni* cecal colonization in a seeder model using 2 week old broilers.

In conclusion: despite the marked bactericidal effect of butyrate towards *C. jejuni* *in vitro*, butyrate coated micro-beads do not protect broilers from cecal colonization with *C. jejuni* in the applied test conditions. This might be partially ascribed to the protective effect of mucus and the rapid absorption of butyrate by the enterocytes.

## Introduction

Consumption of *Campylobacter jejuni* contaminated poultry meat products remains one of the most important sources of food borne illness. A Belgian survey showed that up to 73% of the flocks at the abattoir are contaminated with *C. jejuni* and the withdrawal of antimicrobial growth promoters could aggravate this situation (Rasschaert *et al.*, 2007; Wise *et al.*, 2007). This points to the failure of existing control strategies. While some techniques during carcass processing, such as an increased scalding temperature, improved evisceration methods, crust freezing, forced air-chilling and chemical decontamination, may reduce *Campylobacter* counts on skin and meat (Whyte *et al.*, 2001; Yang *et al.*, 2001; James *et al.*, 2007; Rosenquist *et al.*, 2006), it seems that these interventions are unable to reduce the number of chicken meat consumption related campylobacteriosis cases (EFSA, 2006). It is clear that there is an increasing need for alternative strategies to control *C. jejuni* colonization of broilers.

Carcass contamination is correlated with intestinal *C. jejuni* numbers and it is estimated that a reduction of intestinal *C. jejuni* counts with 2 Log<sub>10</sub> would result in a 30 fold decrease of human campylobacteriosis cases. As such, control measures at farm level are of vital importance in any *C. jejuni* control program (Rosenquist *et al.*, 2003 and 2006; Arsenault *et al.*, 2007; Messens *et al.*, 2007; Smith *et al.*, 2007).

Organic and short chain fatty acids are known for their antimicrobial characteristics due to their ability to cross bacterial membranes in their undissociated form. Different intervention strategies at the primary production level using fatty acids have been investigated for their impact on *C. jejuni* colonization of broilers, such as the supplementation in drinking water, which is a prominent source of horizontal *Campylobacter* transfer. None of these measures have proven to be successful (Chaveerach *et al.*, 2004). L-Lactate has been used for

the acidification and fermentation of feeds, but the effect was only a reduction of crop contamination and there was no effect on cecal *C. jejuni* numbers (Byrd *et al.*, 2001; Heres *et al.*, 2003 and 2004). A possible reason is the premature degradation and/or resorption of the bactericidal components in the intestinal tract and consequently their inability to reach the cecum. Micro-beads coated with SCFA and L-lactate can bypass this problem and their usefulness has been demonstrated for *Salmonella* control in poultry (Van Immerseel *et al.*, 2005). Interestingly, it seems that butyrate acts through downregulation of the pathogenicity island 1 of *Salmonella*, resulting in a reduced invasive capacity (Gantois *et al.*, 2006).

*C. jejuni* colonization of broilers occurs at the age of two to three weeks, which coincides not only with a drop in maternal antibodies, but also with a shift in L-lactate and SCFA composition in the chick's intestinal lumen (Van der Wielen *et al.*, 2000). Since *C. jejuni* is able to invade chicken primary cells, which could play a role in the persistent colonization of broilers (Byrne *et al.*, 2007; Van Deun *et al.*, 2007a), we wanted to investigate the bactericidal properties of SCFA and L-lactate and their effect on the invasive potential of *C. jejuni*. Based on these *in vitro* obtained data, one acid was retained for use as a feed supplement to reduce cecal colonization in broilers.

## Materials and Methods

**Experimental animals.** Day of hatch broilers from a local farm were kept in cages and provided with food and water ad libitum. Husbandry, euthanasia methods, experimental procedures and biosafety precautions were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University. All chicks were examined for the presence of *Campylobacter* in the feces and proved to be *Campylobacter* free.

**Cell culture and reagents.** Caco-2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 1% non essential amino acids and 10% fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37 °C in a 5% CO<sub>2</sub> atmosphere. All cell culture reagents were from Gibco (Invitrogen, Merelbeke, Belgium). For all experiments 24 well plates were used. Cells were seeded at a density of 1 x 10<sup>5</sup> cells/well in 1 ml medium and allowed to attach. Three days after seeding, cells reached confluence and were used for the invasion assay.

**Bacterial strains and culture conditions.** *Campylobacter jejuni* strain KC40 from poultry origin, kindly provided by Dr. M. Heyndrickx (ILVO, Belgium), efficiently invades primary chicken epithelial cecal crypt cells, T84 and Caco-2 cells and colonizes chickens to a high degree (Van Deun *et al.*, 2007a; Van Deun *et al.*, 2007b). Bacteria were routinely cultured in Preston broth (Oxoid, Basingstoke, England) supplemented with *Campylobacter* specific growth supplements (SR117 and SR0232, Oxoid) at 42°C under microaerobic conditions (5% O<sub>2</sub>, 5% CO<sub>2</sub>, 5% H<sub>2</sub>, 85% N<sub>2</sub>). For the enumeration of *C. jejuni* bacteria, tenfold dilutions were plated on modified charcoal cefoperazone deoxycholate agar (mCCDA) plates supplemented with *Campylobacter* specific growth supplements (SR155 and SR0232, Oxoid).

**Collection of chicken intestinal mucus.** Commercial 20-week old brown laying hens were euthanized and the small intestine was collected and gently rinsed with PBS to remove fecal material. The mucus was scraped from the mucosa with a scalpel, diluted 1/3 with N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid (HEPES, 25 mM; pH 7.4) and vortexed. The solution was centrifuged three times at 1000 x g for 10 min by 4°C. The mucus was filter sterilized by passage through a 0.45 µm pore size filter (IWAKI, International Medical, Brussels, Belgium) and stored at -80°C. Protein content was determined with a Biorad protein assay kit (Biorad, Nazareth, Belgium).

**Effect of butyrate, acetate, propionate and L-lactate on the growth of *C. jejuni* strain KC40 in BHI broth and chicken intestinal mucus.** To determine the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) for the *C. jejuni* strain KC40, twofold dilutions of the sodium salts of butyrate, acetate, propionate or L-lactate were made in Brain Heart Infusion (BHI) broth supplemented with 5% lysed horse blood and brought to pH 6.0 or pH 7.5. The MIC and MBC in intestinal mucus was determined by preparing twofold dilutions of the sodium salts of butyrate, acetate, propionate or L-lactate in phosphate buffered saline (PBS), supplemented with 5 mg/ml chicken intestinal mucus and brought to pH 6.0.

The media were inoculated with  $1 \times 10^5$  cfu/ml *C. jejuni* in a 96 well plates in a total volume of 200 µl per well. After incubation in microaerobic conditions at 42°C for 24 hours, titration was done on mCCDA plates to determine the number of cfu/ml.

**The effect of butyrate, acetate, propionate and L-lactate on the invasion of *C. jejuni* strain KC40 in Caco-2 cells.** *C. jejuni* was inoculated in BHI containing 3.13 mM butyrate,



6.25 mM acetate, 6.25 mM propionate or 25.00 mM L-lactate at pH 6.0. After 18 hours of bacterial growth, the bacteria were resuspended in cell culture medium and Caco-2 cell monolayers were exposed to *C. jejuni* at an MOI of 200. Plates were centrifuged for 10 minutes at 600 g at 37°C to deposit bacteria onto the surface of the monolayer. After three hours, cell layers were washed and incubated with 100 µg/ml gentamicin for 2 hours to kill extracellular bacteria. To enumerate intracellular bacteria, cells were lysed using 0.25% sodium deoxycholate and bacterial counts were performed by titration on mCCDA plates.

**The effect of butyrate on the cecal colonization of *C. jejuni* in broilers.** Butyrate was the most bactericidal component of all components tested *in vitro* and was therefore used for the *in vivo* trial. To assess the influence of butyrate on the colonization of chickens, micro-beads coated with butyrate were used in order to limit premature degradation and absorption of butyrate in the upper intestinal tract. One day old commercial broiler chicks were kept in a *C. jejuni* free environment and divided in six groups of 9 chicks. Three groups received standard feed and acted as negative controls, while the other three groups received feed supplemented with 0.05% butyrate coated beads (Greencab 70, Sanluc International NV, Gijzenzele, Belgium). Butyrate coated beads consisted of 16% fat coating and 84% butyrate. After two weeks, 3 chicks from each of the six groups were orally inoculated with  $1 \times 10^9$  cfu/ml and served as seeders. At 5 days post infection, all chicks were euthanized and the ceca were removed for bacteriological analysis. Samples were diluted 1/10 in buffered peptone water (BPW, Oxoid), homogenized and a volume of 120 µl was titrated on mCCDA plates. After 24 hours incubation at 42 °C in microaerobic conditions, colonies were counted.

**Statistical analysis.** All experiments were independently repeated three times. For all results,  $\text{Log}_{10}$  transformation was performed to obtain normally distributed data. Data were analysed with two sided Student-*t* test.

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## Results

**Effect of butyrate, acetate, propionate and L-lactate on the growth of *C. jejuni* strain KC40 in broth and chicken intestinal mucus.** Figure 1 shows the effect of different concentrations of butyrate, acetate, propionate or L-lactate at pH 6.0 (filled triangles) or pH 7.5 (filled squares) with (circles) or without mucus on the growth of *C. jejuni*. The effects were dependent on the pH of the medium: none of the components affected bacterial growth at pH 7.5 at the maximum concentration tested (50 mM). Butyrate was the most active with a bactericidal concentration of 12.5 mM at pH 6.0. Acetate and propionate inhibited growth at 50 mM. L-Lactate did not affect growth at any of the concentrations tested.

The addition of mucus to the medium containing butyrate shifted the minimum bactericidal concentration of 12.5 mM to 50 mM. With mucus, 12.5 mM butyrate had only a bacteriostatic effect ( $\text{Log}_{10} 5.0 \pm 0.6$  cfu/ml) instead of a bactericidal effect. The bacteriostatic concentrations of propionate or acetate were not influenced by mucus.

**Invasive capacity of *C. jejuni* in Caco-2 cells is not influenced by SCFA.** When *C. jejuni* was grown in 3.13 mM butyrate, 6.25 mM acetate, 6.25 mM propionate or 25.00 mM L-lactate, none of the SCFA, nor L-lactate could reduce *C. jejuni* invasion in Caco-2 cells ( $p > 0.05$ ). Data is presented in Figure 2.

**Butyrate coated micro-beads are unable to reduce *C. jejuni* cecal colonization in 2-week old broiler chicks.** The results are summarized in Figure 3. *C. jejuni* was able to colonize the ceca of butyrate fed chickens as efficiently as those of the control group ( $p > 0.05$ ; average control (n = 23):  $\text{Log}_{10} 8.0 \pm 0.1$  cfu/g, circles; butyrate treated (n = 19):  $\text{Log}_{10} 8.3 \pm 0.1$  cfu/g, triangles).

## Discussion

In this study, the effect of SCFA and L-lactate on *C. jejuni* growth and its invasive capacities in Caco-2 cells was studied together with an evaluation on the use of butyrate as a means to control *C. jejuni* infections in broilers.

To investigate minimal growth inhibitory and bactericidal concentrations of SCFA and L-lactate, a maximum of 50 mM of SCFA and 100 mM of L-lactate was used at pH 6.0 or pH 7.5, with pH 6.0 being of physiologic relevance for the chicken's cecum (van der Wielen *et al.*, 2000). The effects of SCFA were dependent on the acidity of the medium: this is due to the increased dissociation of the acid in a neutral environment (Davidson, 1997). It is generally believed that it is the undissociated form of these acids that can penetrate the bacterial membrane and that the bactericidal and bacteriostatic effects are caused by the dissociation of these acids in the near neutral environment of the bacterial cytoplasm, causing a lethal accumulation of anions (Russell, 1992). Butyrate was the only component with bactericidal activity at the concentrations tested. An interesting observation was the lack of any effect by L-lactate, even at high concentrations. Possibly, the bactericidal effect of L-lactate as described in other studies is merely due to the increased acidification. Indeed, *C. jejuni* is sensitive to the pH of the environment and unable to grow at a pH below 5.1 (Roberts *et al.*, 1996)

Mucus is known to elicit a specific transcriptional response in *C. jejuni*, which results in the transcription of virulence associated *Cia* genes (Biswas *et al.*, 2007). Nothing is known of the capacity of chicken mucus to promote *C. jejuni* survival and resistance to stress by altering its transcriptome and it was therefore interesting to notice that mucus had a limited protective effect on butyrate exposed *C. jejuni* bacteria by increasing the bactericidal concentration to more than 50 mM. This effect was in sharp contrast compared to the other

SCFA: for propionate and acetate, mucus did not affect the bacteriostatic concentrations. This butyrate specific protective effect of intestinal mucus is particularly interesting with regard to the niche occupied by *C. jejuni*: *C. jejuni* in the ceca of chickens is found deep in the crypts within the mucus layer (Beery *et al.*, 1988).

Unlike their effect on the virulence properties of *Salmonella*, the SCFA and L-lactate were not able to influence the invasive properties of *C. jejuni* in a Caco-2 model.

Since a strong bactericidal effect of butyrate on *C. jejuni* was observed, butyrate coated micro-beads were used in an attempt to decrease *C. jejuni* counts in the ceca of experimentally infected broiler chicks. Only the cecum was considered for analysis since it is the main site of colonization and poses a risk of carcass contamination during evisceration (Beery *et al.*, 1988; Rosenquist *et al.*, 2006). The use of butyrate coated micro-beads was to limit the possible premature absorption of butyrate in the proximal part of the intestine, but despite this, supplementation of feed with butyrate coated micro-beads was not able to decrease the final cecal *C. jejuni* count at the end of the experiment. This could be due to the rapid absorption of butyrate from the cecal lumen: *C. jejuni* occupies a niche close to the enterocytes and deep in the crypts. The rapid consumption of butyrate by the enterocytes creates a strong concentration gradient, possibly resulting in an insufficient butyrate concentration at the site of *C. jejuni* colonization. Furthermore our *in vitro* results suggest that the close association of *C. jejuni* with the mucus layer could protect *C. jejuni* from the bactericidal effects of butyrate. Therefore, we cannot exclude that, if butyrate concentration could be elevated locally in the mucus layer, a decrease of *C. jejuni* could be achieved. It should be noted that butyrate has a protective effect on the Caco-2 cells themselves: incubation of differentiated Caco-2 monolayers with 5 mM butyrate resulted in a significant decrease of translocation across the cell layer by *C. jejuni* (Van Deun *et al.*, 2008).

In conclusion, despite a strong bactericidal effect of butyrate on *C. jejuni in vitro*, it seems that *C. jejuni* infection in broilers cannot be controlled using feed supplementation with butyrate coated micro-beads at the dosage used.

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## Acknowledgments

This study was financed by the Federal Public Service for Health, Food Chain Safety and Environment project R-04/002-CAMPY.

Research in the author's laboratory was further sponsored by Sanluc International NV (Gijzenzele, Belgium).

## Figure legends

**Figure 1.** *The number of cfu of C. jejuni at 16 hours post inoculation of  $1 \times 10^5$  cfu/ml in BHI either at pH 6.0 (filled triangles) or pH 7.5 (filled squares) or in PBS with intestinal mucus at pH 6.0 (circles) containing butyrate, acetate, propionate or L-lactate. Data are expressed as  $\text{Log}_{10}$  cfu of the means  $\pm$  standard error.*

**Figure 2.** *Intracellular C. jejuni counts after invasion in Caco-2 cells. C. jejuni was grown in sub-bacteriostatic concentrations of SCFA or 50 mM L-lactate. Data are expressed as  $\text{Log}_{10}$  of the means  $\pm$  standard error.*

**Figure 3.** *Values of C. jejuni counts in individual ceca of controls (circles) and butyrate fed chickens (triangles). The  $\text{Log}_{10}$  of the means  $\pm$  standard error are given above each group.*

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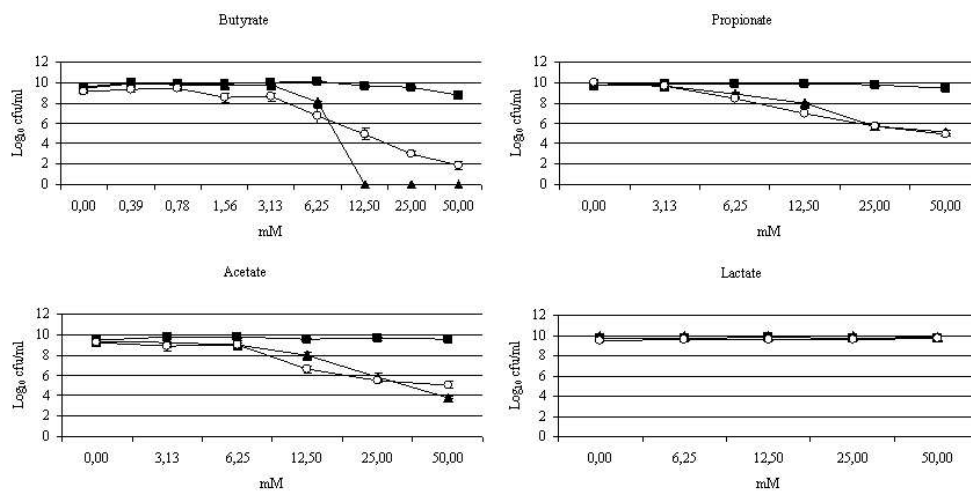


Figure 1

The number of cfu of *C. jejuni* at 16 hours post inoculation of 1 x 10<sup>5</sup> cfu/ml in BHI either at pH 6.0 (filled triangles) or pH 7.5 (filled squares) or in PBS with intestinal mucus at pH 6.0 (circles) containing butyrate, acetate, propionate or L-lactate. Data are expressed as Log<sub>10</sub> cfu of the means ± standard error.

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Figure 2

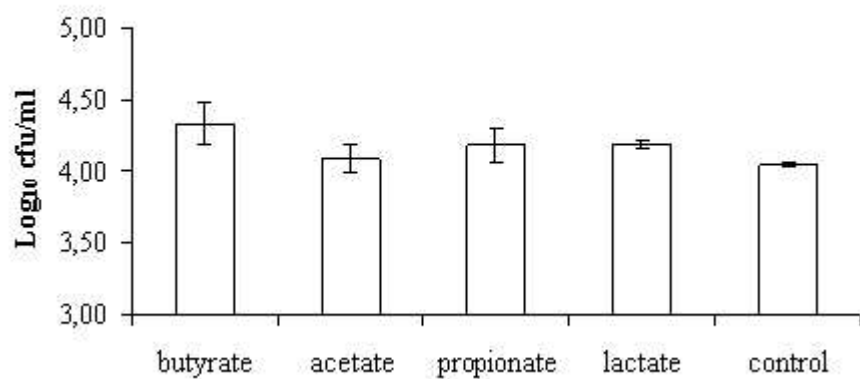


Figure 2

Intracellular *C. jejuni* counts after invasion in Caco-2 cells. *C. jejuni* was grown in sub-bacteriostatic concentrations of SCFA or 50 mM L-lactate. Data are expressed as Log<sub>10</sub> of the means ± standard error.

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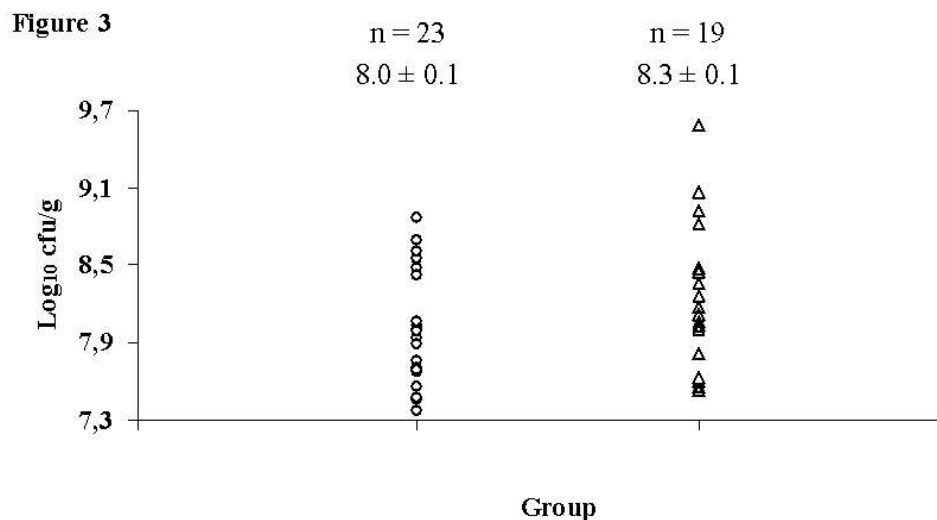


Figure 3  
Values of *C. jejuni* counts in individual ceca of controls (circles) and butyrate fed chickens (triangles). The Log<sub>10</sub> of the means ± standard error are given above each group.

71x38mm (300 x 300 DPI)

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