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Short communication

Purified natural pig immunoglobulins can substitute dietary zinc in reducing piglet post weaning diarrhoea

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Abstract

Enteric infectious disease in weaner piglets, including postweaning diarrhoea (PWD), are usually treated and/or prevented with antibiotics and/or zinc oxide in the piglet feed.
However extensive use of antibiotics and zinc oxide in intensive animal production is unwanted as it may promote microbial antibiotic resistance and pose environmental problems. Recently, in an experimental model of PWD, we observed that oral administration of purified porcine immunoglobulin G (pplgG) from pooled natural pig plasma could reduce enteric infection. In the present study we were able to reproduce these results as it was observed that oral pplgG accelerated clearance of faecal haemolytic bacteria in pigs challenged with E. coli in comparison with pigs not receiving pplgG. This effect was observed upon feeding pplgG for seven days postweaning suggesting that pplgG does not have to be used prophylactically for several days preweaning. Furthermore, the effect of oral administration of pplgG for seven days postweaning was equal to or better than that of dietary zinc oxide in reducing diarrhoea symptoms and in clearing faecal haemolytic bacteria for 14 days postweaning. These observations warrant future trials of dietary pplgG in intensive swine production units to establish its performance as an alternative to dietary antibiotics and zinc oxide for preventing PWD.

Keywords

Postweaning diarrhoea; Immunoglobulins; IgG; Zinc oxide; Antibiotics; ETEC; E. coli F4; Feed supplement; Feacal score

1. Introduction

After parturition, lactogenic immunity provides offspring with oro-gastric protection against infectious pathogens (Hedegaard and Heegaard, 2016). In the conventional swine production systems piglets are weaned at an immunologically immature age (3–4
weeks) depriving them of the continued supply of protective lactogenic antibodies at the same time as they are placed in a new environment with increased risk of enteric bacterial infections that may lead to postweaning diarrhoea (PWD) (Madec et al., 1998). PWD is characterised by diarrhoea caused by enteric infection by Enterotoxigenic Escherichia coli (ETEC) usually within three days postweaning (Fairbrother et al., 2005). PWD, like other enteric infections in pigs, can be treated with dietary antibiotics (primarily tetracyclines, penicillins and macrolides (Becker, 2010; DANMAP, 2015; Wageningen University, 2012)), and/or zinc oxide (Pluske, 2013). Indeed, antibiotics and zinc can improve average daily growth (ADG) in weaner piglets (Cromwell, 2002; Molist et al., 2011), and dietary zinc can reduce frequency of PWD in weaner piglets (Owusu-Asiedu et al., 2003; Pluske, 2013). The mechanisms by which zinc oxide increases ADG and reduces diarrhoea in weaner piglets are not fully understood but seem to involve both restoring plasma zinc levels to normal in piglets after weaning (Davin et al., 2013) and improving intestinal homeostasis (Liu et al., 2014a; Liu et al., 2014b; Shen et al., 2014).

However, the widespread use of dietary zinc oxide in intensive animal production can result in pollution of farmlands and groundwater through repeated fertilisation with zinc-containing residual manure (Hill et al., 2005). In addition, zinc oxide in combination with antibiotics appears to accelerate microbial antibiotic resistance by increasing the rate of the exchange of antibiotic-resistance-gene containing plasmids in the microbiota community in both soil and piglet intestines (Lin et al., 2016; Pal et al., 2015; Vahjen et al., 2015); thus, there is a need to reduce both kinds of interventions.

As a sustainable and economically feasible alternative to antibiotics and zinc for treating PWD, we have previously investigated the use of natural purified porcine
First, it was established that natural ppIgG does indeed contain immunoglobulin dependent anti-E. coli activity. Secondly it was shown that a dietary ppIgG supplement in a model of PWD led to faster clearance of an ETEC challenge infection than seen in a comparable control group not provided with dietary ppIgG (Hedegaard et al., 2016). This prompted us to further investigate this effect of ppIgG in E.coli challenge models. A long term, low ppIgG dose experiment and a short term, high ppIgG dose experiment were performed. In both experiments dietary ppIgG resulted in reduction of diarrhoea and in number of faecal haemolytic bacteria; moreover, in the second trial we observed that ppIgG reduced diarrhoea and cleared the enteric infection faster than dietary zinc oxide. These observations warrant future experiments investigating the use of dietary ppIgG postweaning as an alternative to dietary zinc oxide and antibiotics in treating/preventing PWD.

2. Materials & methods

The study comprised two infection experiments, both using E. coli O149 challenge at two consecutive days post weaning. In experiment 1 (long term, low IgG dose pilot study) 750 mg/day of ppIgG was provided orally for five days before and 10 days post weaning. In experiment 2 (short term, high IgG dose) 1.9 g of ppIgG was given orally twice daily for seven days after weaning/challenge. Experiment 2 also comprised a group receiving dietary zinc oxide for 10 days after weaning.

2.1. Purified porcine immunoglobulin G (ppIgG)
The ppIgG was prepared from pooled pig plasma by expanded bed chromatography (EBA) at Upfront Chromatography A/S (Copenhagen), as described previously (Hedegaard et al., 2016). Concentrated porcine blood plasma was obtained from Daka SARVAL A/S (Lunderskov, Denmark). The batch of ppIgG used in first experiment (experiment 1, see below) was the same as in (Hedegaard et al., 2016), whereas a new batch of ppIgG was prepared prior to the second (experiment 2, see below).

2.2. ELISA

The IgG concentration in the batches of ppIgG was measured by a sandwich ELISA (Hedegaard et al., manuscript in preparation), utilising a goat anti-pig IgG (Fc) antibody (AAI41, Nordic Biosite ApS, Copenhagen) both for capture and detection. The IgG-concentration of ppIgG used in experiment 1 was 37.5 mg/ml and in experiment 2 the ppIgG concentration was 75 mg/ml.

Anti-E. coli activity was found in both batches of ppIgG, used in this study, by indirect whole-E. coli cell ELISA previously reported (Hedegaard et al., 2016); briefly 96 wells flat bottom microtiter plates (Maxisorp, NUNC, Thermo Scientific, Denmark) were coated with 100 μl fixed E. coli O138 (in-house strain isolated from piglet with diarrhoea) in 0.1 M sodium carbonate buffer pH 9.6 (OD546 = 0.25) at 4 °C overnight. All subsequent operations were performed at room temperature. Next day wells were washed four times in PBS with 0.05% Tween 20 (PBS-T), and blocked with 200 μl PBS-T with 1% bovine serum albumin (BSA; Sigma-Aldrich, Brøndby, Denmark) for 30 min when shaking then followed by four times wash as above. The ppIgG was added in 2-fold dilution series (diluted in PBS-T 1% BSA from 10 to 0.02 mg/ml). After 1 h of incubation with shaking and 4 washes in PBS-T, detection antibody HRP-conjugated goat anti-pig IgG
(GGHL-5P; ICL, SMS Gruppen; Rungsted, Denmark) diluted 1/2000 in PBS-T 1% BSA was added and incubated for 1 h with shaking. After washing, plates were developed by TMB Plus substrate (Kem-En-Tec, Taastrup, Denmark), 100 μl/well, and stopping colour development by 100 μl/well 0.5 M H2SO4 (VWR—Bie & Berntsen A/S). Optical density was measured by a Thermo Scientific Multiscan EX microplate reader at 450 nm subtracting background absorbance at 650 nm.

Using the antigen specific ELISA it was found that the ppIgG batch used in experiment 1 had lost 14% of activity in comparison to the original plasma pool, whereas the batch used in experiment 2 had lost no activity (data not shown).

2.3. Experiments

2.3.1. Experimental procedure

Two separate factorial experiments at Aarhus University, Foulum, involving 12 (10.8 ± 1.8 kg BW, from 2 litters) and 18 (7.3 ± 1.0 kg BW, from 2 litters) pigs for the first and second experiments, respectively, were conducted (see Table 1). In both experiments, piglets were weaned from sows that had been tested to be homozygote carriers of the dominant gene encoding for intestinal F4 fimbriae receptors (Van Haeringen Laboratorium, b.v., Wageningen, The Netherlands) on DNA extracted from hair sample. Piglets were weaned from the sows at day 28 of age, and were challenged with E. coli O149:F4 on two consecutive days (d 29 and 30 of age). Within each experiment, piglets from different litters were equally distributed among treatments.

In the first experiment, half of the piglets (Table 1; Exp. 1, Group 1 + 2) received once daily for 15 days (5 days preweaning and 10 days postweaning) 20 ml (750 mg) of ppIgG,
provided by a 20 ml syringe; a small plastic tube was connected to the syringe and the
pplgG was slowly dipped in and piglets willingly lapped up, ensuring that no pplgG was
lost.

The other half of the pigs received 20 ml 0.9% NaCl (Table 1; Exp. 1, Group 3 + 4). Pigs
were provided the immunoglobulin product before feeding.

In the second experiment, pigs were allotted into three challenge-groups (Table 1; lower
part): two groups received no immunoglobulins but piglets were provided with 25 ml of
0.9% NaCl and provision of feed (from day of weaning) based on wheat, barley and
dehulled soybean, and with either 2500 ppm zinc oxide (Hammershøj Pharmacy,
Hammershøj, Denmark) for 14 days postweaning (Table 1; Exp. 2, Group 1), or no zinc
oxide (Table 1; Exp. 2, Group 2). The third group received pplgG via 20 ml syringe on the
morning before weaning (day 27), and on the morning of weaning (day 28). The pplgG
feeding was continued twice daily by drench gun for 7 days postweaning with a
provision of 25 ml (1.9 g) of pplgG twice daily (Table 1; Exp. 2, Group 3).

The animal experiment was conducted according to the personal license (Charlotte
Lauridsen, J. nr. 2012-15-2934-00125) obtained by the Danish Animal Experiments
Inspectorate, Ministry of Food, Agriculture and Fisheries, Danish Veterinary and Food
Administration, and animals were followed by proper veterinary surveillance throughout
the experiment.

2.3.2. Animals, feeding and housing
All piglets were tested susceptible to E. coli O149:F4 by using a DNA marker genotyping-based test (van Haeringen laboratorium b.v., Wageningen, The Netherlands) on DNA extracted from hair samples. From weaning and onwards, the pigs had ad libitum access to feed and water. The feed was a standard diet for weaners prepared at the facilities at Aarhus University, Foulum. Two littermates of similar weight were housed in pairs in 1.45 m × 1.7 m pens with concrete flooring and sawdust bedding. In experiment 1, the unchallenged (Groups 1 + 3) and challenged pigs (Groups 2 + 4) were housed in different rooms to avoid cross contamination with the challenge E. coli strain. A door separated the rooms between the challenged and unchallenged piglets, and personnel changed clothes/shoes before entering any of the rooms. Environmental conditions including temperature (∼24 °C), humidity (∼50%) and bedding (sawdust) were similar in the two rooms. Experiment 2 was conducted in the same rooms, but all pigs were challenged with E. coli, and there was free passage between the rooms throughout the entire experiment.

2.3.2.1. E. coli challenge

In both experiments, pigs were orally inoculated with 1.0 × 10⁹ colony-forming units (cfu) of E. coli O149:F4 in 5 ml 0.9% NaCl on day 1 and 2 after weaning (day 0 was the day of weaning) using a syringe. After inoculation, the tube was flushed with approximately 10 ml 10% NaHCO₃ in order to neutralize gastric acid and increase the survival rate of the challenge strain in the stomach, and to ascertain that all the E. coli suspension had been given to the piglets. The control pigs (exp. 1) received equivalent amounts (approximately 5 ml) of 0.9% NaCl and 10 ml 10% NaHCO₃ in order to obtain
an equal level of stress associated with the oral inoculation as for the challenge treatment.

2.3.3. Performance and diarrhoea assessment

Diarrhoea assessment was based on the consistency of the faeces (1 = hard, dry and cloddy, 2 = firm, 3 = soft with shape, 4 = soft and liquid, 5 = watery and dark, 6 = watery and yellow, 7 = foamy and yellow) from the day prior to challenge until 7 days after. A faecal consistency score >3 was defined as a clinical sign of diarrhoea (Carstensen et al., 2005). Before the E. coli challenge, and on day 2 after challenge, and daily until day 5 after challenge, and thereafter every second day during the second week after weaning, faecal samples were collected from the rectum of the pigs and 1 g faeces was suspended in a (1:10, wt/wt) peptone solution and homogenized by bag mixer (BagMixer100, Interscience, St. Nom, France). Serial dilutions of the slurry were done prior to enumeration of haemolytic E. coli on blood agar (BA; Oxoid) after aerobic incubation at 37 °C overnight. From each BA plate, five haemolytic E. coli colonies were selected and tested for O149 and O138 type reactions by O-seroagglutination (Statens Serum Institut, Copenhagen, Denmark). In addition, faecal samples were analysed for dry matter by freeze-drying (ScanVac Coolsafe 55, Labogene Aps, Lynge, Denmark).

Feed intake was recorded daily for each pen and body weight of the pigs was recorded at the beginning and weekly thereafter until the end of the experiment. Average daily feed intake (ADFI) and gain (ADG) were determined based on pen by dividing the total feed intake or total weight gain of pigs in each pen by days of feeding.
The effects of pIgG, dietary zinc oxide or no treatment for seven days postweaning on diarrhoea symptoms and bacterial count in both experiments were statistically analysed using either Mann-Whitney test or Two-way ANOVA followed by Tukey’s post-test, in GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

3. Results and discussion

In this study we performed two infection experiments using an E. coli O149 challenge strain at both day one and two after weaning. The administration of pIgG was different in the two experiments: In experiment 1, 750 mg of pIgG was provided orally once daily prophylactically for five days preweaning and then 10 days postweaning, whereas in experiment 2, 1.9 g of pIgG was administered orally twice daily for seven days postweaning. Two different pIgG batches were used for the two experiments, for experiment 1 a 37.5 mg/ml batch and for experiment 2 a 75 mg/ml batch.

3.1. Disease

In both experiments diarrhoea was observed within one day after inoculation due to the O149 ETEC challenge as observed by faecal scoring (Fig. 1A + B) and faecal dry matter measurement (Fig. 1C + D). We observed in both experiments that the +pIgG +challenge group (group 2 in exp. 1 and group 3 in exp. 2) had, at day five, significantly less diarrhoea as compared to the control challenge groups (group 4 in exp. 1 and group
227 2 in exp. 2) that were not provided with pplgG as observed by a lower faecal scoring and
228 a higher percentage of faecal dry matter (p < 0.03; Fig. 1, days 1–5). In experiment 1 the
229 O149 ETEC challenge strain began to cease at day 7, however an unintended enteral
230 infection, starting around day 5–7 after weaning and lasting the remainder of the study
231 period was observed (Fig. 1A + C, days 5–21), coinciding with the appearance of an O138
232 ETEC strain in faeces (detected by PCR; data not shown). The presence of this
233 unintended infection somewhat obscured experiment 1, however the data from day 0 to
234 five were promising and prompted us to conduct a second experiment in which the daily
235 IgG dose was quadrupled but then only provided seven days post weaning. This batch of
236 pplgG had an IgG concentration of 75 mg/ml, and was administered twice daily,
237 amounting to a daily dose of 3.8 g.
238
239 Experiment 2 proceeded without any unintended infection, and group 3 (+pplgG
240 +challenge group) had significantly less diarrhoea than both group 1 and 2 (+zinc
241 +challenge and no treatment groups) within the first week after weaning (p < 0.02 Two-
242 way ANOVA; Fig. 1B + D); demonstrating that pplgG significantly reduced diarrhoea in
243 the PWD model within the first week post weaning to the same level or lower than
244 dietary zinc.
245
246 Moreover, our results indicate that it is not necessary to use pplgG prophylactically for
247 several days preweaning as there was a clear effect of pplgG in experiment 2 on
248 diarrhoea without the five days preweaning pplgG treatment applied in experiment 1
249 (Fig. 1). This also corroborates a previous study (Hedegaard et al., 2016) where pplgG
250 was mixed into the feed that was only available from weaning and decreased
251 diarrhoeagenic Enterobacteriaceae in comparison to a non-pplgG diet. Also, it has been
252 shown (Foged et al., 1986) that monoclonal anti-E. coli F4 fimbriae antibodies
administered at as well as after challenge, but not prophylactically, protected neonatal piglets from an otherwise lethal challenge with F4+ ETEC. On the other hand, it seems to be important to maintain a high administration frequency and an adequately high antibody dose for limiting diarrhoea.

3.2. Microbiology

In both experiments ppIgG helped clear the challenge strain within one week of challenge (Fig. 2, days 1–7). However, as noted above, all groups in experiment 1 experienced an unintended O138 ETEC infection from day 4–9, as seen by an increase in numbers of faecal haemolytic bacteria (Fig. 2A, days 4 + 7 + 9). Group 1 (+ppIgG no challenge) experienced this infection at day 4 while it was observed in group 2 (+ppIgG +challenge) on day 9. Thus, the daily administration of 750 mg of natural IgGs did not provide protection against the unintended infection in neither of the two +ppIgG groups (Fig. 2A, groups 1 + 2, days 4–21), even though in vitro data support that ppIgG binds to O138 (and O149) ETEC and can inhibit their adhesion to intestinal epithelial cells in vitro (Hedegaard et al., 2016). This might indicate that the unintended infection was multifactorial, and/or that the daily dose of 750 mg natural ppIgG used in this experiment was not adequate to prevent this type of infection. Data from dose-response field trials are needed before any further conclusions can be made on this matter.

In experiment 2, only infection with the challenge-strain was observed. Confirming previous results (Hedegaard et al., 2016), ppIgG caused a faster clearance of diarrhoeagenic (haemolytic) bacteria in group 3 than was observed in the other two groups (Fig. 2B). Thus day 7 was the last day on which faecal haemolytic bacteria were
detected in group 3 (+pplG), while these bacteria could still be detected on day 9 in group 1 (+zinc group), and in group 2 (control) one piglet still had faecal haemolytic bacteria on day 15 (Fig. 2B). Taken together, pplG appears to intervene with the colonization by the ETEC challenge strain shortening the period of infection significantly (Fig. 2B, Day 5, p < 0.02) and was as efficient as dietary zinc in reducing the infection with the ETEC challenge strain. Although this suggests that pplG can shorten the duration of diarrhoea in weaner piglets by decreasing the number of faecal diarrhoeagenic haemolytic bacteria, this should ideally take place without a perturbing the composition of the normal intestinal microbiota. In experiment 1, no change in the faecal non-haemolytic bacteria were initially observed (Fig. 2C, days 1–5) however, as the unintended infection emerged the number of faecal non-haemolytic bacteria began decreasing (Fig. 2C, days 7–21). In group 2 (+pplG +challenge) the faecal non-haemolytic bacteria actually were not recovered for 3 days at the height of the unintended infection (Fig. 2C, days 11–14), which coincided with the termination of administration of pplG. However in experiment 2, no changes in the count of faecal non-haemolytic bacteria in any of the three other groups were observed (Figs. 2D), indicating that pplG does not intervene with the intestinal non-haemolytic commensal microbiota and that the microbiota changes observed in experiment 1 was probably due to the unintended infection. These observations are supported by preliminary next generation sequencing data on the faecal microbiota composition of piglets fed pplG showing no change in non-haemolytic commensals (unpublished data).

Disease (diarrhoea) frequency, growth and feed conversion are primary end points for swine producers however the two experiments described here comprised low numbers of piglets (n = 12/18) and pens (n = 4/3) making it very difficult to analyse growth data statistically. Also, the data on growth and feed intake showed a very large pig-to-pig
variation. For example, in experiment 2 half of the piglets in group 3 (+pplG), for
unknown reasons, became anorexic during the third week resulting in almost no weight
gain for the group. Therefore it will be interesting to observe how pplG
supplementation will influence disease resistance, growth and feed conversion in field
trials incorporating an adequate number of pigs to allow for appropriate statistical data
analysis to be performed. In spite of being a small preliminary study the results shown
here do however demonstrate the ability of dietary pplG to clear an enteric ETEC
infection and thus pplG could be used as an alternative to dietary zinc.

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technical assistance during the animal experiments.
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Natural pig plasma immunoglobulins have anti-Bacterial effects: potential for use as feed supplement for treatment of intestinal infections in pigs


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Effects of manure and mineral fertilization strategies on soil antibiotic resistance gene levels and microbial community in a paddy-upland rotation system

Environ. Pollut., 211 (2016), pp. 332–337

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Owusu-Asiedu et al., 2003

A. Owusu-Asiedu, C.M. Nyachoti, R.R. Marquardt

Response of early-weaned pigs to an enterotoxigenic Escherichia coli (K88) challenge when fed diets containing spray-dried porcine plasma or pea protein isolate plus egg yolk antibody, zinc oxide, fumaric acid, or antibiotic

Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential

BMC Genomics, 16 (2015), p. 964

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Shen et al., 2014

Coated zinc oxide improves intestinal immunity function and regulates microbiota composition in weaned piglets

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Wageningen University

Antibiotic Usage in Pigs

### Table 1: Study setup

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ppIgG</td>
<td>+ppIgG+E.coli</td>
<td>Control</td>
<td>+E.coli</td>
</tr>
<tr>
<td>Number of piglets</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><em>E. coli</em> F4+ challenge</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>750 mg ppIgG (20 ml)</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>20 ml 0.9% NaCl solution</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Avg. weight at weaning (kg)</td>
<td>11.2±3.1</td>
<td>10.6±1.7</td>
<td>10.4±1.9</td>
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<table>
<thead>
<tr>
<th>Experiment 2</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ E.coli +Zn</td>
<td>+E.coli</td>
<td>+E.coli +ppIgG</td>
</tr>
<tr>
<td>Number of piglets</td>
<td>6</td>
<td>6</td>
<td>6 (5)¹</td>
</tr>
<tr>
<td><em>E. coli</em> F4+ challenge</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>2x1.9 g ppIgG (2x25 ml)</td>
<td>No</td>
<td>No</td>
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<tr>
<td>25 ml 0.9% NaCl solution</td>
<td>Yes</td>
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</tr>
<tr>
<td>Zinc oxide in feed</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
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<tr>
<td>Avg. weight at weaning (kg)</td>
<td>6.9±1.1</td>
<td>7.9±0.9</td>
<td>7.0±0.9</td>
</tr>
</tbody>
</table>

¹ One piglet was euthanized on day 3 post infection due to serious illness.
459 Figure legends

460 **Figure 1. Faecal analysis:** Faecal diarrhoea score (A+B) and faecal dry matter (C+D); (●)
461 no treatment, no E. coli; (●) no treatment, +E. coli: (-■-) +pplG, no E. coli; (▲) pplG
462 treatment, +E. coli; (■) dietary zinc, +E. coli. Each data point is plotted and curves
463 outline the mean for each group. Vertical dotted line indicates the last day of pplG
464 administration. Pairwise comparisons between groups on each day were tested for
465 statistical significance using Mann-Whitney test: a = IgG vs. Zinc (p<0.03); b = IgG vs.
466 Control (p<0.03); c = IgG vs. Zinc (p<0.02); d = IgG vs. Control (p<0.01); e = Zinc vs.
467 Control (p<0.02).

468

469

470 **Figure 2. Faecal bacterial analysis:** Content (CFU/ml) of haemolytic bacteria (A+B) and
471 non-haemolytical bacteria (C+D). Symbols as for Figure 1. Each data point is plotted and
472 curves outline the mean for each group. Vertical dotted line indicates the last day of
473 pplG administration. Pairwise comparisons between groups on each day were tested
474 for statistical significance using Mann-Whitney test: a = IgG vs. control (p<0.02).
Figures

Figure 1