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Oral Administration of Citrus Pulp Reduces Gastrointestinal Recovery of Orally Dosed *Escherichia coli* F18 in Weaned Pigs

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Abstract: The effects of Citrus Pulp (CTP) on the immune and cortisol responses to *E. coli* F18 inoculation and subsequent *E. coli* recovery were evaluated in newly weaned pigs (23.3±1.8 days of age). Barrows were assigned to 1 of 2 treatment groups with (CTP; n = 15) and without (Control; n = 15) the in-feed inclusion of CTP (10% rate as fed) for 13 days. On day 13, all pigs were orally dosed with Novobiocin (Nov) and Nalidixic Acid (Nal) resistant *E. coli* F18 (10 mL 7×10^8 CFU) at 0 h. Serial blood samples were collected via an indwelling jugular catheter inserted on day 12 at hourly intervals from 0-8 h and then at 12, 24, 36 and 48 h. Differential blood cell populations were enumerated hourly from 0-8 h and at 12, 24, 36 and 48 h. Serum cortisol, interleukin-1 beta (IL-1β), IL-6, Tumor Necrosis Factor-alpha (TNF-α) and interferon-gamma (IFN-γ) concentrations were determined via porcine-specific ELISAs at all time points. After 48 h, all pigs were euthanized and samples collected from ileal, cecal and rectal contents for selective *E. coli* F18 standard plate counts on Nov and Nal-treated media. White blood cells, lymphocytes, neutrophils and macrophages were decreased ($p<0.05$) from baseline equally in both treatments by 48 h. A more rapid cortisol suppression ($p<0.05$) was observed in CTP-treated piglets after inoculation with a subsequent return to baseline in both treatments. The production of IL-1β, IL-6, TNF-α and IFN-γ were unaffected by treatment or inoculation. However, the inclusion of CTP suppressed ($p<0.05$) ileal and cecal *E. coli* F18 recovery compared to controls and completely eliminated rectal recovery of the pathogen. These results demonstrate that the potentially therapeutic effects of CTP are the result of direct microbial modulation independent of an immune response. Therefore, supplementation of CTP could potentially be used to enhance growth in weaned pigs by suppressing chronic and acute pathogenic challenges; consequently preventing the diversion of energy towards maintaining innate and adaptive immune responses and liberating it for growth related processes.

Key words: Citirus pulp, cortisol, cytokine, *Escherichia coli* F18, pig, baseline

INTRODUCTION

Current market conditions, pharmaceutical resistance and unsupported human population increases impact animal producers on a global scale. It is therefore increasingly important to meet these challenges by devising economically viable alternative to antibiotics capable of improving animal health and sustaining increased production. The inclusion of various dietary feedstuffs is a potential method to modulate intestinal microbial populations to control pathogen-associated suppression of growth and production (Weber *et al*., 2008; Opapeju *et al*., 2009; Trevisi *et al*., 2009; Wells *et al*., 2009). Current research demonstrates the possible efficacy of the dietary inclusion of Citrus Pulp (CTP) for a variety of production species (Scerra *et al*., 2001; Arthington *et al*., 2002; Mourao *et al*., 2008; Weber *et al*., 2008). Preliminary evidence indicates that the potential growth advantages imparted by citrus pulp inclusion are due to antimicrobial characteristics (Ariza *et al*., 2001; Callaway *et al*., 2008; Nannapaneni *et al*., 2008; Weber *et al*., 2008; Wells *et al*., 2009) however, the possible regulatory role of citrus pulp on the immune response and resultant production improvement are ill-defined. Therefore, the effects of CTP on the immune and cortisol responses to *E. coli* F18 inoculation and subsequent *E. coli* recovery were evaluated in newly weaned pigs.

MATERIALS AND METHODS

Animals and experimental design: All experimental procedures were in accordance with the Guide for the Care and Use of Agriculture Animals in Agricultural Research

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and teaching and approved by the Institutional Animal Care and Use Committee of the USDA-ARS. Thirty terminal cross PIC Line 201 barrows were obtained from Murphy Brown LLC, Dalhart, TX upon weaning at days 23.3±1.8 of age. The pigs were transferred to the USDA Livestock Issues Research Unit nursery building where they were weighed assigned to individual pens (4×2 ft) and allowed *ad libitum* access to food supplemented with (CTP; n = 15) and without (Control; n = 15) the inclusion of dried citrus pulp (10% rate as fed; University of Florida, Range Cattle Research and Education Center, Ona, FL) and water. Fiber content was normalized across both treatments via the inclusion of soybean hulls (Hi-pro feeds, Friona, TX) to the Control diet. The pigs were given 2 weeks to adjust to their surroundings and diet. One day prior to *E. coli* F18 dosing (day 12), all 30 pigs were non-surgically fitted with an indwelling jugular catheter according to Carroll *et al.* (1999). Pigs were then given 24 h to recover from the cannulation procedure before challenge and collection. Two CTP-treated pigs could not be cannulated and were removed from the study. Prior to collection of the initial sample an extension was attached to the catheter to allow for remote sample collection without handling the pigs.

***Escherichia coli* F18 immune challenge:** *Escherichia coli* strain F18 was taken from the FFSRU culture collection and was cultivated in anoxic Tryptic Soy Broth (TSB) medium at 37°C. *Escherichia coli* strain F18 was resistant to novobiocin and nalidixic acid (20 and 25 μg mL^{-1}, respectively) and this mutation was stable for several generations in the absence of selective pressure. Overnight cultures (1000 mL each) were harvested by centrifugation (7,500×g, 24°C, 10 min) and the cell pellet was re-suspended in TSB medium (150 mL). On day 13, each pig was inoculated with 3×10^9 CFU of *E. coli* via oral gavage.

Serum collection and analysis: Blood samples were collected via jugular catheter from 0 h prior to *E. coli* dosing to establish baseline values within each animal as previously validated (Carroll *et al.*, 2005; Reuter *et al.*, 2008). Serial blood samples were then collected at hourly intervals from 0-8 h and then at 12, 24, 36 and 48 h. Approximately 5 mL of blood were drawn at each time point into a serum tube and allowed to clot for 1 h at room temperature. Tubes were then centrifuged (1400×g for 20 min at 20°C), serum collected and aliquoted into microcentifuge tubes and stored at -80°C for later analysis. Total white blood cell and white blood cell differential counts were performed on whole blood samples using a Cell-Dyn (Aushon Biosystems; Billerica, MA, USA) within 1 h of collection. Serum concentration of cortisol (ng mL^{-1}) was determined by RIA (Coat-a-Count Assay, Diagnostic Products Corp.; Los Angeles, CA, USA) as previously performed and validated (Frank *et al.*, 2003). Concentration of serum cytokines interleukin-1 beta (IL-1β), IL-6, Tumor Necrosis Factor-alpha (TNF-α) and interferon-gamma (IFN-γ) was determined according to the manufacturer's protocol using a porcine specific ELISA kit for proinflammatory cytokines (SearchLight Porcine Inflammatory Cytokine Array # 84664; Pierce, Rockford, IL). All assays were performed in duplicate and intra and inter-assay Coefficient of Variance (CV) values calculated. The intra and inter-assay Coefficients of Variation (CV) were 9 and 11%, respectively for cytokine analyses and 7 and 10%, respectively for the cortisol.

Bacterial enumeration: After 48 h, all pigs were euthanized before aseptic collection of intestinal luminal contents and epithelial tissues from the ileum, cecum, colon and rectum. Intestinal and fecal contents were serially diluted (10 fold increments) in sterile Phosphate Buffered Saline (PBS). Dilutions were plated on MacConkey's agar supplemented with novobiocin (20 μg mL^{-1}) and nalidixic acid (25 μg mL^{-1}) and incubated overnight at 37°C. Colonies that grew on agar plates after 24 h incubation were directly counted (quantitative enumeration). To qualitatively confirm the presence of inoculated *E. coli* intestinal contents and epithelial tissue samples were incubated overnight in TSB at 37°C and were streaked on antibiotic supplemented MacConkey's agar. Plates that contained colonies after 24 h incubation were classified as positive for the inoculated *E. coli*.

Statistical analysis: All data from canulated pigs (n = 28) were subjected to analysis of variance specific for repeated measures using the mixed procedure of SAS (SAS Inst., INC., Cary, NC). Sources of variation included litter, time and their interactions. Specific treatment comparisons were made using Fisher's Protected Least Significant Difference with comparisons of $p<0.05$ considered significant. In almost all cases the strongest relationships were found when analyses were conducted among the magnitudes of the response and these coefficients are presented.

RESULTS AND DISCUSSION

Piglet growth: Cumulative piglet weight was greater ($p<0.05$) in control piglets when compared to CTP-treated piglets (9.38±0.23 vs. 8.09±0.17 kg, respectively). However, the ADG of piglets supplemented with CTP did not differ ($p>0.05$) from control piglets

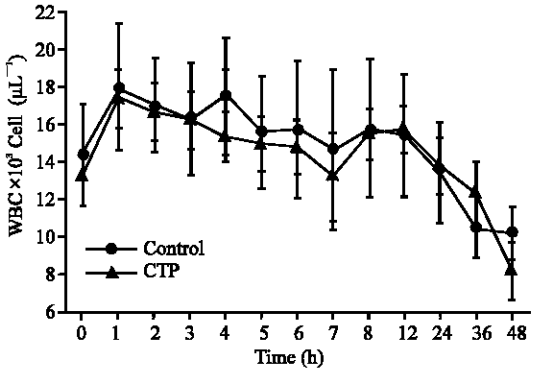

Fig. 1: Effect of CTP supplementation (10% rate as fed) for 13 day on the numerical density of White Blood Cells (WBC) in pigs orally dosed with Novobiocin (Nov) and Nalidixic acid (Nal) resistant *E. coli* F18 (10 mL 7×10^8 CFU) at 0 h. Values represent the mean±SEM for barrows assigned to 1 of 2 treatment groups with (CTP; n = 15) and without (Control; n = 15) the in-feed inclusion of CTP for 13 day. On day 13, all pigs were dosed orally with Nov and Nal-resistant *E. coli* F18 at 0 h. Blood samples were subsequently collected via catheters at 1 h intervals from 0-8 h and at 24, 36 and 48 h post-challenge

(0.10±0.022 vs. 0.12±0.28 kg day^{-1}, respectively) through the entire study. Feed consumption did not differ ($p>0.05$) between control and CTP-treated piglets (2.7+0.45 vs. 3.35+0.29 kg, respectively). The initial weight of control piglets was greater ($p<0.05$) than CTP-treated piglets (8.52±0.26 vs. 7.61±0.22 kg, respectively) and could partially explain the difference in cumulative weight independent of treatment effect.

Differential cell populations: Total white blood cell populations trended upwards after 0 h before returning to baseline by 24 h and dropped below baseline by 48 h (Fig. 1). However, WBC populations were unaffected ($p>0.05$) by CTP administration. Similar trends were observed in lymphocyte and monocyte enumeration. Inoculation with *E. coli* F18 induced elevated ($p<0.05$) basophil counts peaking at 2 h and sustained for 2 h before returning to baseline by 5 h (Fig. 2). The addition of CTP increased basophil counts when compared to control animals at 4 and 5 h.

Circulating cortisol and cytokine concentrations: Cortisol concentrations in CTP-treated piglets decreased below 0h baseline more rapidly than Control animals reaching maximal suppression by 2 h while Controls were maximally suppressed at 5 h (Fig. 3). Both treatments

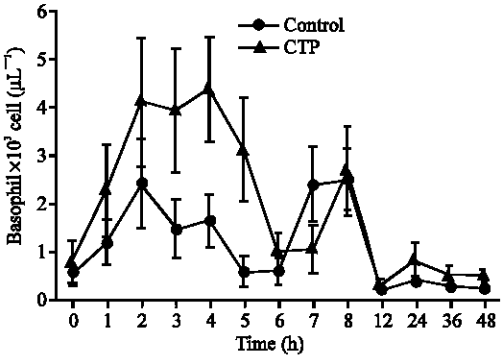

Fig. 2: Effect of CTP supplementation (10% rate as fed) for 13 days on the numerical density of basophils in pigs orally dosed with Novobiocin (Nov) and Nalidixic acid (Nal) resistant *E. coli* F18 (10 mL 7×10^8 CFU) at 0 h. Values represent the mean± SEM for barrows assigned to 1 of 2 treatment groups with (CTP; n = 15) and without (Control; n = 15) the in-feed inclusion of CTP for 13 day. On day 13, all pigs were dosed orally with Nov- and Nal-resistant *E. coli* F18 at 0 h. Blood samples were subsequently collected via catheters at 1 h intervals from 0-8 h and at 24, 36 and 48 h post-challenge

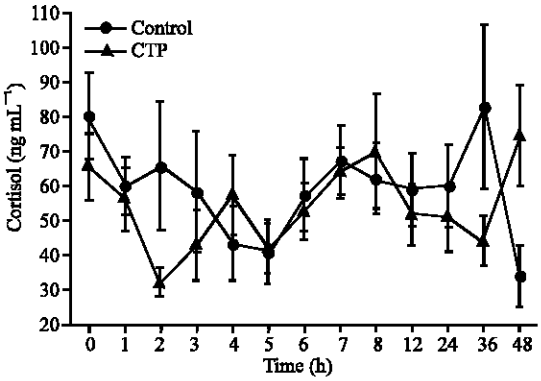

Fig. 3: Effect of CTP supplementation (10% rate as fed) for 13 days on the cortisol concentration in pigs orally dosed with Novobiocin (Nov) and Nalidixic acid (Nal) resistant *E. coli* F18 (10 mL 7×10^8 CFU) at 0 h. Values represent the mean±SEM for barrows assigned to 1 of 2 treatment groups with (CTP; n = 15) and without (Control; n = 15) the in-feed inclusion of CTP for 13 days. On day 13, all pigs were dosed orally with Nov and Nal- resistant *E. coli* F18 at 0 h. Blood samples were subsequently collected via catheters at 1 h intervals from 0-8 h and at 24, 36 and 48 h post-challenge

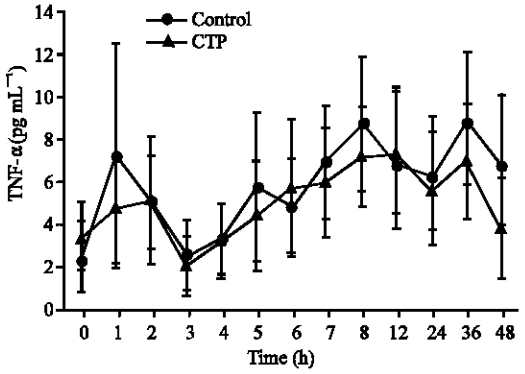

Fig. 4: Effect of CTP supplementation (10% rate as fed) for 13 days on the TNF-α concentration in pigs orally dosed with Novobiocin (Nov) and Nalidixic acid (Nal) resistant *E. coli* F18 (10 mL 7×10^8 CFU) at 0 h. Values represent the mean±SEM for barrows assigned to 1 of 2 treatment groups with (CTP; n = 15) and without (Control; n = 15) the in-feed inclusion of CTP for 13 days. On day 13, all pigs were dosed orally with Nov and Nal- resistant *E. coli* F18 at 0 h. Blood samples were subsequently collected via catheters at 1 h intervals from 0-8 h and at 24, 36 and 48 h post-challenge

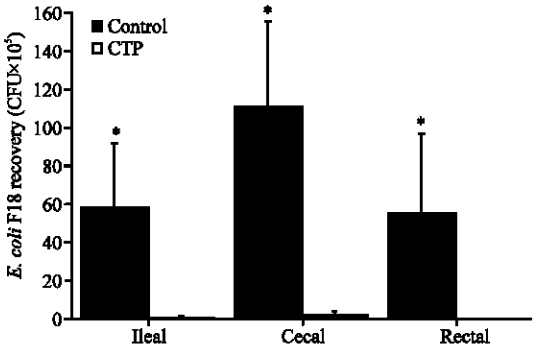

Fig. 5: Esffect of CTP supplementation (10% rate as fed) for 13 days on the recovery of novobiocin (Nov.) and Nalidixic acid (Nal) resistant *E. coli* F18 (10 mL 7×10^8 CFU) dosed at 0 h. Values represent the mean±SEM for barrows assigned to 1 of 2 treatment groups with (CTP; n = 15) and without (Control; n = 15) the in-feed inclusion of CTP for 13 days. On day 13, all pigs were dosed orally with Nov and Nal-resistant *E. coli* F18 at 0 h. After 48 h, all pigs were euthanized before aseptic collection of intestinal luminal contents and epithelial tissues from the ileum, cecum, colon and rectum. Dilutions were plated on MacConkey's agar supplemented with novobiocin (20 μg mL^{-1}) and nalidixic acid (25 μg mL^{-1}). Colonies that grew on agar plates after 24 h incubation were directly counted (quantitative enumeration). *denotes $p<0.05$ within intestinal section

returned to baseline by 5 h. Detectable concentrations of circulating IL-1β and IL-6 were not found. While not statistically significant, TNF-α concentrations increased by 1 h post inoculation before returning to 0 h baseline at 3 h (Fig. 4). The concentration of TNF-α then increased again peaking at 8 h before returning to baseline. Treatment-induced differences were not observed with the addition of CTP. The concentration IFN-γ were similar to patterns observed with TNF-α.

***Escherichia coli* F18 recovery:** The inclusion of CTP suppressed ($p<0.05$) ileal and cecal *E. coli* F18 recovery and completely eliminated rectal recovery of the pathogen when compared to controls (Fig. 5).

Currently, little is known about the mechanisms by which CTP as well as many other feed additives, imparts health and growth improvements to production animals. Here, unlike sub-therapeutic antibiotic use (Gaskins *et al*., 2002), CTP did not increase growth or production parameters. However, the inclusion of CTP increased peak basophil populations and accelerated cortisol suppression after *E. coli* F18 inoculation. While the inclusion of CTP did not alter the other *E. coli* F18 induced immune responses measured here, it did suppress *E. coli* F18 recovery in the ileum and cecum of treated animals while completely eliminating recovery from the feces. Current research detailing the effects of dietary inclusion of citrus pulp on animal health and growth performance remains sparse. Similar to the absence of growth performance improvement shown here, other studies have shown minimal impact on growth via CTP inclusion (Callaway *et al*., 2008; Jaramillo *et al*., 2009; Nazok *et al*., 2010). However, some evidence suggests that tertiary growth parameters not typically associated with production animal growth calculations could possibly be improved via feeding of CTP. For example, the anti-oxidative properties associated with CTP have been demonstrated to improve bone mass in a rat model of osteoporosis (Morrow *et al*., 2009). These anti-oxidative properties may also have multi-target anti-metastatic properties (Glinsky and Raz, 2009).

To date, this research is among the first to detail possible immuno and cortisol-modulating properties associated with CTP feeding. One other reference outlines the use of 15% CTP feed inclusion to increase the activity of T cell killers in tumors and higher level of proteins

associated with apoptosis (Kossoy *et al*., 2001). Here, WBC and lymphocyte counts were elevated and then suppressed in response to *E. coli* F18 inoculation but CTP did not alter those responses. Peak basophil production was greater in CTP-treated piglets when compared to controls (Reese *et al*., 2007; Voehringer, 2009; Ohnmacht and Voehringer, 2010).

As basophils are typically associated with immune reactions to parasites or allergies, the reasoning for their increased concentration is unclear. However, the increased basophil counts may help modulate the innate immune response against oral *E. coli* F18 dosing via increasing mucus production (Voehringer *et al*., 2004, 2006). Pro-inflammatory cytokine concentrations were unaffected by addition of CTP. These researchers are not aware of any other research outlining the effects of CTP on cortisol concentrations. While CTP accelerated *E. coli* F18-induced cortisol suppression, these results cumulatively indicate that alterations to the pig physiology were independent of immune modulation.

The recovery of *E. coli* F18 from the ileum, cecum and rectum were diminished in CTP-treated piglets. Previous results from this lab and others demonstrate the efficacy of citrus pulp to decrease the growth of *E. coli* 0157:H7 and *Salmonella typhimurium* in pure culture and in fermentation with mixed ruminal microorganisms *in vitro* (Callaway *et al*., 2008; Yuk *et al*., 2008).

CONCLUSION

Cumulatively, these results demonstrate that the potentially therapeutic effects of CTP are primarily the result of direct microbial modulation independent of an immune response. Therefore, supplementation of CTP could potentially be used to enhance growth in weaned pigs by suppressing chronic and acute pathogenic challenges.

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